

Cloning viral dsRNA genomes: Analysis and application

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“Here, ons God, U is waardig
om die heerlikheid
en die eer
en die mag te ontvang
omdat U alles geskep het;
deur u wil het alles ontstaan
en is dit geskep.”

Openbaring 4:11

Aan my ouers, broer en suster

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SOLI DEO GLORIA

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SUMMARY

Double-stranded RNA viruses occur in a large number of hosts in nature ranging from bacteria to mammals. Molecular studies of the double-stranded RNA viruses have greatly enhanced man's understanding of this large group of viruses as far as structure and function of their genes and epidemiology is concerned. However, one of the major prerequisites of obtaining this information is the ability to clone the genomes of these viruses for nucleotide sequencing and recombinant protein expression studies. In the dsRNA field, cloning viral genomes has historically been difficult and time consuming and created a bottleneck that hampered molecular studies. The main aim of this investigation was to optimise a method for cloning viral dsRNA genomes to the extent that it would be easy and fast as well as applicable to most dsRNA viruses.

In this study a sequence-independent, oligo-ligation mediated dsRNA cloning procedure for large genes (up to 6.8 kb) was perfected and tailored for routine use to amplify and clone complete genome sets or individual genes. Complete genome sets could be amplified and cloned from as little as 1 ng dsRNA. The method was shown to be simple and efficient compared to other methods and is currently the only method that allows the amplification of complete genomes in a single PCR reaction.

Complete gene sets of seven genomes from the Reovirus family, one from the Cystovirus family and one mycovirus, have been amplified and cloned. The full-length VP2 genes of all 9 AHSV and 24 BTV serotypes were also cloned. Phylogenetic analysis of VP2-genes revealed the same grouping of AHSVs and BTVs as serology. Several cloned genes of AHSV, rotavirus and EEV have been utilised for recombinant protein production establishing that the cloned cDNAs have full open reading frames. The nine AHSV VP2 genes have been developed as serotype-specific probes which allowed serotyping of AHSV isolates within 4 days compared to 2-4 weeks needed with the traditional serological serotyping.

The new cloning procedure finally opens the bottleneck that hamstrung the development of complete repertoires of recombinant vaccines, molecular diagnostics and epidemiology to combat dsRNA viral diseases. It should now be possible to deliver on many of the expectations that were envisaged for dsRNA virus research and biotechnology since the advent of recombinant DNA technology.

OPSOMMING

Virusse met dubbelstring RNS (dsRNS) genome kom in die natuur voor in 'n wye verskeidenheid gashere, van bakterieë tot soogdiere. Die mens se bestudering van die struktuur en funksie van hierdie groot groep virusse is hoofsaaklik moontlik gemaak deur molekulêre studies veral sover as struktuur, funksie en molekulêre epidemiologie aangaan. Daar is egter 'n voorvereiste verbonde aan molekulêre studies, naamlik die vermoë om die genome van die virusse te kloneer. In die veld van dsRNS virusse was kloneringstegnieke tot dusver altyd moeilik en het baie tyd in beslag geneem met die gevolg dat molekulêre studies van die virusse tot 'n groot mate gekortwiek is. Die hoofdoel van hierdie ondersoek was om 'n tegniek vir die klonering van dsRNS virus genome te optimaliseer tot so 'n mate dat dit doeltreffend, maklik en toepaslik op verskeie virusse sou wees.

In hierdie studie het ons 'n nukleotiedvolgorde-onafhanklike, oligo-ligerings kloneringsmetode ge-optimaliseer tot so 'n mate dat groot dsRNS segmente (tot en met 6.8kb) op 'n roetine basis geamplifiseer en gekloneer kon word. Die metode kan gebruik word vir die klonering van volledige genoomstelle asook individuele gene. Volledige dsRNS genome kon geamplifiseer en kloneer kon word vanaf minder as 1 nanogram beginmateriaal. Die metode is tans die eenvoudigste gepubliseerde metode asook die enigste metode tot dusver wat navorsers toelaat om 'n volledige genoom van 'n dsRNS virus in een PKR-eksperiment te amplifiseer.

Die metode het dit moontlik gemaak om volledige genoomstelle van 7 verskillende virusse uit die familie *Reoviridae* en van 'n Cystovirus te amplifiseer en te kloneer, sowel as die volle genoom van 'n mikovirus. Verder is die vollengte VP2 gene van al 9 perdesiektevirus (PSV) serotipes en 24 bloutong virus (BTV) serotipes gekloneer. Filogenetiese analise van vollengte aminosuurvolgordes van die PSV VP2 proteïene, gene en gedeeltelike aminosuurvolgordes van die BTV VP2 proteïene, het dieselfde groepering getoon as wat serologies verkry word. Verskeie vollengte gekloneerde gene van PSV, EEV en rotavirus is gebruik vir die uitdrukking van rekombinante proteïene in die bakulovirussisteem. Die studies het getoon dat al die gekloneerde gene volledige oop leesrame het. Die vollengte cDNS klone van al 9 PSV serotipes is ook gebruik in die ontwikkeling van peilers om PSV isolate te serotipeer binne 4 dae in vergelyking met die tradisionele serologiese metodes wat 2-4 weke neem.

Die nuwe klonerings metode oorkom die probleem waarmee vir jare gesukkel is om dsRNS virusse se genome te kloneer. Navorsing en ontwikkeling van dsRNS virusse behoort nou voluit te kan deel in die krag van rekombinante DNS tegnologie en te begin voldoen aan die baie verwagtinge wat in die vooruitsig gestel is met die koms van rekombinante DNS tegnologie.

PREFACE

This dissertation is presented in the article format. The dissertation consists of a literature study (Chapter 1) followed by four published articles (Chapters 2-5), a results and discussion chapter and finally a concluding chapter (Chapter 7). All chapters in this study are concerned with the cloning of viral dsRNA genomes and the applications as a result thereof.

My contribution to the first article "Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes" is as follows. All the work presented is my own except for the provision of viruses. All developmental work on the cloning method, the amplification of the genomes, sequence analysis as well as all baculovirus recombinant expression work was done by myself.

My contribution to the second paper entitled "A first full outer capsid protein sequence data-set in the Orbivirus genus (family Reoviridae): cloning, sequencing, expression and analysis of a complete set of full-length outer capsid VP2 genes of the nine African horsesickness virus serotypes" is as follows. All cloning, sequence analysis and baculovirus recombinant expression work was done by myself. While other VP2 genes have been cloned sequenced and expressed by M. Cloete (second author), the cloning of all 9 AHSV VP2 genes were repeated including partial sequencing. Viruses were supplied by the OIE Reference Centre for AHS at OVI.

For the third paper "Development of probes for typing African horsesickness virus isolates using a complete set of cloned VP2-genes", I provided the full-length VP2 cDNA clones of AHSV serotypes 1, 2, 4, 6, 7 and 8 to complete the full set of probes needed in this study. Also previously, probes for serotypes 4 and 7 were prepared by myself after cloning.

My contribution to the fourth paper entitled "Characterization of Φ 12, a bacteriophage related to Φ 6: Nucleotide sequence of the large double-stranded RNA" was mainly the cloning, partial sequencing and provision of the full-length large segment of Φ 12. Virus and host were provided by the first author (P Gottlieb).

All co-authors have given their consent for using the papers in this dissertation.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Mankind has struggled against pathogens of himself, his animals and crops since ancient times. Devastating pandemics of cholera, smallpox, the Bubonic plague (“black death”), polio and the “cattle plague” (rinderpest) are but a few historic examples. In modern times HIV-AIDS, “mad cow disease”, and severe acute respiratory syndrome (SARS) continue to add to the list. To date, smallpox is the only disease that man has been able to eradicate with vaccination worldwide. This milestone was achieved after a worldwide vaccination campaign in the late 1970s (W.H.O., 1980). Also, with proper vaccination strategies the incidence of polio is currently less than 10 per year compared to 21 000 cases in the 1950s (Levine, 1994).

Currently more than 3600 virus species are listed by the ICTV (International committee on virus taxonomy). It is estimated, however, that more than 30 000 viruses, strains and subtypes are being tracked by laboratories and reference centres around the world (Büchen-Osmond *et al.*, 2000). The amounts of viruses found in nature are staggering, for example, some 10 million viruses infecting prokaryotic hosts are present per milliliter of water in aquatic environments alone (Bergh *et al.*, 1989). It is estimated that the same amounts of viruses are present for multicellular organisms. Therefore, viral hosts are estimated to be outnumbered by viruses at least by 10 species of virus per host (Bamford *et al.*, 2002).

The advent of recombinant DNA technology has resulted in spectacular progress in biology and biotechnology in the past two decades. This is most certainly also true where viral studies are considered.

At the Onderstepoort Veterinary Institute (OVI) in South-Africa studies on viral agents that cause disease in animals essentially started in 1901 with studies on African horsesickness virus (AHSV) (Theiler, 1901). This virus and two other viruses studied at Onderstepoort,

namely bluetongue virus (BTV) and equine encephalosis virus (EEV), contain double-stranded RNA (dsRNA) as genetic material. Molecular studies on viruses with dsRNA genomes have been severely hampered by the inability to efficiently clone the large (>2.5 kb) dsRNA genes. This has limited basic as well as applied virus research and the applications that biotechnology offers to viral research. This study describes how this limitation has been largely overcome by improving current cloning technologies and will show the application of this new technology.

1.1.1 Early history

The study of viral pathological agents essentially started in the late 19th century. One of the first observations of a viral agent of disease was that of Adolf Mayer (1886) while studying diseases of tobacco. He reported that inoculating juice extracted from diseased tobacco plants into healthy plants, caused nine out of ten inoculated plants to become "heavily diseased". Dimitri Ivanovsky (1892) showed the sap caused disease in healthy plants even after filtration of the virus through a Chamberland filter. Since the results were so unexpected, it was believed that the filters (which should remove all contaminating bacteria) were either defective or that a toxin was present in the filtrate. Martinus Beijerinck (1851-1931) proved that the filtered sap contained an infectious agent when diluted filtered sap from infected plants "regained its strength" after replication in the plant and it was thus not a toxin. Finally, after a 25 year debate d'Herelle developed a viral plaque assay in 1917 and in 1929 the first micrographs of tobacco mosaic virus were taken. These observations conclusively demonstrated that *viruses* (from Latin meaning slimy liquid or poison) were indeed particles. The study of viruses was not restricted to the agents causing disease in plant hosts. In 1898 Loeffler and Frosch published the first report of a filterable disease causing agent from animals, namely the foot and mouth disease virus. This was followed shortly by Reed *et al.* (1901) who discovered such a disease causing agent in humans, namely yellow fever virus.

In South Africa viral studies were first reported not long after the discovery of viral agents in humans and animals. In 1901 Arnold Theiler published the discovery of a filterable disease causing agent in horses, namely African horsesickness virus, at Onderstepoort in South Africa (Theiler, 1901). In 1906 he reported that bluetongue virus was a filterable agent closely associated with blood that caused disease in sheep. However, it was not until scientists had the ability to culture animal viruses outside their hosts that studies on viral

genomes could be done easily. Initially viruses were cultured in wild animals and later in laboratory animals (like mice) and embryonated chicken eggs (1930s). Viruses were first cultured in cell culture in the early 1950s. Around the same time (1953) Watson and Crick had shown that double-stranded DNA was a double helix which sparked interest in molecular studies. It is essentially during this time that studies on the molecular biology of viruses started. Once researchers had the ability to culture single cells the growth and amplification of some viruses in the laboratory became relatively easy. BTV was first cultured on primary lamb kidney cells (Haig *et al.*, 1956). The first molecular studies of BTV started from the virus cultured on BHK cells. It was shown that BTV contained a genome of double-stranded RNA (Verwoerd, 1969). At the time only one other virus, namely reovirus, had been shown to contain such a genome. Today the study of viruses with dsRNA genomes that cause disease in animals such as BTV, AHSV, EEV and EHDV continues at the Onderstepoort Veterinary Institute.

A lot of information on virus structure, function and epidemiology was gathered from virological and serological studies. The advent of recombinant DNA technology in the early 1970s was a major milestone in biology. The discovery of the enzyme reverse transcriptase (Baltimore, 1970; Temin and Mizutani, 1970) was the breakthrough that opened up the biotechnology for RNA genes. This had a huge impact on man's understanding of RNA viruses.

The studies of viruses containing dsRNA like bluetongue virus, rotavirus and African horsesickness virus followed the trends of science as far as serology and epidemiology was concerned. This was until the age of biotechnology. While it was most certainly possible to study dsRNA viruses at a molecular level these studies proved to be difficult when it came to cloning their genetic material. Firstly, most dsRNA viruses contained genomes with multiple dsRNA segments such as the members of the family *Reoviridae*. Secondly, some of the members of this family have multiple serotypes like bluetongue virus (24), African horsesickness virus (9) and equine encephalosis (7). Other viruses like the rotaviruses have not only serotypes but also groups, types and subtypes.

The need to develop recombinant subunit vaccines against AHS based on the outer capsid protein VP2 was the underlying reason for undertaking this study. It was shown previously that VP2 of BTV was responsible for inducing a protective neutralizing antibody response in

infected sheep (Huimans *et al.*, 1987). Later it was shown that the AHSV outer capsid protein, VP2, could protect horses against disease when injected as a full-length recombinant protein (Roy *et al.*, 1996; Scanlen *et al.*, 2002). The study of the VP2-genes of AHSV and the proteins they encode was, therefore, of great interest to Onderstepoort. This was due to the potential of using them to develop recombinant vaccines as well as new methods for AHSV serotyping and initiating molecular epidemiology.

Historically the cloning of the AHSV VP2 genes has been problematic, not only because of their size (~3.2 kb) but also since the nucleotide sequences of VP2 genes from different serotypes differed substantially. This was shown clearly by the fact that after attempts of more than 20 years the VP2 genes of only two AHSV serotypes (3 and 9) were cloned in South Africa and only two (4 and 6) in Europe and England. This meant that to clone and analyze the full repertoire of AHSV VP2 genes a method had to be developed for cloning these large genes without having prior sequence information. Although a cloning method was to be optimised primarily to enable us to clone AHSV VP2-genes it was envisaged that if the method proved to be successful it would be applicable not only to other dsRNA viruses being studied at Onderstepoort but also to the whole field of dsRNA viruses.

1.2 The double-stranded RNA Viruses

Currently there are 6 families of viruses with dsRNA genomes, namely the *Cystoviridae*, *Reoviridae*, *Birnaviridae*, *Totiviridae*, *Partitiviridae* and *Hypoviridae* (Mertens *et al.*, 2000). DsRNA viruses have a very wide host range including bacteria, fungi, protozoa, plants, invertebrates and vertebrates (Table 1). In addition to their host range, the viruses of the six dsRNA virus families are distinguished by differences in the viral genome organization, virus structure and protein coding strategies and sequences.

Some of these viruses, for example human rotavirus (HRV), bluetongue virus (BTV), African horsesickness virus (AHSV) and epizootic haemorrhagic disease virus of deer virus (EHDV) are disease causing agents and are, therefore, of socio-economical importance. Others that do not cause disease are, however, attractive agents for study, since studying these viruses yields information about their structure and replication that are often applicable to their pathogenic counterparts. This study is mainly concerned with the dsRNA viruses that cause disease in

Table 1. dsRNA Viruses

Family	Genus	Number of genome segments	Hosts
<i>Reoviridae</i>	Turreted *	<i>Orthoreovirus</i>	Mammals, birds, reptiles
		<i>Aquareovirus</i>	Fish, molluscs
		<i>Cypovirus</i>	Insects
		<i>Fijivirus</i>	Plants, insects
		<i>Oryzavirus</i>	Plants, insects
	Nonturreted *	<i>Rotavirus</i>	Mammals, birds
		<i>Orbivirus</i>	Mammals, birds, arthropods
		<i>Coltivirus</i>	Mammals, arthropods
<i>Phytoreovirus</i>		Plants, insects	
<i>Birnaviridae</i>	<i>Aquabirnavirus</i>	Birds	
	<i>Avibirnavirus</i>	Fish	
	<i>Entomobobirnavirus</i>	Insects	
<i>Totiviridae</i>	<i>Totivirus</i>	Fungi	
	<i>Giardiavirus</i>	Protozoa	
	<i>Leishmaniavirus</i>	Protozoa	
<i>Partitiviridae</i>	<i>Partivirus</i>	Fungi	
	<i>Chrysovirus</i>	Fungi	
	<i>Alphacryptovirus</i>	Plants	
	<i>Betacryptovirus</i>	Plants	
<i>Hypoviridae</i>	<i>Hypovirus</i>	Fungi	
<i>Cystoviridae</i>	<i>Cystovirus</i>	Bacteria	

* The *Reoviridae* are divided into two groups - those viruses with spike extension (turrets) on the 12 vertices of the icosahedrons and those who appear smooth and rounded (unturreted)

The table is compiled from those of PPC Mertens in the ICTV book on Virus taxonomy and that of Nibert *et al* (2001) in Fields Virology (Volume 2) from the chapter on Reoviruses

animals, and of socio-economic importance in South Africa, namely AHSV, BTV and EEV as well as rotavirus that causes disease in humans. For this reason the viruses from the *Reoviridae* (*Orbiviruses* in particular) will be described in more detail.

1.3 The *Reoviridae* family

The family *Reoviridae* is the largest and most diverse group of dsRNA viruses based on their wide range of hosts (Table1). The family is currently grouped into nine genera of which the hosts mainly consists of vertebrates, invertebrates and plants. The virions within this family have icosahedral symmetry but may appear spherical in shape. The viruses have one, two or three capsid shells each composed in turn of concentric protein layers. As can be seen from Table 1, the nine genera within the family can be divided into two groups - those viruses containing "turrets" or spikes at the 12 vertices of the capsid (or core icosahedron) and those which appear smooth and spherical.

The virion molecular weight is about 1.2×10^7 with buoyant densities in CsCl of 1.36 - 1.39 g/cm³. Virions in this family are moderately resistant to heat and organic solvents. Resistance to pH and non-ionic detergents vary among the genera. The genomes of members of the *Reoviridae* family contain 10, 11 or 12 segment sets of dsRNA, each segment is packaged as a single molecule per viral particle. There are, therefore, exactly equal molar amounts of each dsRNA segment per virion. These dsRNA segments constitute 15-20% of the total dry weight of the virions. All positive strands of each dsRNA duplex have 5' terminal caps (type 1-^{7M}GpppG^(2-0m)). Viruses from some of the genera contain ssRNA oligonucleotides in intact virions.

The reovirus proteins that constitute the viral particles range in size from M_r 15 to 155×10^3 . They constitute 80 to 85 % of the total dry weight of the viruses. In each virus at least three of the internal capsid proteins are involved in mRNA synthesis and capping, namely dsRNA-dependent ssRNA polymerase, dsRNA unwinding enzyme (helicase), a nucleotide phosphohydrolase a guanylyltransferase and methyl-transferase involved in Cap 1 formation. In some cases one of the three or four proteins has more than one activity. Mature virions contain no lipids but some viruses may acquire some lipids from cell membranes during virus assembly, like rotavirus. These membranes are, however, later lost.

1.4 The *Orbivirus* genus

The genus *Orbivirus* constitutes one of the nine genera within the *Reoviridae* (Table 1). There are currently 21 virus species within the *Orbivirus* genus (Mertens *et al.*, 2003). They are distinguished based on serological cross-reactivity. The viruses within this genus are transmitted to their hosts by hematophagous arthropods (du Toit, 1944). The viruses replicate both in their vertebrate hosts as well as the arthropod hosts which serve as viral vectors. Some orbiviruses can infect humans and can cause febrile illnesses (Calisher and Mertens, 1998). The viruses that do cause disease of economical importance are those that are mainly pathogens of ruminants and horses, and include BTV, AHSV and EHDV. At the OVI two of these viruses have been studied, namely AHSV and BTV. A related virus called equine encephalosis virus (EEV) has also received some attention over the years. Bluetongue and African horsesickness are both OIE A-list diseases (Coetzer and Erasmus, 1994) highlighting the importance of studying these viruses.

1.4.1 Bluetongue virus (BTV)

Bluetongue virus is currently the best studied virus in the genus *Orbivirus* and is considered to be the prototype orbivirus. This group of viruses currently consists of 24 serotypes. The virus infects a number of wild and domestic animals. The disease, namely bluetongue (BT), is of main concern in sheep where it causes high morbidity and in some cases mortality. The virus is transmitted by biting midges of the *Culicoides* species (du Toit, 1944).

Like other members in the family *Reoviridae*, BTV are nonenveloped viruses. The virions consist of two protein shells (Owen and Munz, 1966) with a genome of 10 dsRNA segments contained in the inner shell or core (Verwoerd *et al.*, 1972). The core is composed of two structural proteins VP3 and VP7 which encloses the three minor proteins namely VP1 (RNA-dependent RNA polymerase), VP4 (guanylyltransferase and capping enzyme) and VP6 (helicase).

The structure of complete BTV particles have been studied by cryoelectron microscopy (Hewat *et al.*, 1992; Prasad *et al.*, 1992). More recently the atomic structure of the BTV core has been resolved to 3.6Å resolution (Grimes *et al.*, 1998). The BTV core has a diameter of 69 nm and has icosahedral symmetry. The outer layer of the core is made up of clusters of

VP7 trimers which are mostly arranged in 6 membered rings with 5 membered rings situated at the 5 fold vertices of the icosahedron (hence the name 'Orbivirus' derived from 'orbis' meaning ring or circle). This is a common feature within the cores of the *Orbiviridae*. The inner layer of the cores are made up of a second major protein called VP3. Cores from which the VP7 trimers are removed appear spherical and, therefore, the VP3 structure is relatively featureless.

The outer capsid of the virions are composed of two major proteins namely VP2 and VP5 (Verwoerd *et al.*, 1972). While negative staining of complete virions show the morphology of BTV to have a fuzzy appearance, cryoelectron microscopy revealed the well-ordered morphology of the virions (Roy, 2001). VP2 is present as sail-shaped trimers that almost completely cover the VP7 trimers of the BTV core. The VP2 protein is a hemagglutinating protein that contains virus neutralizing epitopes (Huismans *et al.*, 1987). VP5 proteins are also present as trimers and have a globular appearance. The VP5 globules are underlying to the VP2 proteins (Roy, 2001). Together the VP2 and VP5 proteins form a continuous layer that completely covers the core of the virion except for the fivefold axis of the virions.

The BTV genome consists of 10 dsRNA segments ranging from 0.56×10^6 to 2.7×10^6 daltons (822-3954) basepairs (Roy, 2001). The dsRNA constitutes 12 % of the total molecular mass of complete virions. The genomic dsRNAs are capped at the 5' end of the coding strand. While separation of purified viral dsRNA using agarose gel electrophoresis (AGE) shows very similar patterns within the bluetongue group, differences in segment mobility are apparent when dsRNA segments are separated by PAGE. The RNA terminal sequences of all ten dsRNA segments of BTV are conserved and contain 5' GUU and UAC 3' ends (Roy, 2001). This is similar for most other orbiviruses. The non-coding sequences at the 5' ends of BTV serotype 10 segments range from 8 to 34 bp, while those at the 3' end range from 31-116 bp. These non-translated regions may differ in length for some segments from other BTV serotypes (Mertens *et al.*, 2000).

Currently vaccines are produced for 16 of the 24 serotypes of BTV. Only 15 of these are currently sold in South Africa, namely those against serotypes 1-14 and 19. The vaccine consists of BTV attenuated by serial passage through embryonated chicken eggs followed by plaque purification and propagation on BHK cell culture.

Since outbreaks of the disease started in the Mediterranean (Mellor *et al.*, 2002 and Savini *et al.*, 2003) BTV is receiving a lot of renewed attention.

1.4.2 African horsesickness virus (AHSV)

As mentioned earlier AHSV was one of the first filterable disease causing agents described in South Africa (Theiler, 1901). The virus causes African horsesickness, a fatal disease in horses. AHSV infection causes mild clinical signs in other equids including zebra, donkeys and mules (Davies and Lund, 1974; Erasmus *et al.*, 1978; Coetzer and Erasmus 1994). The virus infects and is transmitted by biting midges of the *Culicoides* species (du Toit, 1944). Currently there are 9 serotypes of AHSV (Mcintosh, 1958; Howell, 1962).

The virus shares many of the structural features of BTV (Oellermann, 1970; Bremer, 1976; Bremer *et al.*, 1990). While the virus structure has not been studied as extensively as that of BTV some studies have been done on virus structure and assembly (Maree *et al.*, 1998). Common features shared between AHSV and BTV are their coding assignments and morphology (Bremer, 1976). While the genes and their products differ slightly from one another the same proteins are present in the cores and outer capsid of both viruses. The genome segments of AHSV range from 764-3965 bp.

Currently live attenuated vaccines for AHSV are available for serotypes 1-4 and 6-8. The disease is mostly confined to sub-Saharan Africa and only live vaccines are currently available. Therefore, discrimination between naturally infected and vaccinated horses is not possible and also animals that are vaccinated with live attenuated viruses are subject to a prescribed quarantine period before export is allowed. To be protected against disease, animals have to be immune to all nine AHSV serotypes. This highlights the importance of the development of AHSV recombinant vaccines in which the cloning of full-length cDNAs for all nine serotypes plays a very important role.

1.5 The *Rotavirus* genus

Rotaviruses are agents associated with gastroenteritis in humans and animals. The viruses cause disease mainly in young children and animals. The disease currently leads to an estimated 870 000 human deaths each year (Arias, 2002). Consequently, rotaviruses in especially humans, but also animals have been studied extensively.

Currently there are seven serogroups of rotavirus, A to G (Saif *et al.*, 1994). Rotavirus isolates from the first three groups (A to C) are predominant in humans. Groups D to G have only been found in animals to date. Within each of the serogroups, rotaviruses are classified into serotypes based on plaque neutralization assays which is a measure of the antibodies against the two major outer capsid proteins (VP4 which is protease sensitive and VP7 which is a glycoprotein). There are currently 14 such serotypes or G types (Estes *et al.*, 1997). Since antibody titres against one of the proteins (VP4) are generally low, classification of VP4 serotypes are done at sequence level which is responsible for the P types of which there are 20 different ones. Different combinations of G and P types have been found.

The viruses are triple layered and have a wheel-like appearance (Hence the name *rota* from Latin meaning wheel - Flewett *et al.*, 1997). The complete infectious virus particles are approximately 100 nm in diameter (Kapikain *et al.*, 1974) and have 60 spikes (VP4) that extend from the smooth surface of the outer shell (Prasad *et al.*, 1988 and 1994). Removal of VP4 and 7 which comprises the outer capsid leads to non-infectious double-layered particles containing proteins VP1-3 and VP6. Removal of the group-specific antigen, VP6, from these particles shows the innermost subcore particles composed of the outer layer, VP2, and the enzymes involved in transcription, VP1 and VP3 (Estes *et al.*, 1979 and 1989).

Packaged within the innermost shell (subcore) of the virus are 11 discrete segments of dsRNA. For rotavirus group A isolates they range in size from 663 to 3302 bp encoding six structural and six non-structural proteins. The dsRNAs are 5' terminally capped with no polyadenylation signal near the 3' end. The complete genome of group A rotaviruses is approximately 18 550 bp.

There are currently no vaccines for rotavirus despite the fact that many live attenuated strains are available. One such vaccine, Rotashield, licensed in 1998 was withdrawn in 1999 due to

possible association with intussusception (Ehresmann *et al.*, 1999). This highlighted the need for the careful study of viral pathogenesis which will be aided immensely if a reverse genetics system for rotavirus were to become available. The development of a rotavirus reverse genetic system will most probably depend on the availability of complete cloned genome sets of homologous rotavirus genes.

1.6 The *Cystoviridae* family

Until recently (1999) bacteriophage phi6 was the only member of the genus *Cystovirus*. Phi6 is a bacteriophage that infects phytopathogenic *Pseudomonas* species (Vidaver *et al.*, 1973). The family derives its name from the Greek word *kystis* meaning "bladder" or "sack" describing the morphology of the virus.

More recently bacteriophages related to phi6 have been isolated, phi7-phi14 (Mindich *et al.*, 1999). The isolates phi7, phi9, phi10 and phi 11 are close relatives of phi6 based on host range and cDNA identity while phi8, phi12 and phi13 are distant relatives (Mindich *et al.*, 1999). The genome sequences of phi12 are presented as part of this study.

The bacteriophage phi6 has been studied extensively and can be described as the prototype virus in the genus. Like all the other members of the family *Cystoviridae*, phi6 contains a genome of three dsRNA segments. The three dsRNA segments have sizes of 6.4 kb, 4.1 kb and 2.9 kb respectively. The sizes of the dsRNA segments of the other members of the genus is slightly different (Mindich *et al.*, 1999). The virions contain approximately 10 % RNA.

The genome of phi6 codes for 12 proteins. The proteins encoded by the L-segment (P1, P2, P4 and P7) make up the polymerase complex of the virus. The S-segments encodes the only nonstructural protein as well as the nucleocapsid proteins P5 (endopeptidase) and P8 (nucleocapsid surface protein). The M-Segment encodes 4 proteins P3, P6, P10, P13 of which P10 and P13 reside in the envelope (Bamford, 2000).

The virus is similar in many ways to the members of the family *Reoviridae*. The structure and function of the polymerase complex is similar and the polymerase particle is surrounded by two layers that are involved in host specificity (as with the reoviruses).

The bacteriophage has been subjected to considerable investigation as far as its life cycle and mechanisms of genome packaging are concerned (Mindich, 1988 and 1999; Qiao *et al.*, 1997; Gottlieb *et al.*, 1990; Olkkonen *et al.*, 1990; van Dijk *et al.*, 1995) to name but very few of the studies.

Phi6 was the first segmented dsRNA virus to be rescued by reverse genetics (Olkkonen *et al.*, 1990) and recently became the first dsRNA virus to be self-assembled from its purified protein and RNA constituents (Poranen *et al.*, 2001). These studies may prove very useful when it comes to studying the mechanisms of replication of viruses classified within the family *Reoviridae* since the architecture of their polymerase complexes are strikingly similar (Cheng *et al.*, 1994; Butcher *et al.*, 1997; Grimes *et al.*, 1998; Reinisch *et al.*, 2000).

1.7 History of cloning viral dsRNA

Efforts to clone viral RNA genes commenced soon after the discovery of the enzyme reverse transcriptase (Baltimore, 1970; Temin and Mizutani, 1970). The first report of the molecular cloning of dsRNA genes were only published more than ten years later (Cashdollar *et al.*, 1982). At the time when this study commenced, several methods for cloning viral dsRNA genes existed. These methods were generally difficult, time consuming and/or required prior sequence information as well as large amounts of highly purified starting material.

Unlike viruses with single-stranded RNA genomes, the two strands of dsRNA viruses have to be separated before reverse transcription can be performed. This has historically been done either by denaturing the double-strands with heat/DMSO or chemically with methyl-mercuryhydroxide (MMOH).

The presence of both the positive and negative strands of RNA is actually beneficial to cDNA synthesis, since there is no need for "second strand synthesis" as is the case with mRNA and RNA from ssRNA viruses. Both strands are thus synthesized at the same time. Once the cDNAs of both strands have been prepared the cDNAs can be annealed to form double-stranded cDNA that is suitable for cloning.

Reverse transcription with reverse transcriptase depends on hybridization of a DNA oligonucleotide to the 3' end of the single-stranded RNA to be transcribed. This is easily

performed with eukaryotic messenger RNA (mRNA) which contains 3' polyadenylated ends. Reverse transcription is achieved with an oligo(dT) primer which binds to the polyadenylated 3' end. However, most viral dsRNA does not contain polyadenylated 3' ends. Therefore, before reverse transcription can be achieved specific sequences have to be added to the 3' ends of the dsRNA before or after strand separation. This is also the case with the genomes of single stranded RNA viruses that do not contain polyadenylated ends. Methods for cloning dsRNA employed two ways of adding sequence to the 3' ends of dsRNA. The one way is polyadenylation and the other oligo-ligation.

1.7.1 Polyadenylation mediated methods

The first methods describing the cloning of viral dsRNA using polyadenylation was that of Cashdollar *et al.* (1982) - See Figure 1.1. They described cloning of the S2 gene of Reovirus serotype 3 and later the whole genome of the same virus (Cashdollar *et al.*, 1984). The method was based on the fact that purified *Escherichia coli* poly (A) polymerase could be used to polyadenylate single-stranded RNA. cDNA was cloned as follows: Reovirus dsRNA (150 µg) was denatured and polyadenylated. After purification of polyadenylated dsRNA by oligo(dT)-cellulose chromatography, cDNA was prepared using oligo(dT) priming and MuMLV reverse transcriptase. RNA templates were then removed by incubation with 0.5 M KOH. Resulting cDNAs (containing transcripts from both polarities of cDNA) were subsequently annealed under high salt concentration followed by filling in of the overhanging cDNA ends using *E. coli* DNA polymerase I. The resulting double-stranded cDNAs were oligo(dC) tailed and cloned into PstI digested oligo(dG) tailed plasmid pBR322. This method, although complicated and requiring large amounts of dsRNA permitted the cloning of the whole genome of reovirus serotype 3 as full-length cDNA copies of each of the 10 dsRNA segments.

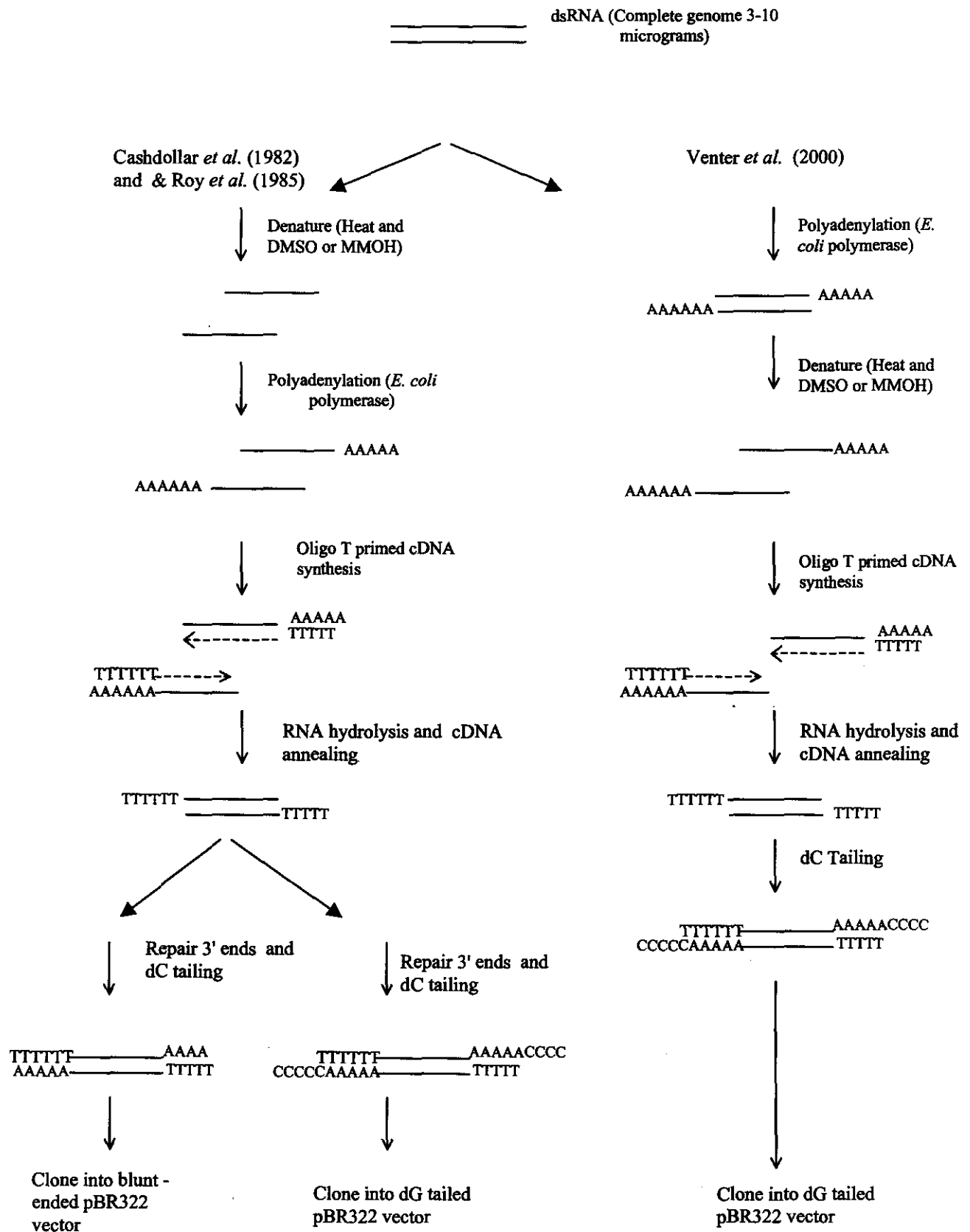


Figure 1.1 Polyadenylation mediated dsRNA cloning methods as described by Cashdollar *et al.* (1985), Roy *et al.* (1985) and Venter *et al.* (2000)

Later Roy *et al.* (1985) described cloning of the BTV VP3 gene using the same method with some minor modifications (Figure 1.1). Firstly, individual dsRNA species were isolated from the dsRNA genome separated by agarose gel electrophoresis. The cDNAs produced in the same way as that of Cashdollar were cloned as blunt ended products into plasmid pBR322, that were Hind III digested and blunt ended. This method ensured that no oligo(dC) tails were present in the cDNA clones. The oligo(dC) tails hampered sequencing reactions and in many cases cDNAs had to be "lifted" from plasmid cloned cDNA by PCR with primers complementary to their true 5' and 3' ends. The method employed by Roy *et al.* (1985) thus also facilitated subcloning of cDNA into other vectors for expression purposes. The clones did, however, still contain long oligo(T) tails at either end.

Venter *et al.* (2000) cloned the VP2 gene of AHSV serotype 9 using the same method as Cashdollar *et al.* (1982) with some minor differences. To obtain pools of predominantly large, medium and small dsRNAs, large amounts (10 µg) of dsRNA were separated by centrifugation on 5-40% sucrose gradients. Gradient fractions of the different size pools of dsRNAs were then polyadenylated using dsRNA as template and not ssRNA as was done with other methods (Figure 1.1). They showed an improvement of cDNA synthesis using this method. It was, however, noted that dsRNA preparations had to be of very high quality for this procedure to be successful.

The cloning methods based on polyadenylation had the following aspects in common: Very large amounts of highly purified dsRNAs were needed for the procedures to be successful. In most cases dsRNAs of the larger segments had to be pooled either by purification from agarose gels or dsRNAs separated by sucrose gradient centrifugation. A drawback was that plasmid cloned cDNAs contained both poly(dA) tails and /or oligo(dC) tails at either end of the cloned cDNA fragments. These hampered sequencing, expression as well as subcloning efforts.

1.7.2 Oligo-ligation mediated methods

Oligo-ligation mediated methods for cloning dsRNA were first employed by Imai *et al.* (1983). The method was based on the fact that T4 RNA ligase could catalyze the addition of an oligo (C)₁₅ to the 3' end of dsRNA (Figure 1.2). Complementary DNAs were prepared by incubation of denatured oligo(C)-tailed RNA with reverse transcriptase and oligo(dG)₁₀. RNA templates were removed by incubation with 0.3 M NaOH followed by cDNA annealing at 68°C. Partial cDNA duplexes were filled in with reverse transcriptase. Resulting full-length cDNAs were dC-tailed and cloned into dG-tailed pBR322 as described by Cashdollar *et al.* (1982). This method allowed the cloning of several genes from human reovirus and human rotavirus including the full-length gene of segment 11 of human rotavirus.

A substantial improvement of the oligo-ligation mediated technique was attained when PCR was used in the technique to amplify cDNA. Lambden *et al.* (1992) used T4 RNA-ligase mediated ligation of an oligo-nucleotide with specific nucleotide sequence to the dsRNA of rotavirus isolates (Figure 1.2). Oligomerization of the primer by T4 RNA ligase was prevented by introduction of an amino group to the 3' end of the primer. cDNA of the whole rotavirus genome was produced from denatured RNA, reverse transcriptase and a primer complementary to the one used for oligo-ligation. After RNA hydrolysis, cDNA annealing and filling of partial cDNA duplexes, the cDNA could be amplified using the same primer as was used for cDNA synthesis. Although this method required a 1000 times less (20 nanograms) dsRNA compared to other techniques, only cDNA smaller than 2.5 kb could be efficiently amplified by PCR. The method did, however, prove to be very useful for cloning of dsRNA genes of rotavirus up to 2.5 kb. Also, dsRNA genes could be cloned from dsRNA extracted directly from stool samples of rotavirus infected patients.

Further improvements of this technique for sequence determination of larger dsRNA segments were introduced by Bigot *et al.* (1995). Partial cDNA clones of the larger segments were sequenced and primers complementary to the sequences as well as primers used for amplification of oligo-ligated RNA were used to amplify the remainder of the large dsRNA genes.

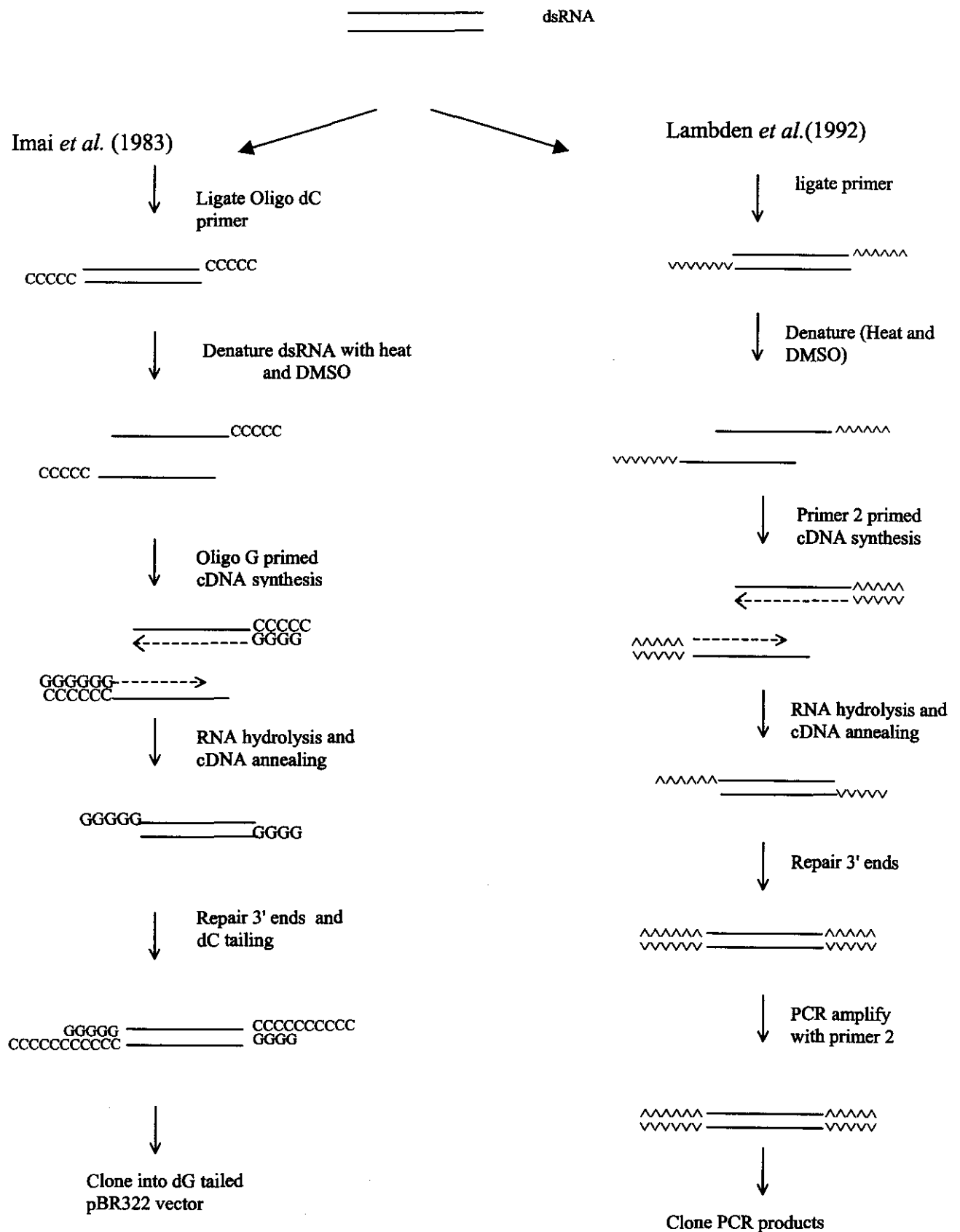


Figure 1.2 Oligo-ligation mediated dsRNA cloning methods of Imai *et al.* (1983) and Lambden *et al.* (1992)

This allowed Bigot *et al.* (1995) to amplify the complete genome of *Diadromus pulchellus* reovirus (DpRV) with four segments ranging from 0.98 kb to 4.23 kb. The large dsRNA segment (4.23 kb) could, however, not be cloned as a full-length cDNA segment but was sequenced from overlapping cDNA clones.

Similar strategies were followed by Attoui *et al.* (2000a) for sequencing several dsRNA genomes (Figures 1.2 and 1.3). While genes of up to 2.5 kb could be cloned efficiently using Lambden's protocol, large dsRNA genes were amplified by introduction of the SMART™ technology to the oligo-ligation method. SMART™ technology (formerly known as cap-finder - Clontech) is based on the fact that MuMLV reverse transcriptase acts as a deoxycytidine-specific terminal transferase. When the RT-enzyme reaches the capped 5' end of the template RNA, the enzyme's terminal transferase activity adds a few deoxycytidine (poly C) residues to the 3' end of the cDNA. In the presence of a DNA oligo with a poly-(dG) tail the hybridization of the primer to the poly (dC) at the 3' end of the cDNA provokes template switching that is dependent on the 7-methylguanosine cap structure of the RNA (see Figure 1.3). This technique permits direct sequencing of the resulting amplicon since dissimilar primers are used for PCR and only the leading strand of the dsRNA is 5' capped. Also full-length amplicons of large genes could be cloned and sequenced. It is, however, necessary for the dsRNAs to be separated and purified prior to this procedure. Also, at least 20 nanograms of purified dsRNA per segment is needed for this procedure. A drawback of this method is that only dsRNA that are 5' capped can be amplified using this method.

Vreede *et al.* (1998) managed to amplify and clone the large dsRNA genes (3-4 kb) of AHSV using another modification of the Lambden technique (Figure 1.4). The oligonucleotides used for ligation to the 3' ends of dsRNA were poly(dA) tailed, followed by oligo(dT) reverse transcription of denatured ligated dsRNA. Sucrose gradient ultracentrifugation of dsRNA was employed to pool the large dsRNA segments. Excess RNA was hydrolyzed after reverse transcription by electrophoresis on alkaline agarose gels. cDNAs were purified and concentrated from the alkaline agarose gels, annealed, the partial duplexes were filled in and PCR amplification of the cDNAs were performed using a Taq polymerase with the ability to amplify large DNA targets (Dynazyme). These improvements to Lambden's method made it possible to clone the largest AHSV dsRNA segment, namely the 4.0 kb AHSV1 VP1-gene.

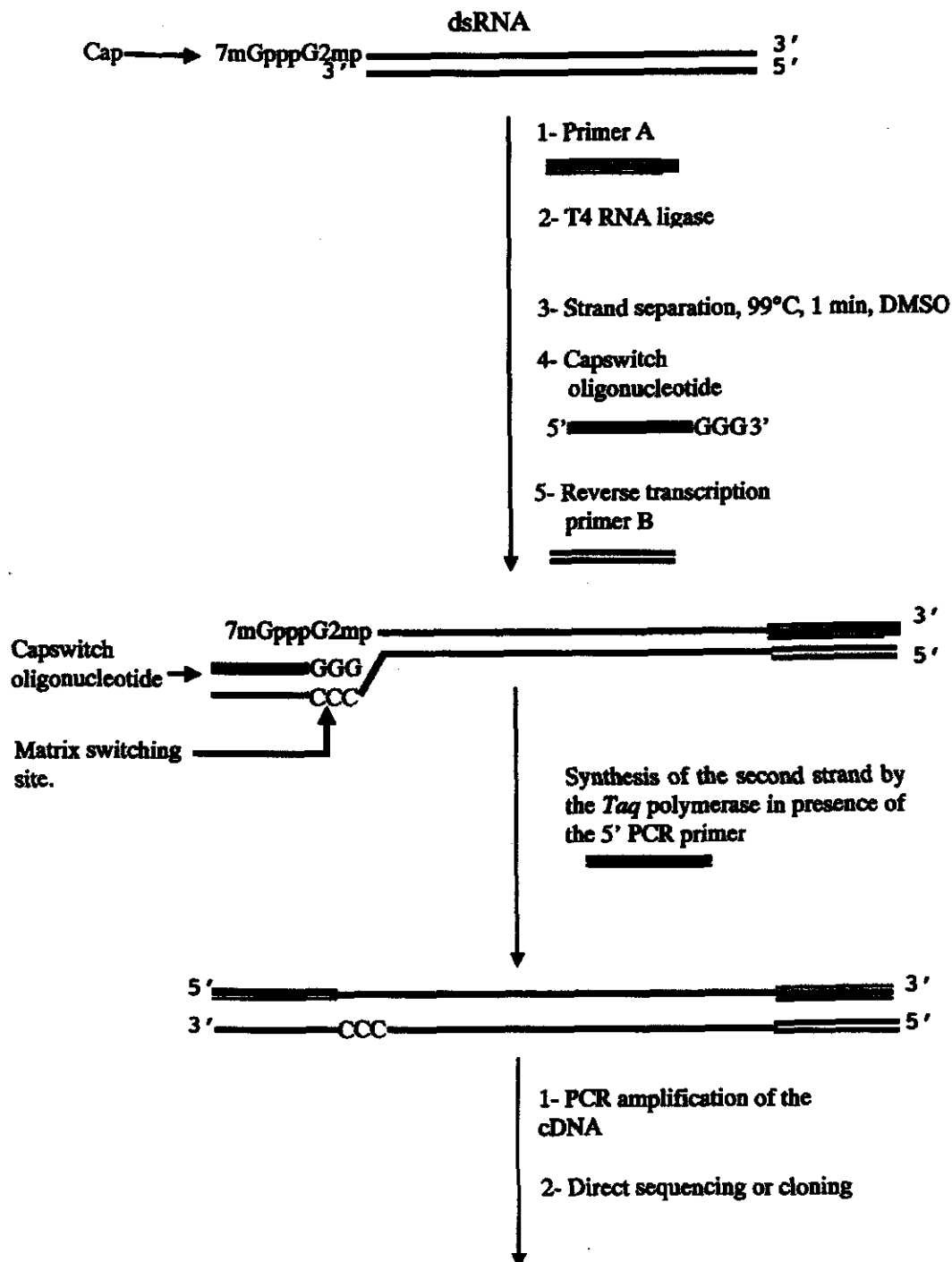


Figure 1.3 Schematic representation of the SMART™ technique used for dsRNA segments.
(with permission from Houssam Attoui)

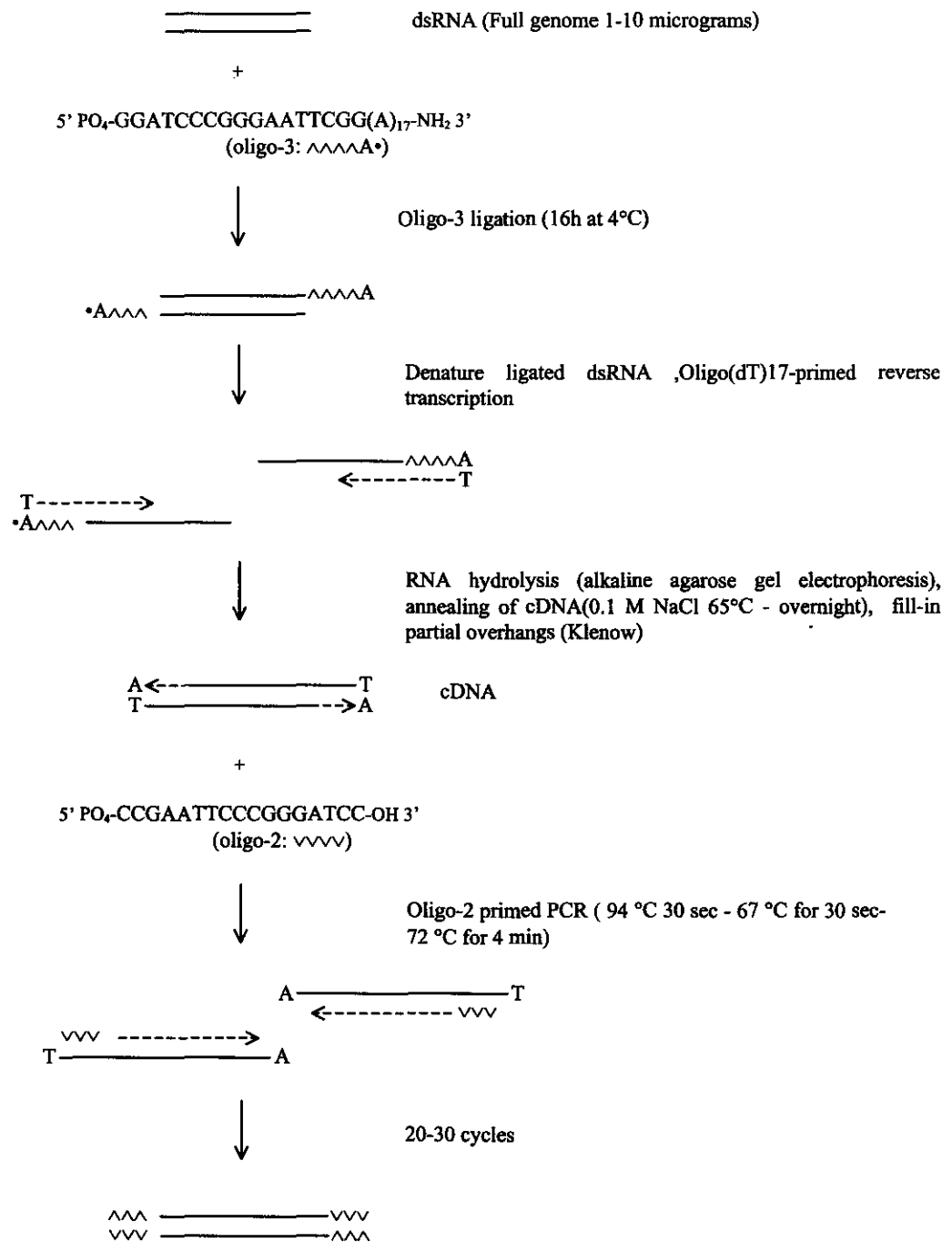


Figure 1.4 The oligo-ligation mediated method of Vreede *et al.* (1998) - with permission from Frank Vreede

The major advantage of this method over that of Lambden *et al.* (1992) is that it only allows PCR amplification of full-length cDNA since different primers are used for cDNA synthesis and PCR amplification.

1.7.3 Sequence specific procedures

The procedures described above are all sequence-independent procedures where no sequence is needed prior to cDNA synthesis. While it is possible to sequence amplified dsRNA segments directly from cDNA templates (Drs P. P. C. Mertens and S. Rao - personal communication) these procedures do not yield cloned full-length cDNAs that can be utilised for expression purposes. However, once the sequence of a specific gene or dsRNA segment has been determined, it is possible to prepare cDNA from dsRNA with exactly the same or even just similar nucleotide sequence. This is done by simply designing primers on either ends of the region that the researcher wants to clone or amplify. When the dsRNA is denatured these primers will bind to their complementary sequence and cDNA is made from the dsRNA template using a reverse transcriptase. These regions, whether it be complete dsRNA segments or specific regions within dsRNA segments, can be amplified by PCR using specific sets of primers. In cases where copious amounts of dsRNA are available the cDNA can be cloned directly after annealing of the two strands of cDNA. These procedures are, however, dependent on prior sequence information that can only be attained from sequencing amplified cDNA directly, or cloning cDNA from RNA with sequence-independent procedures and sequencing cDNA clones.

1.8 AIMS

The advent of recombinant DNA technology offered researchers in the field of virology vast new opportunities in basic research as well as the prospect of developing novel applications in control, prevention and monitoring of viral diseases. These studies are, however, largely dependent on the ability to clone, sequence and express genes from the genomes of viruses. The field of dsRNA viruses has lagged behind for the simple reason that procedures have been greatly hampered by the technical difficulties of the original methods described for cloning and sequencing of dsRNA viral genomes. This is especially true for the larger dsRNA segments (>2.5 kb) which are present in most dsRNA genomes. These large dsRNA

genes generally encode viral proteins of great importance for vaccine development and immunological, epidemiological and viral replication studies. Cloning these genes using traditional methods requires large amounts of viral dsRNA and time consuming cloning procedures. Therefore, there existed a definite need for an efficient, fast, robust and user friendly cloning procedure, with high fidelity for large dsRNA genes.

For this study, the sequence-independent dsRNA cloning methods of Lambden *et al.* (1992) and Vreede *et al.* (1998) based on oligo-ligation was chosen as a starting point for the development of a procedure to clone complete viral dsRNA genome sets as full-length PCR amplicons. The rationale for choosing to further develop the ligation-mediated method was threefold: Firstly, the method had been shown previously to allow cloning of large dsRNA segments as full-length PCR amplicons (Vreede *et al.*, 1998). Secondly, the incorporation of PCR in the method, makes it extremely sensitive for small amounts of starting material. Finally, the cloned cDNA does not have any homopolymer oligo(dT) or oligo (dC) tails, but the synthesized cDNAs are flanked by sequences of choice built into the primers used for ligation and PCR amplification. This facilitates subcloning into expression vectors.

The following aims were identified for this study:

1. The primary objective was to develop a procedure for efficient sequence-independent amplification and cloning of dsRNA genes larger than 3 kb. The approach defined to attain this goal was based on the sequence-independent dsRNA cloning method of Lambden *et al.* (1982) and Vreede *et al.* (1998). The following features were envisaged for the method:

The method should

- be appropriate for cloning viral dsRNA from most sources.
 - at least cover the size range of dsRNA genes of the family *Reoviridae* (0.8 – 4.5 kb).
 - yield clonable amounts of full-length cDNA.
 - be efficient, robust, repeatable and relatively simple so as to allow any researcher to apply it to their own field of interest.
2. The second aim of this study was to demonstrate how such a new method will open up a major bottleneck in dsRNA virology by means of describing a variety of applications

using the cloned cDNAs. It should be shown that the cloned cDNA obtained using the method can be utilised for the following:

- sequence determination of complete dsRNA genomes
- phylogenetic analysis and molecular epidemiology
- expression of viral proteins from their corresponding cDNA
- development of recombinant vaccines
- development of molecular detection and diagnostic procedures based on expressed proteins and cDNA sequences
- advancing basic molecular research on dsRNA viruses.

CHAPTER 2

Paper 1

Potgieter A.C., Steele A. D. & van Dijk A.A., 2002. Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes. *Journal of General Virology* 83 (9), 2215-2223

Instructions to the authors for this journal may be found at the following website

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Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes

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Cloning full-length large (> 3 kb) dsRNA genome segments from small amounts of dsRNA has thus far remained problematic. Here, a single-primer amplification sequence-independent dsRNA cloning procedure was perfected for large genes and tailored for routine use to clone complete genome sets or individual genes. Nine complete viral genome sets were amplified by PCR, namely those of two human rotaviruses, two African horsesickness viruses (AHSV), two equine encephalosis viruses (EEV), one bluetongue virus (BTV), one reovirus and bacteriophage Φ 12. Of these amplified genomes, six complete genome sets were cloned for viruses with genes ranging in size from 0.8 to 6.8 kb. Rotavirus dsRNA was extracted directly from stool samples. Co-expressed EEV VP3 and VP7 assembled into core-like particles that have typical orbivirus capsomeres. This work presents the first EEV sequence data and establishes that EEV genes have the same conserved termini (5' GUU and UAC 3') and coding assignment as AHSV and BTV. To clone complete genome sets, one-tube reactions were developed for oligo-ligation, cDNA synthesis and PCR amplification. The method is simple and efficient compared to other methods. Complete genomes can be cloned from as little as 1 ng dsRNA and a considerably reduced number of PCR cycles (22–30 cycles compared to 30–35 of other methods). This progress with cloning large dsRNA genes is important for recombinant vaccine development and determination of the role of terminal sequences for replication and gene expression.

Introduction

There are six virus families with dsRNA genomes, namely *Birnaviridae*, *Cystoviridae*, *Hypoviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae*. Several viruses of the family *Reoviridae* are aetiological agents for disease in humans and animals. Advances in recombinant DNA technology continue to raise expectations of generating a range of new vaccine candidates to combat infectious diseases. For diseases caused by viruses of the family *Reoviridae*, the key to tapping into this powerful technology is the ability to clone full-length dsRNA genes. In the case of the *Rotavirinae* and *Orbivirinae*, the main focus of our research, this requires cloning of dsRNA genes that are classified as large, namely genes of 3–4 kb (Sabara *et al.*, 1991; Crawford *et al.*, 1994; Brussow *et al.*, 1990; McNeal *et al.*, 1992; Madore *et al.*, 1999; Roy *et al.*, 1996, 1990; Martinez-

Torrecedrada *et al.*, 1996; Stone-Marschat *et al.*, 1996; du Plessis *et al.*, 1998; Scanlen *et al.*, 2002). Although dsRNA cloning has progressed steadily, routine cloning of large dsRNA genes remains problematic, especially where dsRNA template is limited, e.g. in cases where viruses have not been cultured, or where no sequence information is available.

Difficulties and limitations of existing dsRNA cloning methods are as follows: the first dsRNA cloning methods based on polyadenylation of genomic dsRNA, oligo(dT)-primed reverse transcription, followed by blunt-ended cloning or dC-tailing and cloning into dG-tailed pBR322 (Cashdollar *et al.*, 1982, 1984) were generally technically complicated, the various steps were very inefficient and required relatively large amounts (> 1 μ g) of dsRNA starting material. The homopolymeric tails of cloned genes presented difficulties for subsequent sequencing and expression. The second generation cloning procedures used PCR amplification of cDNA, which made it possible to clone from much smaller amounts of starting material. Initially, PCR-based procedures depended on the

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Table 2. Characteristics of dsRNA segment 2 and VP2 of the reference strain of each of the nine AHSV serotypes

AHSV serotype	Segment length (bp)	Open reading frame (nt)	Size of protein (aa)	Predicted protein molecular mass (Da)	Terminal-end sequences
AHSV1	3218	13-3183	1056	122 966	5'-GUUUUUUUCAGCauggcguc..UCCAUGCUGAUCACCCUUAC-3'
AHSV2	3221	13-3186	1057	122 962	5'-GUUUUUUUCAGCauggcguc..UCUAUGCUGAAUACACAUAC-3'
AHSV3*	3221	13-3186	1057	122 905	5'-GUUUAAUUCACCauggcuuc..CUCCAUGUGAAUCAACUUAC-3'
AHSV4*	3229	13-3195	1060	123 898	5'-GUUUAAUUCACUauggcguc..CUCCAUGUGAAUACACAUAC-3'
AHSV5	3217	13-3186	1057	122 731	5'-GUUUUUUUCAUauggcuuc..CUCCAGAUGAAUACACUUAC-3'
AHSV6*	3203	13-3168	1051	122 169	5'-GUUUAAUUCACCauggcuuc..UCAUGGUGAUUCAACUUACC-3'
AHSV7	3222	13-3186	1057	123 497	5'-GUUUAAUUCACUauggcuuc..UCCAAGUGAAUCAGCCGUAC-3'
AHSV8	3220	13-3189	1058	123 077	5'-GUUUAAUUCAUauggcguc..CUCAUGAUGAAUACACUUAC-3'
AHSV9*	3205	13-3174	1053	123 414	5'-GUUUAAUUCACCauggcguu..CUCCAUGUGAAUACACUUAC-3'

*AHSV serotypes that have been cloned and sequenced previously.

black). Within two of these regions, there was very low identity between the amino acid sequences of the different serotypes. Furthermore, we established that the amino acid sequences of serotypes that show serological cross-neutralization also shared regions of high identity that are not present in other serotypes. Some of these regions are present within the regions with low identity. Phylogenetic and homology trees compiled from multiple alignments of the VP2 amino acid sequences clearly showed that the VP2 sequences of the serotypes that show serological cross-reaction have higher identity with each other and group together (Fig. 4). Phylogenetic analysis including the VP2 amino acid sequences of three other orbiviruses, bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV) and Chuzan virus, showed that, based on the VP2 sequences, AHSV and Chuzan virus were more closely related than AHSV, BTV and EHDV. Hydrophobic and hydrophilic analysis of the protein sequences encoded by the nine VP2 genes indicated that the proteins had very similar hydrophobicity profiles, even in regions where there was very

little amino acid sequence identity. Fig. 5 shows the hydrophobicity profiles of the 220-450 amino acid regions of all nine AHSV VP2s, which have little amino acid identity between serotypes and where antigenic sites have been identified.

DISCUSSION

In this paper we have described the cloning of full-length VP2 genes of the reference strain of each of the nine AHSV serotypes. Baculovirus recombinants expressing the cloned VP2 genes of six serotypes, namely serotypes 1, 2, 4, 6, 7 and 8, confirmed that they all had full open reading frames. The cloned VP2 genes of serotypes 1, 2, 5, 7 and 8 were sequenced and their amino acid sequences were deduced. The data presented here completes the cloning, sequencing and expression of the first representative of each of the nine AHSV outer capsid VP2 genes. Our sequencing data, together with that of published data for the VP2 genes of serotypes 3, 4, 6 and 9 (Iwata *et al.*, 1992; Sakamoto *et al.*, 1994; Vreede *et al.*, 1994; Williams *et al.*, 1998; Venter *et al.*, 2000), has allowed us to perform the first complete sequence analysis of all the (sero)types for a species of the *Orbivirus* genus.

Phylogenetic analysis of the nine AHSV VP2s grouped together VP2s of serotypes that show serological cross-reaction. Phylogenetic analysis, which included the VP2 amino acid sequences of some other orbiviruses (BTV, EHDV and Chuzan virus), showed very low homology between AHSV VP2 amino acid sequences and the VP2 sequences of these orbiviruses. Chuzan virus was, however, more closely related to AHSV than BTV and EHDV based on the VP2 amino acid sequences (Fig. 4B). Low identity between serotypes was demonstrated for specific regions within the VP2 amino acid sequences that have been shown to be antigenic and play a role in virus neutralization

Table 3. Homology matrix for the amino acid sequences of the full-length VP2 proteins of the nine AHSV reference serotypes

The highest homology (AHSV serotypes 1 and 2) and the lowest homology (AHSV serotypes 2 and 9) are shown in bold.

AHSV1	100								
AHSV2	71.4	100							
AHSV3	51.6	51.7	100						
AHSV4	51.1	52.1	50.2	100					
AHSV5	50.1	51.0	49.7	53.0	100				
AHSV6	49.5	48.0	48.0	50.5	51.8	100			
AHSV7	53.5	53.7	64.8	50.0	50.2	50.3	100		
AHSV8	48.9	49.2	48.8	51.0	69.3	50.3	49.8	100	
AHSV9	48.8	47.6	48.6	51.2	51.5	60.5	51.0	51.8	100

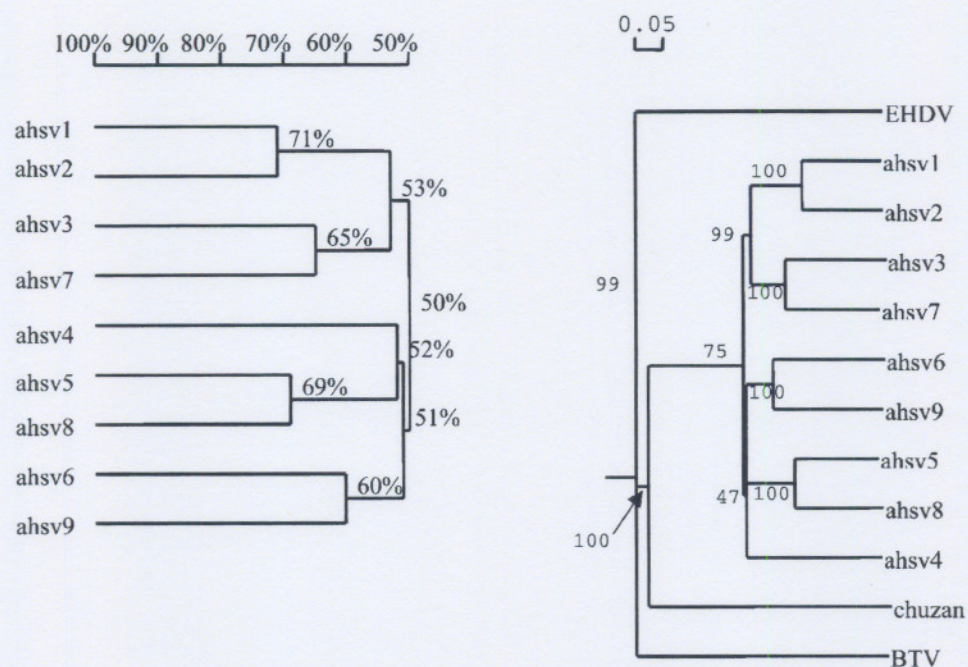


Fig. 4. Homology and phylogenetic trees compiled from the multiple amino acid alignment of the VP2 genes of all nine serotypes of AHSV. (A) Homology tree. The percentage homology is indicated. (B) Phylogenetic tree including the VP2 amino acid sequences of BTV, Chuzan and EHDV. Bootstrap values are indicated.

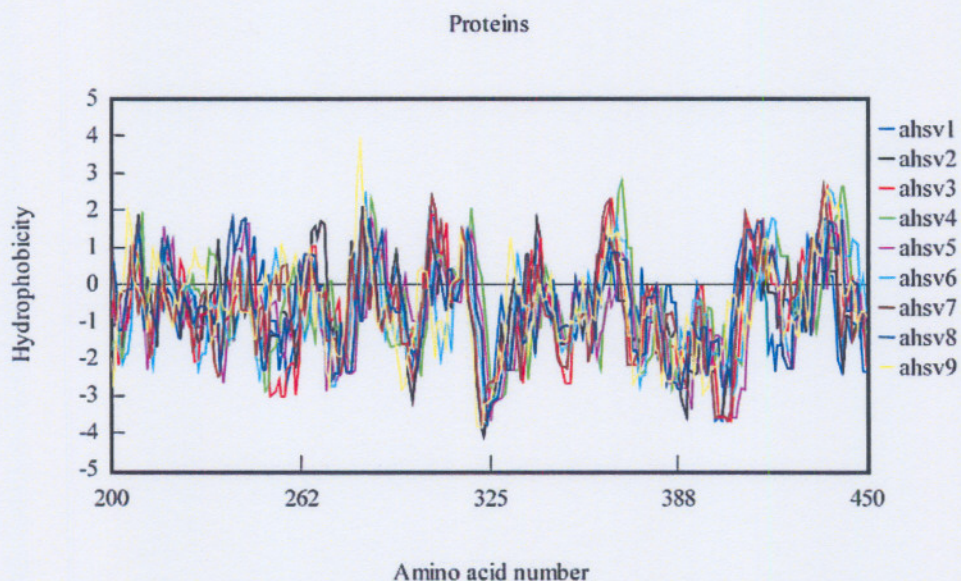


Fig. 5. Hydrophobicity profiles of the 220–450 amino acid region of all nine AHSV VP2s that has little amino acid identity between serotypes and where antigenic sites have been identified. The hydrophobic regions are shown above zero and the hydrophilic regions below zero.

VP2s between aa 252 and 488. This region not only shows significant low identity between the nine AHSV serotypes but is also mostly hydrophilic, suggesting that these sites

could be located on the surface of the virion. Furthermore, within these antigenic regions there is higher identity between the VP2 amino acid sequences of serotypes that

show serological cross-reaction. In general, there is also more homology between the VP2s of the serotypes that show serological cross-reaction (Fig. 4A). This may explain the serological cross-reaction between serotypes, since the VP2 protein determines serotype. However, it should be noted that the abovementioned studies were performed with linear peptides and not with full-length VP2 in its natural conformation. Bentley *et al.* (2000) also found a non-continuous epitope on AHSV-3 VP2 using a random peptide library. Since BTV VP2 contains a serotype-specific antigenic region at approximately the same amino acid residues, namely aa 328–335 (Gould & Eaton, 1990) and aa 327–402 (Demaula *et al.*, 1993), it seems possible that antigenic determinants in different orbivirus VP2s might be located in approximately the same region. The use of recombinant peptides from these regions from each serotype for diagnostic applications could, therefore, be an informative area for further investigation.

The importance of the availability of a complete set of full-length AHSV VP2 cDNA clones for each of the nine serotypes for recombinant vaccine development is underscored by the fact that thus far full protection against disease has only been achieved with full-length, soluble baculovirus-expressed AHSV VP2, notably for serotypes 4 and 5 (Roy *et al.*, 1996; Martinez-Torrecuadrada *et al.*, 1996; Du Plessis *et al.*, 1998; Scanlen *et al.*, 2002). We are now developing recombinant VP2-based vaccines for all nine serotypes of AHSV using our set of full-length cDNA copies of the VP2 genes described in this paper.

The value of the diagnostic and epidemiological applications of this first full set of AHSV VP2 clones and sequence data includes the possibility of speeding up and extending procedures for serotyping and topotyping of isolates, serum samples and midge collections. It comprises the development of molecular methods and reagents for serotyping, such as serotype-specific probes and RT-PCR procedures, as well as generating phylogenetic data sets for molecular epidemiology. In fact, the VP2 gene set generated in this study has already enabled us to demonstrate proof of concept for the development of serotype-specific probes (Koekemoer *et al.*, 2000). Using an incomplete VP2 sequence data set, Sailleau *et al.* (2000) described the development of a serotype-specific RT-PCR for AHSV. Their method was based on small regions of sequence within the VP2 nucleotide sequences and required eight separate PCR reactions to be performed to determine the serotype of one sample. We envisage that multiple alignment of the full-length nucleic acid sequences of all nine AHSV VP2 genes will enable us to develop a set of primers to amplify a specific region within the VP2 gene of all nine serotypes of AHSV by RT-PCR in a one-step single reaction and generate sequence data sets for the VP2 genes of field strains for phylogenetic and topotyping analyses.

The data described in this paper are the first for a full set of VP2 genes of any orbivirus. The completion of expression, sequencing and phylogenetic analysis of this set of AHSV

VP2 genes sets the scene for the development of complete repertoires of new vaccines, the identification and characterization of antigenic regions and the development of molecular diagnostic and epidemiological tools to improve the prevention, control, diagnosis and surveillance of AHS.

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CHAPTER 4

Paper 3

JJO Koekemoer., Potgieter AC., JT Paweska and AA van Dijk. (2000). Development of probes for typing African horsesickness virus isolates using a complete set of cloned VP2-genes. *J Virol Methods* **88**, 135-144

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Development of probes for typing African horsesickness virus isolates using a complete set of cloned VP2-genes

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Abstract

A set of cloned full-length VP2-genes from the reference strain of each of the nine serotypes of African horsesickness virus (AHSV) was used to develop probes for typing AHSV isolates. The VP2-gene probes hybridised serotype-specific to purified viral dsRNA from its corresponding serotype. No cross-hybridisation was observed between the different AHSV serotypes or with RNA from equine encephalosis virus or bluetongue virus (BTV) which are related viruses within the genus *Orbivirus* that co-circulate with AHSV in South Africa. The probes were able to detect AHSV isolates from recent field cases of AHS in South Africa, despite being derived from historical reference strains. With regard to sensitivity and time considerations: radioactive ^{32}P -labelling resulted in a marginal increase in sensitivity over digoxigenin-labelled probes. By infecting cell cultures at different multiplicities of infection (m.o.i.) and harvesting at various times post infection, it was established that AHSV RNA could be detected 16 h post infection (p.i.) at a m.o.i. of 1.00 pfu per cell and 48 h p.i. at a m.o.i. of 0.01 pfu per cell. Typing of AHSV isolates by means of VP2-gene probe hybridisation can be completed in 4 days, which is less than half the time required for conventional isolation and serotyping. This report on the use of a complete set of cloned AHSV VP2-gene probes is the first demonstration of typing for a whole specie (serogroup) in a genus of the family *Reoviridae*. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: dsRNA viruses; African horsesickness virus; AHSV serotype-specific probes; AHSV typing

1. Introduction

African horsesickness (AHS) is an arthropod-borne disease of equines that results in a non-contagious infection which has a mortality rate of more than 90% in susceptible horses. It is an

Office International des Epizooties (OIE) list A disease that is endemic in most of sub-Saharan Africa. The disease occurs sporadically in north Africa, Mediterranean countries and the Middle East. The aetiological agent, African horsesickness virus (AHSV) belongs to the genus *Orbivirus* in the family *Reoviridae* (Murphy et al., 1995). There are nine serotypes of AHSV (McIntosh, 1958; Howell, 1962). Since protection to AHS is serotype-specific, horses need to be immune to all

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the nine serotypes in order to be fully protected against disease (Van Dijk, 1999). Outbreaks of AHS outside enzootic regions have major zoonotic and economic repercussions (Powell, 1985).

For many viruses of medical and veterinary importance, the era of molecular diagnostics is already well established. For viruses for which this technology is not yet in place, molecular methods are increasingly being developed and used for detection, identification and characterisation. Serotyping of AHSVs is important for the rapid implementation of effective vaccination strategies, particularly when outbreaks occur outside enzootic regions, as well as, for proper diagnosis and surveillance of the disease. When the live attenuated polyvalent vaccine produced by Onderstepoort Biological Products (OBP) is to be used in an outbreak, it is important to administer the vaccine that contains the relevant serotype first, since the two AHS vaccine doses have to be administered at least 3 weeks apart (the OBP live attenuated AHS vaccine groups the seven AHSV vaccine strains in two separate bottles, one contains serotypes 1, 3, and 4 and the other serotypes 2, 6, 7 and 8). In specific cases where authorities prefer to administer a monovalent vaccine, such as during the 1987 AHS serotype 4 outbreak in Spain, vaccination can only start once serotyping of isolates is completed. Currently, laboratory confirmation and serotyping of an AHS diagnosis takes about 14 days, since it requires viral isolation and typing. The only methods for serotyping AHSVs to date remains virus neutralisation tests (Howell, 1962; Hopkins et al., 1966; Huismans and Erasmus, 1981).

African horsesickness virions consist of a double-layered capsid comprised of seven structural proteins, VP1–VP7, enclosing a dsRNA genome of ten segments. The outer layer of the capsid is made up of two structural proteins, VP2 and VP5, that surround the inner core particle composed of viral proteins VP1, VP3, VP4, VP6 and VP7 (Bremer, 1976; Van Dijk and Huismans, 1982). The first correlation between the serotype of an orbivirus and the genome date back to cross-hybridisation analyses with bluetongue virus (BTV), the prototype orbivirus, which established that genome segment 2 was the major genome segment

that was serotype-specific on hybridisation (Huismans and Howell, 1973). Reassortment studies indicated that the other outer capsid protein, VP5, is also involved in the determination of virus serotype, possibly by imposing conformational constraints on VP2 (Cowley and Gorman, 1989; Mertens et al., 1989). Cross-hybridisation was subsequently used in the characterisation of an Australian BTV isolate (Huismans and Bremer, 1981). An investigation of the antigens involved in serological variation between BTVs demonstrated that BTV VP2 immune precipitated only with the sera obtained from animals infected with homologous serotypes, thus indicating BTV VP2 as the main determinant of serotype-specificity (Huismans and Erasmus, 1981). Similarly, for AHSV, neutralising epitopes were identified on VP2 (Burrage et al., 1993). Subsequently, BTV genome segment 2 was shown to encode VP2 (Van Dijk and Huismans, 1988). Molecular investigations confirmed and extended the data. Gene-assignment studies of cloned fragments from shotgun cloning of BTV dsRNA segments (Huismans and Cloete, 1987) and AHSV3 (Bremer et al., 1990) revealed that VP2-genes hybridised serotype-specific under high stringency conditions. Analyses of nucleic acid sequence data from cloned genes revealed that the VP2-genes of *Orbiviruses* are the most variable genes between serogroups, as well as between serotypes of a serogroup (Iwata et al., 1992; De Mattos et al., 1994a). Investigations on sequence variation between the VP2 of AHSV serotypes are still in their infancy. Thus far, the VP2 genes of only four isolates have been sequenced, namely, one each of AHSV serotypes 3, 4, 6 and 9 which revealed that they have 49% amino acid identity and 67% similarity (Williams et al., 1998).

Group-specific genomic probes of conserved viral genome segments have been developed for several orbivirus serogroups including AHSVs (Bremer et al., 1990), BTVs (Roy et al., 1985; Huismans and Cloete, 1987), equine encephalosis viruses (Viljoen and Huismans, 1989) and epizootic haemorrhagic disease viruses, EHDVs, (Nel and Huismans, 1990). In addition, new serogroup-specific tests such as a capture ELISA (Hamblin et al., 1991) and several RT-PCRs (Stone-Mar-

chat et al., 1994; Zientara et al., 1994, 1995a; Bremer and Viljoen, 1998) have been developed for the AHSV serogroup.

Initial investigations into developing molecular typing methods for at least three *Orbivirus* genera have yielded promising results. In the case of BTV it has been found that VP2 sequences of the BTV isolates are characteristic for each serotype and can assist in typing (Wilson and Chase, 1993). Nucleotide sequence analyses grouped BTV serotypes into nucleotypes in broad agreement

Table 1
AHSV reference strains

Serotype	Year of isolation
AHSV 1	1962
AHSV 2	1961
AHSV 3	1963
AHSV 4	1962
AHSV 5	1962
AHSV 6	1963
AHSV 7	1962
AHSV 8	1962
AHSV 9	1961

Table 2
1997–1998 field isolates of AHS cases in South Africa

Isolate	Serotype	Passage history	Source	Year
M322	AHSV3	1 MB ^a , 2 Vero ^b	Equine spleen	1997
HS39	AHSV4	2 MB, 1 Vero	Equine blood	1997
HS6	AHSV6	2 Vero	Equine lung	1998
HS7	AHSV8	1 MB, 1 Vero	Equine blood	1998
HS14	AHSV3	1 MB, 2 Vero	Equine spleen	1998
HS33-4	AHSV6	1 MB, 2 Vero	Equine spleen	1998
HS37	AHSV4	2 Vero	Equine spleen	1998
HS43 ^c	AHSV7	2 Vero	Equine lung	1998
HS45 ^c	AHSV7	2 Vero	Equine lung	1998

^a MB, suckling mouse brain.

^b Vero, vero cell culture.

^c Both the HS43 and HS45 isolates were made from Gauteng Province during 1998, but from different horse studs and were not part of the same outbreak.

with the inter-relationships determined using plaque reduction neutralisation tests (Pritchard and Gould, 1995). For epizootic EHDV1, the concept of VP2 gene-based typing has been demonstrated using an approach combining RT-PCR and probing (Aradaib et al., 1995). The first effort to differentiate AHSV serotypes with a molecular approach was by comparing restriction fragment length polymorphisms (RFLPs) of RT-PCR cDNA prepared from AHSV genome segment 7. A panel of eight restriction enzymes separated the nine AHSV serotypes in two groups based on electrophoretic migration patterns (Zientara et al., 1993). By combining the RFLP data from AHSV genome segment 7 and genome segment 10 cDNA, all the nine AHSV serotypes could be differentiated (Zientara et al., 1995b). However, diagnostic development of this RFLP typing for AHSV was abandoned because neither of the protein products of these segments exerts any detectable influence on virus serotype. Phylogenetic analysis of nucleic acid sequencing data confirmed the relatively low degree of similarity of segment 10 within the AHSV serogroup reflected by RFLP analysis and revealed that the genetic variability between segment 10 of different isolates within a serotype showed extensive divergence compared with the situation amongst BTV serotypes (Sailleau et al., 1997; Zientara et al., 1998).

The aim of this investigation was to extend the observation that a cloned AHSV3 VP2-gene hybridised serotype-specific (Bremer et al., 1990) to a complete set of cloned AHSV VP2-genes and lay the foundation for the diagnostic application of probes for typing AHSV isolates.

2. Materials and methods

2.1. Viruses and cells

AHSV reference strains of serotypes 1–9 (Table 1), AHSV isolates from 1997 to 1998 field cases of AHS in South Africa (Table 2), as well as SA-BTV4 and EEV-Kyalami were obtained from the Onderstepoort OIE Reference Laboratory for Bluetongue and African horsesickness, South

Africa. The passage history of the reference viruses used in this investigation is as follows; virulent isolates from field cases were passaged two to three times in suckling mouse brains, plaque purified twice in Vero cell cultures and freeze-dried. This material was subsequently passaged two to five times in BHK and/or a chicken fibroblast-hamster kidney hybridoma cell line (CER) in modified Eagle's medium supplemented with 10% bovine serum. Viral stocks were titered using Vero cell monolayers as described by Oellermann (1970).

2.2. Preparation of DNA probes

Cloned VP2 genes from AHSV3 (Vreede and Huismans, 1994) and AHSV9 (Venter et al., 2000) were obtained from H. Huismans (Department of Genetics, University of Pretoria, South Africa), the cloning of the AHSV5 VP2-gene used was described in Vreede et al. (1998), while full-length cloned VP2-genes of AHSV serotypes 1, 2, 4, 6, 7 and 8 were generated recently (Potgieter et al., manuscript in preparation). Full-length cloned VP2-genes, were excised from plasmids after large scale preparation and isolated by gel electrophoresis. VP2-DNA (0.5 µg) was denatured by boiling and snap cooling on ice. This was used as template to prepare ³²P- or digoxigenin-labelled probes by incorporating either α-³²P-dCTP (Multiprime DNA labelling system, Amersham Life Sciences) or digoxigenin-11-dUTP (DIG High Prime, Boehringer Mannheim) during random primer extension reactions. After the reactions were completed the probes were denatured by boiling for 5 min.

2.3. Preparation of template RNA

Template total viral dsRNA was purified essentially as described by Bremer (1976). Briefly, monolayer cultures of CER cells were infected with virus stocks and harvested at 48–60 h p.i., or earlier when CPE reached 80–100%. The cells were collected by centrifugation, resuspended in STE buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA) and ruptured using a dounce homogeniser. The cells were lysed further with 1%

SDS in the presence of Na-acetate buffer (0.01 M Na-acetate pH 5.1, 0.01 M EDTA). The pH of the lysate was adjusted to 5.0 with glacial acetic acid. This was followed by phenol protein extraction and ether extraction to remove residual phenol. RNA was precipitated from the aqueous phase by mixing it with two volumes of ethanol and pelleted by centrifugation at 10 000 × g for 45 min. The RNA pellet was dissolved in 0.001 M EDTA and LiCl was added to a final concentration of 2 M to precipitate the ssRNA which was removed by centrifugation (10 000 × g for 45 min). The LiCl concentration in the supernatant was adjusted to 4 M to precipitate the dsRNA which was collected by centrifugation as described above, dissolved in RNase-free water and stored at –20°C. The concentration of the dsRNA was determined by UV-spectrophotometry at 260 nm.

In the case of total RNA extraction from viral infected cell culture, the commercial reagent Trizol® (GIBCO-BRL Life Technologies) was used according to the manufacturer's instructions. The method is a modification of the acid guanidinium-phenol extraction procedure described by Chomczynski and Sacchi (1987). Cell cultures were infected as indicated in the text. When CPE reached 80–100% in the cell cultures, total RNA extractions were done. The RNA that was prepared was electrophoresed on a 1% TBE-gel to visually compare the relative amounts of dsRNA between the preparations. The total RNA was denatured with methyl mercury II hydroxide (MMOH) before it was spotted and hybridised with the VP2 gene-probes.

2.4. Dot-spot nucleic acid hybridisation

RNA samples were denatured in 20 mM MMOH for 30 min at room temperature and spotted on positively charged nylon membranes (Hybond N+, Amersham) in 200 µl volumes using a 96-well dot-spot apparatus. Unless otherwise indicated, the amount of denatured dsRNA that was applied to the membrane was in the order of 200–600 ng per spot. The membrane was then placed on a filter paper saturated with a solution containing 50 mM NaOH and 1.5 M NaCl for 5 min and neutralised on a filter paper saturated

with a solution containing 1.5 M NaCl, 1 mM EDTA and 0.5 M Tris-HCl, pH 7.2. The RNA was fixed to the membrane by exposure to UV-light (312 nm) for 5 min. Prehybridisation was carried out for 4 h at 42°C in a mixture of 50% formamide, 5 × SSC, 5 × Denhardt's solution (0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin (BSA)) and 0.1% SDS, to which 0.01 mg/ml denatured herring sperm DNA had been added. The membrane was then cut into strips that were hybridised separately with each of the VP2-gene probes as indicated in the text. Generally, as a control, one strip was hybridised with an AHSV group-specific probe, prepared from the AHSV9 NS2-gene (Van Staden et al., 1991). The ³²P-labelled probes were diluted in hybridisation mixture (50% formamide, 5 × SSC, 1 × Denhardt's solution and 0.1% SDS) and hybridisation was carried out at 42°C for 16 h. When digoxigenin-labelled probes were used, both the prehybridisation and hybridisation steps were carried out in DIG Easy Hyb hybridisation solution (Boehringer Mannheim) at 42°C. Where necessary, probes were diluted (in general 10-fold) to avoid non-specific high background hybridisation to the membrane.

2.5. Post hybridisation washes and probe detection

Blots were washed twice for 10 min under conditions of high stringency (about 90%), namely in a 0.1 × SSC buffer containing 0.1% SDS, at 65°C to eliminate cross-hybridisation between serotypes of AHSV (Bremer et al., 1990). Radioactive blots were exposed overnight to Hyperfilm MP X-ray film (Amersham Life Sciences) at -70°C with an intensifying screen. Hybridised digoxigenin labelled probes were detected with the use of an anti-DIG-alkaline phosphatase-conjugate antibody and the chemiluminescent substrate, CSPD (DIG High Prime DNA Labelling and Detection Starter Kit II, Boehringer Mannheim). Chemiluminescence was detected on an X-ray film (Hyperfilm MP, Amersham Life Sciences) or with a Lumi-Imager™ F1 workstation.

3. Results

3.1. Specificity of VP2 gene-probes

The observation that a cloned AHSV3 VP2 gene hybridised serotype-specific to dsRNA from AHSV (Bremer et al., 1990) together with the recent cloning of an AHSV VP2-gene from each of the nine AHSV serotypes (Potgieter et al., manuscript in preparation) offered the first opportunity to investigate the possibility of developing a complete set of probes for typing AHSV isolates. Specificity of the cloned full-length AHSV VP2-gene probes was investigated by hybridising the individual probes to membrane strips containing purified viral dsRNA from the reference strains of AHSV serotypes 1–9 from which the probes were derived, as well as to dsRNA from SA-BTV4 and EEV-Kyalami which are members of two closely related orbivirus species that co-circulate with AHSVs in South Africa. The membranes were washed under conditions that required about 90% stringency, since for BTV the nucleic acid similarity between field isolates, vaccine and reference strains of one serotype is as high as 93.8–99.1% for serotype 17 (De Mattos et al., 1994a), and between 95.2 and 99.9% for serotype 10 (De Mattos et al., 1994b).

The AHSV VP2-gene probes all hybridised to denatured RNA from their corresponding serotypes and no cross-hybridisation was observed between the heterologous AHSV serotypes or with RNA from SA-BTV4 or EEV-Kyalami (Fig. 1). The AHSV serogroup-specific NS2 gene-probe hybridised to RNA from all of the nine AHSV serotypes, but not to RNA from BTV or EEV (Fig. 1). The differences in signal intensities can most likely be ascribed to the variation in amounts of template RNA (200–600 ng) spotted on the membrane referred to in Section 2.

3.2. Detection of recent field isolates of AHSV

Since the AHSV VP2-gene probes were all generated from historical AHSV reference strains isolated several decades ago, it was important to determine whether they would detect AHSV isolates from recent AHS field cases in South Africa.

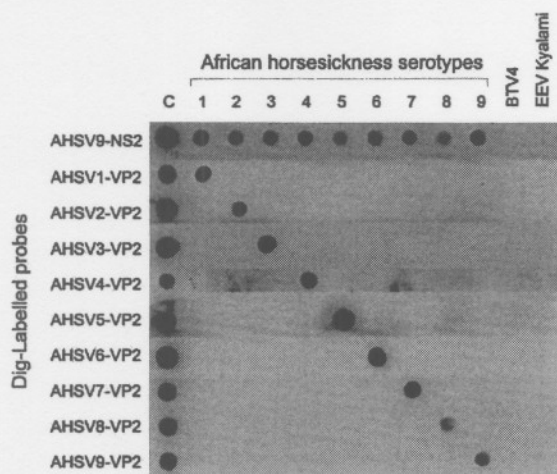


Fig. 1. Chemiluminescent image of a set of membrane strips, each containing denatured dsRNA from the nine serotypes of AHSV and BTV4 and EEV-Kyalami and each hybridised with a different probe as indicated to the left. Probes were prepared by labelling full-length cDNA of the VP2-gene of each of the nine serotypes of AHSV as well as cDNA of the NS2-gene of AHSV9. The NS2-probe was included as an AHSV group-specific control. The positive controls (C) consisted of homologous cloned VP2-DNA.

Two different isolates from the 1997 to 1998 period for each of AHSV serotypes 3, 4, 6 and 7 and one of serotype 8 were available (Table 2).

Total RNA was extracted from cell cultures infected with the respective viruses and probed with the different AHSV VP2-gene probes of the serotypes involved.

The AHSV VP2-gene probes were able to detect the recent AHSV field isolates of their corresponding serotypes (Fig. 2). The signals for serotypes 4 and 6 are weaker than the rest, since the probes were used at a 10-fold dilution to reduce background on the nylon membrane.

3.3. Sensitivity of VP2-probes

The sensitivity of the type-specific AHSV probes was investigated by two different experimental approaches. The first approach determined the threshold of detection for purified AHSV dsRNA and compared non-radioactive digoxigenin-labelling to ^{32}P -labelling of the probes under optimised conditions. A dilution series of AHSV7 dsRNA was prepared in RNase-free water and spotted in two rows. The blots were hybridised with a non-radio-active probe and radio-active probe, respectively. The chemiluminescent signal was detected with a Lumi-ImagerTM F1 workstation.

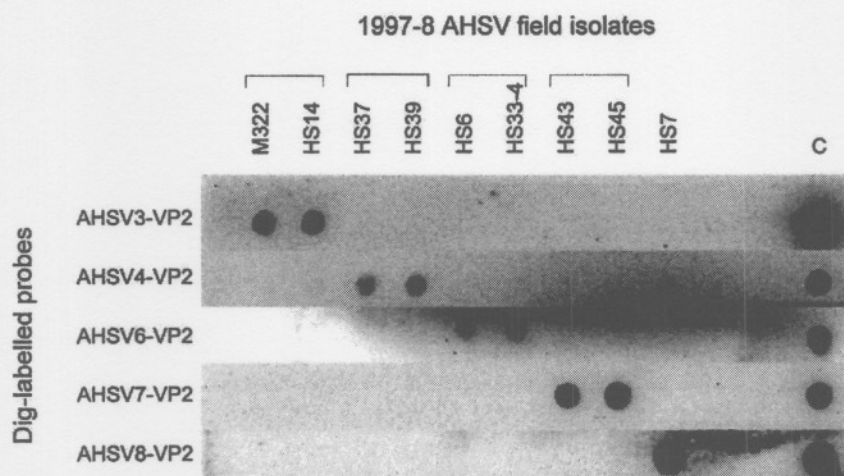


Fig. 2. Chemiluminescent image of VP2-probes obtained from clones of historical AHSV isolates, hybridised with RNA from recent isolates of AHSV (Table 2). Total RNA was extracted from infected cultures and hybridised with each of the VP2-gene probes. The positive controls (C) consisted of homologous cloned VP2-DNA. The serotype from which the digoxigenin-labelled VP2-probes were derived is indicated at the left.

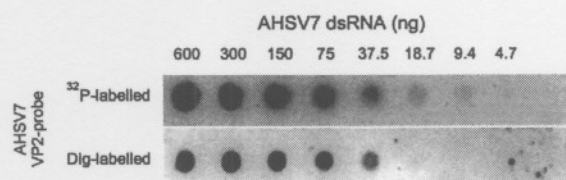


Fig. 3. Autoradiogram and chemiluminescent image of a duplicate dilution series of denatured AHSV7 dsRNA hybridised with ^{32}P - and digoxigenin-labelled AHSV7 VP2-probes, respectively.

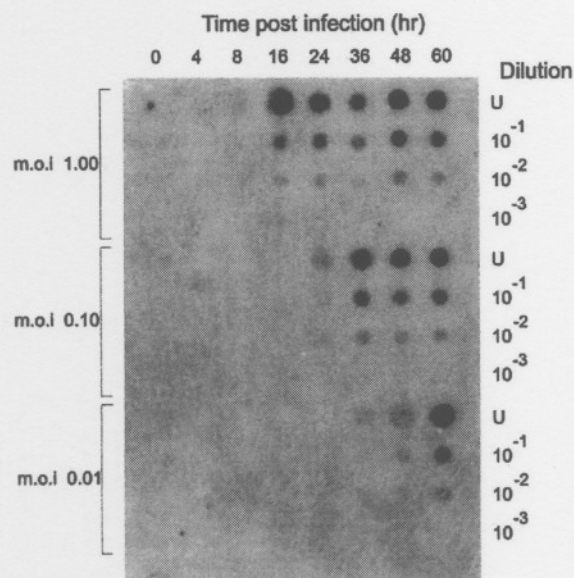


Fig. 4. Autoradiogram of RNA samples extracted from AHSV7 infected cell cultures at different times post infection that were hybridised with an AHSV7 ^{32}P -labelled VP2-probe. Three sets of infections were done at m.o.i. 1.00, 0.10 and 0.01, respectively. Each RNA sample was denatured and blotted at the different dilutions indicated. The dilution factor of the harvested samples are indicated at the right, U, undiluted.

The digoxigenin-labelled probe could detect 37.5 ng dsRNA, whereas the radio-active ^{32}P -labelled probe was about four-fold more sensitive, being able to detect in the order of 9.4 ng dsRNA (Fig. 3).

The second approach involved determining the sensitivity of the serotype-specific AHSV probes for viral isolates in cell culture by establishing how soon p.i. at different multiplicities of infection (m.o.i.) there would be enough viral RNA for detection. Three sets of Vero cell cultures were

infected using the same AHSV7 inoculum. The tissue cultures were infected at m.o.i. of 1.00, 0.10 and 0.01 plaque forming units (pfu) per cell in 25 cm^2 tissue culture flasks. The inoculum was removed after 30 min at 37°C and replaced with fresh medium. At various times p.i. as indicated in Fig. 4, the supernatant was removed from the tissue cultures, discarded and a total RNA extraction was done on the infected cells. At high m.o.i. and at more than 48 h p.i., it was necessary to first collect the cells by low speed centrifugation before the RNA could be extracted from the cells. The RNA was denatured and a 10-fold dilution series of each sample was prepared. The samples were blotted as shown in Fig. 4 and hybridised to the ^{32}P -labelled AHSV7 VP2-probe.

At m.o.i. of 1.00, viral RNA could be detected 16 h p.i. When the m.o.i. was 0.10, RNA could be detected 24 h p.i. and when the m.o.i. was 0.01, the probe could detect viral RNA from 48 h p.i.

4. Discussion

This investigation amounts to proof of concept for the feasibility of genomic typing of AHSV isolates based on a complete set of cloned full-length VP2-gene probes of the nine serotypes of AHSV. It is the first VP2-gene probe procedure to span a whole species (serogroup) in a genus of the family *Reoviridae*. The finding that type-specific AHSV VP2-gene probes derived from historical reference strains detect AHSV isolates from recent field cases of AHS in South Africa and can identify the type in less than half the time needed for current virological procedures, augers well for the future application of the approach as a useful expansion of the diagnostic technology repertoire.

The threshold of detection of radio-active ^{32}P -labelling of VP2 gene-probes was determined to be about 9 ng total AHSV dsRNA which is in the same range as was reported for the dot and northern blot hybridisation methods for rotavirus detection (Larralde and Flores, 1990; Parwani et al., 1993). ^{32}P -labelling of probes resulted in a two-fold increase in sensitivity compared to non-radioactive digoxigenin-labelled probes (Fig. 3). When cells were infected with AHSV at a m.o.i. of

0.01 pfu per cell, viral RNA could be detected and typed by 48 h p.i. using a ^{32}P -labelled probe (Fig. 4). The minimum number of cells needed for detection has not been accurately determined, but is estimated to be in the order of 1×10^7 cells. Cellular RNA does not affect the specificity of the VP2-probes, since no signal was detected in virus-infected cells up to 8 h p.i. (Fig. 4). Further technical refinement can now follow to optimise, evaluate, validate and standardise the procedure for diagnostic applications. Of particular interest for diagnostics will be to cut template RNA extraction and purification requirements to the minimum.

Further improvement of sensitivity of molecular AHSV typing will have to await development of RT-PCR based methods. In the case of rotavirus, a semi-nested RT-PCR method could detect dsRNA from 10 to 10 000 virus particles, which is 200–20 000 times more sensitive than the hybridisation procedure of gene 4 typing (Gentsch et al., 1992; Arista et al., 1999). RT-PCR based typing methods have been initiated for some of the orbiviruses, but none have thus far been developed and tested on all the viral serotypes of a serogroup. For BTV, a multiplex PCR type-specific assay has been developed based on the VP2-gene of the five USA BTV serotypes, generating a specific product which is identified by size difference (Wilson and Chase, 1993). The diagnostic potential of the concept of VP2-based serotyping using a combined RT-PCR/probe approach has also been demonstrated for EHDV1. The sensitivity of the EHDV1 PCR assay was 1.0 fg of virus RNA, the equivalent to 60 virus particles (Aradaib et al., 1995). For AHSV RT-PCR based typing methods to become a reality, a sizeable AHSV VP2-gene sequence reference databank will have to be developed. However, although PCR has numerous advantages for epidemiological studies, production of vaccines, viral evolution investigations, production of non-infectious diagnostic antigens, evaluation of mutagens and can be used for rapid and specific detection and identification of viral RNA, the fact that it can be too specific for some aspects of diagnostics must always be taken into account.

The advantage of orbivirus VP2 gene-probe based typing over RT-PCR-based methods might well prove to be that nucleotide sequence variations which are typical of VP2-genes of BTVs, EHDVs and AHSVs should not affect it in the way it can be expected to impact on RT-PCR based methods where conservation of nucleotide sequences used as primers is crucial. An illustration of the latter problem is the report of a Nigerian rotavirus serotype G8 which could not be typed by PCR due to nucleotide mutation at the 3' end of the primer binding site (Adah et al., 1997). In order to determine the diagnostic potential of the probe approach, it will thus be necessary to investigate the extent of genetic variability of AHSV VP2-genes within and between serotypes thoroughly. Once this is known, a comprehensive comparison of the merits of molecular and serological serotyping should be undertaken to determine the situation of choice for the application of the various methods.

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CHAPTER 5

Paper 4

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Characterization of $\Phi 12$, a Bacteriophage Related to $\Phi 6$: Nucleotide Sequence of the Large
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Characterization of $\phi 12$, a Bacteriophage Related to $\phi 6$: Nucleotide Sequence of the Large Double-Stranded RNA

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The isolation of additional bacteriophages besides $\phi 6$ containing segmented double-stranded RNA genomes (dsRNA) has expanded the *Cystoviridae* family to nine members. Comparing the genomic sequences of these viruses has allowed evaluation of important genetic as well as structural motifs. These comparative studies are resulting in greater understanding of viral evolution and the role played by genetic and structural variation in the assembly mechanisms of the cystoviruses. In this regard, the large double-stranded RNA genomic segment of bacteriophage $\phi 12$ was copied as cDNA and its nucleotide sequence determined. This genome's organization is similar to that of the large segment of bacteriophages $\phi 6$, $\phi 8$, and $\phi 13$. In the amino acid sequence of the viral RNA-dependent RNA polymerase (P2), similarity was found to the comparable proteins of $\phi 6$, $\phi 8$, and $\phi 13$. Amino acid sequence similarity was also noted in the nucleotide triphosphate phosphorylase (P4) to the comparable proteins of $\phi 8$ and $\phi 13$. © 2002 Elsevier Science (USA)

INTRODUCTION

Bacteriophage $\phi 12$ was isolated from the leaves of the Sweet Basil plant (*Ocimum basilicum*) (Mindich *et al.*, 1999), and it is among the nine known members of the *Cystoviridae* family. Bacteriophage $\phi 6$, which until recently has been alone in the genus *cystovirus* (Van Regenmortel *et al.*, 1999), was isolated from bean straw infested with *Pseudomonas syringae* pv. *phaseolicola* (Vidaver *et al.*, 1973). $\phi 12$ is similar in structure to bacteriophage $\phi 6$ in that it also contains a genome of three segments of double-stranded RNA (dsRNA) (Semancik *et al.*, 1973) packaged inside a procapsid that is covered by a protein shell and a lipid-containing membrane with additional proteins (Vidaver *et al.*, 1973). It has recently been shown that the $\phi 6$ active polymerase subunit is very similar in structure to that of the polymerase of hepatitis C virus, suggesting an evolutionary link between dsRNA viruses and the flaviviruses (Butcher *et al.*, 2001).

The genome of $\phi 6$ has been cloned and sequenced and the replication cycle and structure of the virus have been extensively investigated (Butcher *et al.*, 1997; de Haas *et al.*, 1999; Mindich, 1999). Study of genomic packaging in $\phi 6$ led to the development of a detailed pack-

aging model (Mindich, 1999; Onodera *et al.*, 1998; Qiao *et al.*, 1997). Specifically, it is proposed that the binding of the small (plus sense) single-stranded RNA segment (s) to sites on the outside of an empty procapsid and positioning its 5' end at an entry portal initiate packaging. When segment s is packaged, the binding sites are lost and then new sites for the middle segment (m) appear on the outside of the particle. The packaging of segment m results in the loss of its binding sites, and sites for the large segment (l) are revealed. Each of the viral RNA segments contains a packaging sequence of about 200 nucleotides near the 5' ends of the plus strands (Gottlieb *et al.*, 1994).

The isolation of additional bacteriophages other than $\phi 6$ containing three segments of dsRNA revealed some to be very similar to $\phi 6$ and others only distantly related (Mindich *et al.*, 1999). Eight additional cystoviruses, $\phi 7$ to $\phi 14$, were isolated and are being characterized, particularly in regard to their relationship to $\phi 6$. The close relatives of $\phi 6$ include $\phi 7$, $\phi 9$, $\phi 10$, and $\phi 11$. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of this group with primers derived from $\phi 6$ sequences was possible, and subsequent genomic sequence analyses demonstrated 80 to 85% sequence similarity. Within open-reading frames (*orfs*), the base sequence changes were concentrated in the third base of codon triplets so that the amino acid sequences remained highly conserved. The 5' ends of the genomic plus strands were found to contain the *pac* sequences, which are about 200 nucleotides in length and unique and specific for the packaging of each segment. The sequences of the *pac*

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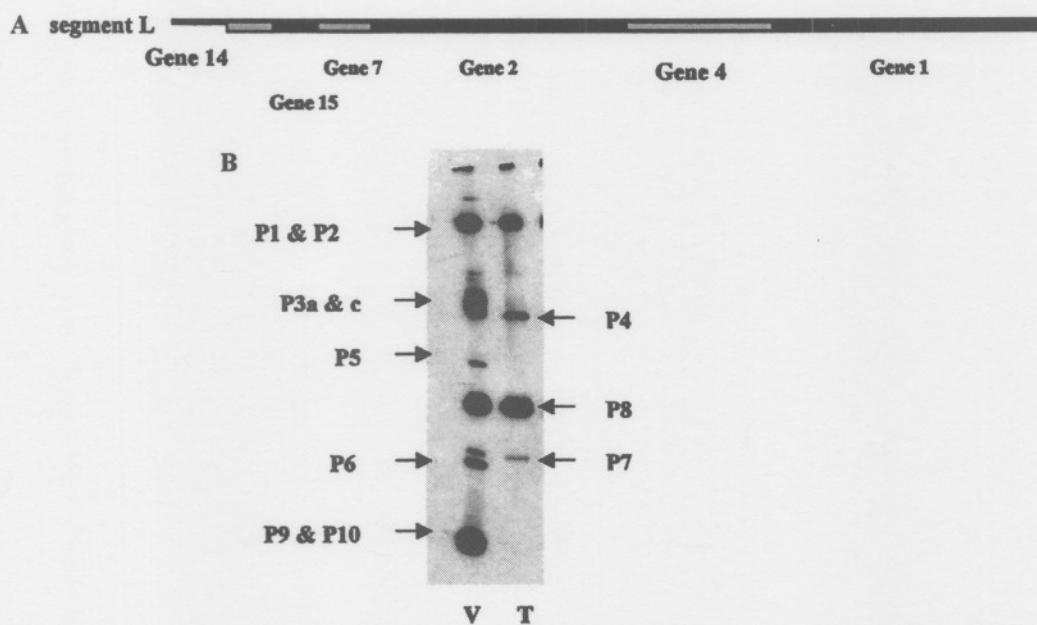


FIG. 1. (A) cDNA copy of the L genomic segment of ϕ 12. The genes are numbered to correspond to those of ϕ 6, ϕ 8, and ϕ 13. (B) Autoradiogram of PAGE analysis of whole virus (V) and Triton X-100-treated virus (T).

regions in this group of phages were about 90% identical, and it was also found that they were capable of accepting both the m and s segment of ϕ 6 (Mindich *et al.*, 1999).

Bacteriophages ϕ 8, ϕ 12, and ϕ 13 were judged to be distantly related to ϕ 6 (Mindich *et al.*, 1999). These phages, while unable to infect the normal host of ϕ 6, *P. syringae* pv. *phaseolicola* HB, can infect a mutant, LM2333. This latter strain is resistant to several common phages, most of which contain DNA genomes. The three phages were also able to infect both a derivative of LM2333 lacking type IV pili and a rough strain of *P. syringae*, Ro49da1. It is likely that these phages attach directly to the LPS. RT-PCR on the RNA segments of these phages using primers derived from ϕ 6 was unsuccessful (Mindich *et al.*, 1999). Previous studies have determined the nucleotide sequence of both the ϕ 8 and ϕ 13 genomes, showing that the overall genetic organization is similar to ϕ 6, although for the most part there is no similarity in either the nucleotide sequences or amino acid sequences. An exception is that the amino acid motifs characteristic of viral RNA polymerases are present in the protein sequences of the polymerase complex. In the case of bacteriophage ϕ 8, it was noted that protein P8, which constitutes a shell around the procapsid in ϕ 6, is part of the membrane in this phage. The ϕ 8 and ϕ 13 host attachment proteins consist of two peptides rather than the one found in ϕ 6 (Hoogstraten *et al.*, 2000; Qiao *et al.*, 2000).

We have recently determined the nucleotide sequence of the small and middle segments of bacteriophage ϕ 12 and have seen that the host cell attachment proteins (P6, P3a, P3b, and P3c) have marked similarity to the com-

parable proteins of ϕ 13. In contrast, we found significant similarity of the ϕ 12 lysis cassette proteins (P5 and P10) to those of bacteriophage ϕ 6 (Gottlieb *et al.*, 2002). In this paper, we present the cDNA cloning and complete sequencing of the large genomic segment of ϕ 12. We discuss the implications of this work on the evolutionary history of the cystoviruses.

RESULTS AND DISCUSSION

RNA sequence

The cDNA copies of the ϕ 12 large genomic segment were sequenced and the arrangement of the genes was determined. The size of the L segment was found to be 6751 bp (Fig. 1A) and its base composition is 54.1% GC. The corresponding size of the L segment in ϕ 6 is 6374 bp with a base composition of 55.5% GC (Mindich, 1988). The sizes of the corresponding segment in ϕ 8 and ϕ 13 were found to be 7051 and 6458 bp at 54.0 and 58.4% GC, respectively (Hoogstraten *et al.*, 2000; Qiao *et al.*, 2000).

Identification of genes

The genes in the L segment were named for the genes in the same position as ϕ 6 and are also arranged in a similar manner to those of ϕ 8 and ϕ 13 (Fig. 1A and Table 1). The assignments were made on the basis of chromosomal position, size, and amino acid similarity or identity with those of the other cystoviruses. The gene assignments were also integrated with the results of SDS-polyacrylamide gel analysis (SDS-PAGE) of the viral proteins, both from purified virions and detergent-extracted

TABLE 1
List of *orf*s and Ribosome-Binding Sites for the ϕ 12 L Segment

<i>orf</i>	Start	End	aa number	MW ^a	IEP ^b	Ribosome-binding sites
						Complement of the 16s rRNA 3' end UAAGGAGGUGAUC
P14	410	709	99	11.5	8.79	GACUAACAGGGAAUCCAUAUUGCGA
P15	714	1016	100	11.1	8.85	GUAUAACAGGAGUAAACGGGAUGUUC
P7	1019	1528	169	18.5	4.32	AUGAAUACCUGGGGUAACUAUGGAC
P2	1512	3491	659	75.4	6.75	UUCGACCACGGAGCGAUCGAUGAUG
P4	3488	4483	331	35.1	6.34	AUUACAAGGUAAUUACGUUAUGAUC
P1	4497	6668	723	79.0	5.70	AACUGAAGGAUAGUUAUCAUGGCA

^a Molecular weight in kDa.

^b Predicted isoelectric point of the protein.

virions. In addition N-terminal analysis of proteins transferred from the SDS-PAGE gels to PVDF filters and subjected to Edman degradation helped in gene assignments.

The viral nucleocapsid proteins were readily identifiable by SDS-PAGE of Triton X-100 treated ³⁵S-Met-labeled virions in that the detergent removed the envelope-associated proteins (Fig. 1B). We were able to visualize three of the four proteins that constitute the procapsid. P2, the viral polymerase, is not visible on this SDS-PAGE analysis and presumably migrates extremely close to protein P1. The polymerase protein was judged to be present in the virus in a lower amount than that of P1 and therefore both proteins appear as one band in the autoradiogram.

Gene 7 is the first procapsid *orf* in the L segment near the 5' end known to encode a protein (Fig. 1A). The AUG initiating codon starts at position 1019 and is preceded by a ribosome-binding site (Woese *et al.*, 1984) (Table 1). N-terminal analysis of the first 5 amino acids of the protein that had been excised from a blot of the gel bands confirmed its identity. The molecular weight of P7 was calculated to be 18.5 kDa. Two genes that we call 14 and 15, respectively, precede gene 7 (Fig. 1A). In ϕ 6, gene 14 is thought to play a role in the expression of gene 7, and it is also found to be present in the L segment of ϕ 8. In ϕ 12, both genes 14 and 15 have a calculated molecular weight of 11.5 and 11.1 kDa, respectively, and are preceded by a ribosome-binding site (Woese *et al.*, 1984) (Table 1), and it remains to be seen whether they are expressed and are part of the virion.

Following gene 7 is gene 2 (Fig. 1A). The aspartate sequence GDD found in conserved segment III of viral RNA-dependent RNA polymerases was present in protein P2 (Koonin *et al.*, 1989). This motif is SDD in ϕ 6 and ϕ 13 (Mindich *et al.*, 1988; Qiao *et al.*, 2000) but is also GDD in ϕ 8 (Hoogstraten *et al.*, 2000). In segment L in ϕ 6, ϕ 8, and ϕ 13, translation stop mutations in gene 7 are polar on gene 2. It has been seen in these other three cystoviruses that the AUG initiating codon for gene 2 overlaps (ϕ 6), directly follows (ϕ 8), or directly precedes

(ϕ 13) the stop codon for P7 (Mindich *et al.*, 1988; Hoogstraten *et al.*, 2000; Qiao *et al.*, 2000). In gene 2 of both ϕ 6 and ϕ 8, no ribosome-binding site was found to precede the gene (Mindich *et al.*, 1988; Hoogstraten *et al.*, 2000). A surprising finding was that the initiating codon for gene 2 of ϕ 12 was found 15 bp prior to the stop codon for gene 7 (Table 1). The gene itself has a ribosome-binding site (Woese *et al.*, 1984) before it (Table 1), and it remains to be determined whether translation stop mutations in gene 7 are polar on gene 2 in this cystovirus.

Gene 4 of ϕ 12 codes for protein P4, which is the NTPase found to be necessary for genomic packaging in ϕ 6 (Gottlieb *et al.*, 1992). N-terminal analysis of the first 8 amino acids confirmed P4's identity. It was found to have a Walker motif A (Walker *et al.*, 1982) for NTP binding that had the amino acid sequence GKGNSGKT. In ϕ 6, ϕ 8, and ϕ 13, this motif is GATGSGKS (Mindich *et al.*, 1988), GTAGGKT (Hoogstraten *et al.*, 2000) and GGTGAGKS (Qiao *et al.*, 2000), respectively. The molecular weight of the protein was calculated to be 35.1 kDa (Table 1), and a ribosome-binding site (Woese *et al.*, 1984) precedes the gene.

Gene 1 follows gene 4 (Fig. 1A), and its identity was also confirmed by N-terminal analysis of the first 5 amino acids of the protein. The initiating codon, AUG, begins at position 4497 and this *orf* ends at position 6668 (Table 1), with the stop codon 83 bp from the 3' end of the RNA segment.

Structural similarity of the proteins to those of other cystoviruses

The sequence of the L segment was analyzed using both the BLAST X and BLAST P software provided at the National Center for Biotechnology Information's web site. We found that the ϕ 12 polymerase P2 displayed significant identity to the comparable proteins of bacteriophages ϕ 6, ϕ 8, and ϕ 13, at 21, 24, and 20%, respectively. Qiao *et al.* report that the ϕ 13 P2 has 50% identity to the polymerase of ϕ 6 and Hoogstraten *et al.* describe the ϕ 8 P2 as having 20% identity to the ϕ 6 P2 (Qiao *et al.*, 2000;

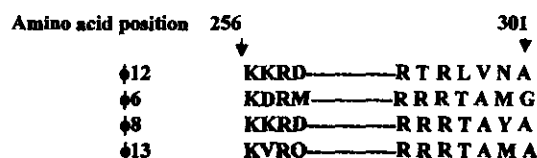


FIG. 2. Amino acids in the immediate vicinity of the key basic residues within the substrate pore of the viral polymerase P2. The amino acid position numbering is that of ϕ 12.

Hoogstraten *et al.*, 2000). Recently, the 2-Å X-ray structure of the active polymerase subunit of ϕ 6 was determined, and sequence motifs characteristic of RNA polymerases were noted (Butcher *et al.*, 2001). The key aspartic acid residues found at positions 324, 453, and 454 in the ϕ 6 P2 are also found at positions 349, 469, and 470 of the ϕ 12 polymerase. The second and third of these conserved amino acids are within the conserved segment III of viral RNA-dependent RNA polymerases (Koonin *et al.*, 1989). The structural analysis of the ϕ 6 polymerase indicates that tyrosine 630 found within the polymerase-template complex might form a platform in which an initiation complex could be constructed (Butcher *et al.*, 2001), and this tyrosine is conserved in the ϕ 12 P2 at position 621. ϕ 13 and ϕ 8 have a tryptophan and tyrosine at this site, respectively (Hoogstraten *et al.*, 2000; Qiao *et al.*, 2000). Crystal soaking experiments of the ϕ 6 P2 with NTPs demonstrated a binding site in the substrate pore that orders triphosphate moieties by attachment to the key basic residues lysine 223 and arginines 225, 268, and 270 (Butcher *et al.*, 2001). The latter three arginine residues are conserved in the ϕ 12 P2 at positions 258, 295, and 297. The ϕ 12 lysine residue is located at position 256 (Fig. 2). The amino acid sequences of the polymerases from ϕ 8 and ϕ 13 show conservation of these basic residues (Hoogstraten *et al.*, 2000; Qiao *et al.*, 2000). The amino acids in the immediate vicinity of the basic residues of the substrate pore are also of interest in defining the relationships among the characterized cystoviruses. Although this region would be expected to be highly conserved, the ϕ 12 sequence near arginines 295 and 297 is most distant in comparison to the analogous regions of the others, yet at the lysine 256, arginine 258 region it resembles that of ϕ 8 (Fig. 2).

The ϕ 12 NTPase, P4, displayed significant identity to the comparable proteins of ϕ 6, ϕ 8, and ϕ 13 showing 23% identity to that of ϕ 6 and 27% to both ϕ 8 and ϕ 13. No significant similarities of the ϕ 12 P1 or P7 proteins with those of the comparable proteins from the other three characterized cystoviruses were noted. Protein P1 is the major structural protein of the procapsid and may be the major determinant of viral RNA packaging specificity. Protein P7 plays a stimulatory role in RNA packaging along with plus- and minus-strand synthesis (Mindich, 1999). Their divergence in ϕ 12 could reflect the variation in the RNA genomic sequences. In ϕ 8, amino acid sim-

ilarity is reported to be absent in genes 1, 4, and 7 when compared to those proteins of ϕ 6 (Hoogstraten *et al.*, 2000). However, the ϕ 13 proteins P1, P4, and P7 showed about 50 and 30% identity with the corresponding ϕ 6 proteins (Qiao *et al.*, 2000).

The observation that some of the components of the phage can display undetectable levels of similarity in proteins with analogous function warrants further investigation. In regard to the RNA polymerase and NTPase of the procapsid, the data suggest that ϕ 12 is closer to ϕ 8 and ϕ 13 than to ϕ 6. Previously, we have shown that the ϕ 12 lysis cassette is related to that of ϕ 6, while the attachment specificity proteins appear closer to those of ϕ 13 (Gottlieb *et al.*, 2002). Therefore, in total, these results are interesting in that evolution of form and function in ϕ 12 may have occurred by acquisition in the exchange of large pieces of genetic information from more than one other virus followed by genetic drift of that sequence. We see that the sequences of P1 and P7 are unrelated to any of the known cystoviruses and may be related to as yet undiscovered members of this virus family.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids

P. syringae pv. *phaseolicola* HB10Y (HB) is the host of ϕ 6 and was utilized as a phenotypic screen in that ϕ 12 is noninfectious on it. LM2333 is a mutant of HB which ϕ 12 productively infects (Mindich *et al.*, 1999). *Escherichia coli* strain XL1-Blue Supercompetent Cells (*recA1*, *end1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, [F', *proAB*, *lac1^q*, *ZΔM15*, Tn10 (Tet^r)]) (Stratagene, La Jolla, CA) were used as hosts for cDNA cloning.

Plasmid pT7T319U (Pharmacia, Peapack, NJ) was used as the cloning vector for the cDNA copies of phage cDNA produced by reverse transcription. Plasmid pCR vector purchased from Invitrogen (Carlsbad, CA) or a pGEM-T vector from Promega Corp. (Madison, WI) were both utilized for the rapid cloning and sequence analysis of the RT-PCR produced cDNA derived from the viral genomic RNA. The pCR recombinants were constructed as directed in the product manual and isolated using Qiagen miniprep columns. The clones using the pGEM-T vector were constructed as described below.

Media, enzymes, and chemicals

The media used were LB and M9. Ampicillin plates contained 200 μ g/ml in LB agar supplemented with 40 μ g/ml of isopropyl-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal) (Maniatis, 1982). Restriction enzymes were purchased from Promega. Poly(A) polymerase was purchased from Perkin-Elmer (Boston, MA). The reagents used for cDNA synthesis of the phage RNA and its cloning to a plasmid vector were all supplied in The Universal Riboclone cDNA Synthesis

System also purchased from Promega. Ready to Go RT-PCR Beads were utilized for the RT-PCR amplification of select portions of the RNA segment (Amersham Pharmacia Biotech, Piscataway, NJ). Buffer ACN was used for the suspension of purified bacteriophage and contains 10 mM KPO₄ (pH 7.5), 1 mM MgSO₄, 200 mM NaCl, and 0.5 mM CaCl₂.

Preparation of pure virions of ϕ 12

Twenty to 30 plate lysates of ϕ 12 were prepared by plating phage dilutions into soft agar with an overnight grown culture of LM2333. These plates were incubated overnight at room temperature. The next day, phage-containing top agar was collected and the cell debris and agar were removed by centrifugation in a Sorvall SS-34 rotor at 15,000 rpm, 15 min at 4°C. Phage was collected by centrifugation in a Beckman TI-75 rotor at 33,000 rpm, 2 h at 4°C. The pellet was suspended in 1 ml of buffer ACN. Purification of the phage was by equilibrium centrifugation in CsCl, average density 1.28 g/ml, using a Beckman SW 50.1 rotor at 33,000 rpm, overnight at 4°C. The next day the phage band was located by light scattering, collected by tube puncture, and dialyzed overnight in buffer ACN. The dialyzed sample was centrifuged in a Beckman TI-75 rotor at 33,000 rpm, 2 h at 4°C, and the collected phage suspended in 300 μ l buffer ACN.

In order to isolate nucleocapsids (NC) free of the lipid envelope, the purified phage were treated with 2% Triton X-100 and the preparation was centrifuged at 33,000 rpm in a Beckman TI-75 rotor, 90 min at 4°C. The pellet was suspended in buffer ACN and stored at -80°C.

Isolation and *in vitro* transcription of the ϕ 12 dsRNA

dsRNA was isolated from viral particles by phenol:chloroform (1:1) extractions. The RNA was precipitated with 10% 7.5 M NH₄Ac and 2.5 vol of ethanol. The RNA was pelleted and resuspended in 50 μ l of sterile water.

Nucleocapsids isolated from virions by Triton X-100 treatment were utilized in *in vitro* transcription, in the presence of manganese ions, to synthesize complete transcripts of the three viral RNA segments (Emori *et al.*, 1983). The synthesized transcripts were extracted with phenol:chloroform, ethanol-precipitated, pelleted, and resuspended in H₂O. dsRNA from both the whole phage and synthesized transcripts served as templates for the cDNA synthesis.

Preparation of cDNA

Poly(A) tailing. Either dsRNA or viral transcripts were denatured by boiling for 5 min and rapidly cooled in a dry ice/ethanol bath. A 5 \times poly(A) polymerase buffer was added to the RNA along with ATP and yeast poly(A) polymerase. The mixture was incubated for 1 min at 30°C, transferred to ice, and brought to a volume of 50 μ l

with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The poly(A)-tailed RNA was then extracted with phenol:chloroform, ethanol-precipitated, pelleted, and resuspended in sterile water.

First-strand synthesis. Phosphorylated oligo(dT) (1–2 μ g) was added to 10 μ l of poly(A) tailed RNA. The sample was left at 70°C for 5 min, after which it was cooled on ice for 5 min. Four microliters of 5 \times first-strand buffer, 3 μ l H₂O, 40 u RNase inhibitor (RNasin), and 30 u of AMV reverse transcriptase were added and incubated at 42°C for 1 h.

Second-strand synthesis. To the reverse-transcribed RNA sample, 40 μ l 2.5 \times second-strand buffer, 37.6 μ l H₂O, 0.8 u RNaseH, and 23 u *E. coli* DNA polymerase I were added and the second-strand synthesis proceeded for 4 h at 14°C. The polymerase I was inactivated at 70°C for 10 min. T4 DNA polymerase was added for 10 min at 37°C to blunt the ends of the cDNA. The reaction was stopped by bringing it to 20 mM EDTA. The sample was then treated with phenol:chloroform, ethanol-precipitated, pelleted, and suspended in 2.5 μ l dH₂O.

Preparation of the vector utilized for cloning. PT7T3 19U was digested with *Sma*I and dephosphorylated with shrimp alkaline phosphatase. The vector was then treated with phenol:chloroform, ethanol-precipitated, and suspended in 10 μ l of H₂O. The ligation mixture contained 5 μ l of the cDNA sample, 0.5 μ l vector, 1 μ l 10 \times ligation buffer (containing ATP), and 2.5 u of T4 DNA ligase. Incubation was overnight at 14°C. The ligation mixture was used to transform supercompetent XL-1 Blue *E. coli*. Transformed cells were spread on LB plates containing 40 μ g/ml of X-gal, 40 μ g/ml IPTG, and 200 μ g/ml of ampicillin. White colonies were picked by toothpick and small plasmid preparations prepared using QIAprep Spin Miniprep columns (Qiagen, Stanford, CA). The plasmids were cut with restriction endonuclease *Pvu*II and plasmids containing inserts were sequenced first with both the T3 and M13r primers. Subsequent sequencing of the cloned cDNA inserts was with oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) designed from the sequences derived using the initial primer set.

RT-PCR synthesis of cDNA. cDNAs of all three dsRNA segments were amplified using a modification of the Lambda method (Lambden *et al.*, 1992). The three amplified cDNAs were separated on a 1% agarose gel and purified using the gel extraction kit of Qiagen. The purified PCR products were then cloned into the pGEM-T vector and positive clones were identified based on insert size and restriction enzyme patterns.

Preparation of radioactively labeled bacteriophage

LM2333 was infected with ϕ 12 at a multiplicity of infection of 20 in M9 medium supplemented with amino acids, metal ions, and glucose. ³⁵S-Met (10 μ Ci/ml) was

added and the culture was allowed to proceed to lysis. The phage was purified as described above.

cDNA sequence analysis

cDNA sequencing was performed at both The Protein/DNA Technology Center of Rockefeller University, New York City, and the RCMI Facility at The City College of New York. The sequences were assembled using the AlignIR Assembly and Alignment Software, Li-Cor Biotechnology Division, Lincoln, Nebraska. The sequence of the L segment was submitted to GenBank and the accession number is AF408636.

Amino acid sequence analysis of the ϕ 12 proteins

The amino acid sequences encoded on the L segment were analyzed for similarity to other protein amino acid sequences using the Basic Local Alignment Search Tool (BLAST X) provided by the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health (Bethesda, MD) (Altschul *et al.*, 1990).

Protein sequence

Proteins were sequenced from the N-terminus using automated Edman degradation on a PE Biosystems 494 protein sequencer at the Protein Chemistry Core Facility, Howard Hughes Medical Institute of Columbia University (New York, NY). PAGE-separated viral proteins were transferred to PVDF membranes by Western blotting and the proteins visualized with Ponceau stain. These were excised from the membrane and sent to the sequencing facility for the analysis.

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CHAPTER 6

RESULTS AND DISCUSSION

The study of dsRNA viruses has been severely hampered by the technical difficulties associated with cloning dsRNA genomes. This study describes the optimization of a cloning method for complete viral dsRNA genomes and demonstrates the application thereof. Examples are presented of how the new method resulted in the development of strategies for genome sequence analysis, phylogenetics and molecular epidemiology, expression of recombinant proteins for research, vaccine development and the development of improved methods for the detection and diagnosis of dsRNA viruses.

6.1 Cloning dsRNA genomes

6.1.1 Amplification of viral dsRNA genomes from various sources

The optimised cloning method developed in this study (Figure 6.3) has been used to amplify and clone dsRNA from viruses infecting mammalian, plant, fungal and bacterial hosts. The method is robust, simple, fast and suited for routine cloning. This was demonstrated by the cloning of the complete dsRNA genome sets of three *Orbiviruses* as well as reovirus from infected cell culture (Chapter 2), two human rotavirus (group A and group C) genomes from stool samples (Chapter 2) and the bacteriophage phi12 genome from infected *Pseudomonas* bacterial cells (Chapter 5). Subsequently, full-length genomes of a phytoreovirus from plant material as well as a mycovirus from infected fungus (*Botrytis cinerae*) have also been amplified with the same method (Figure 6.1 A and B).

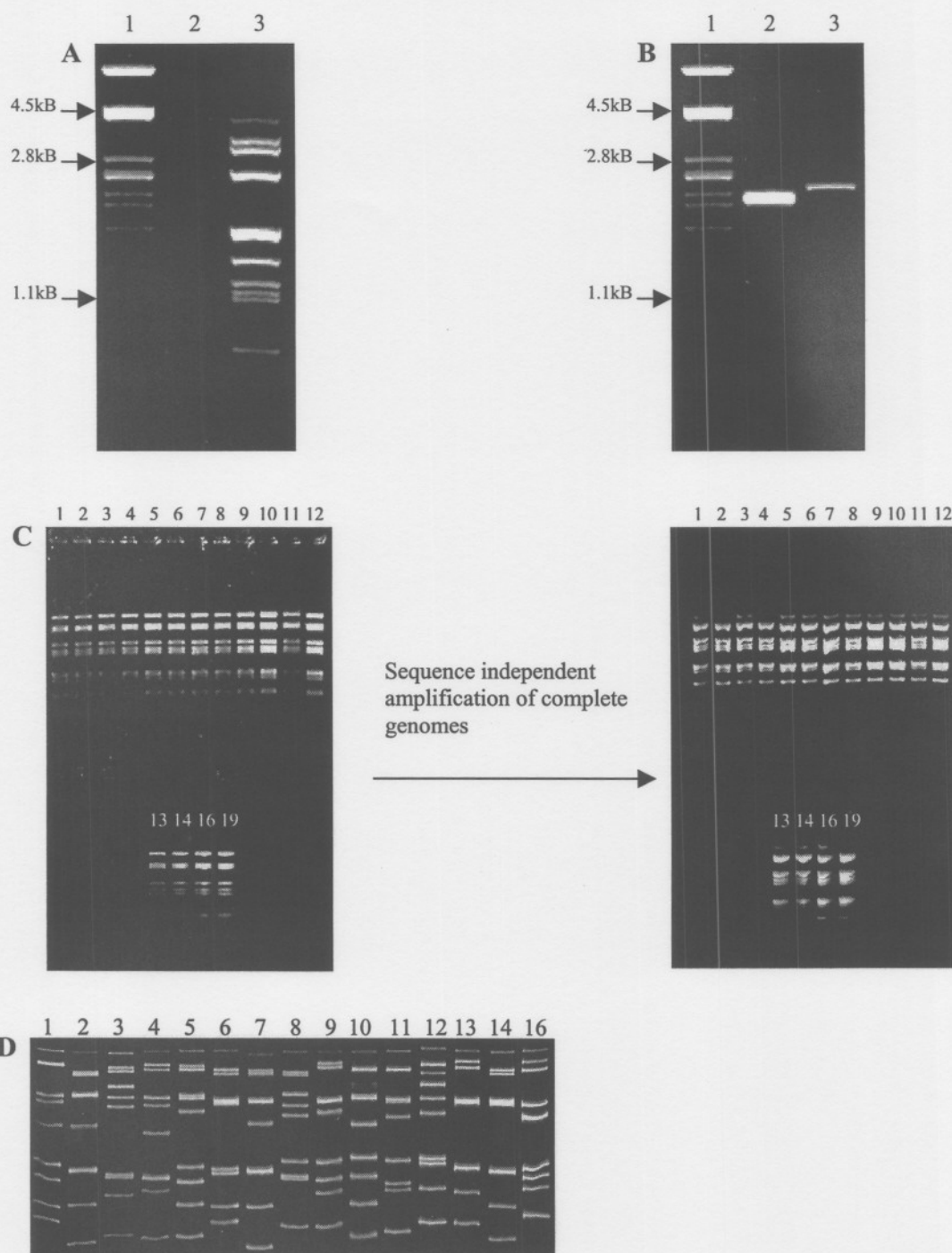


Figure 6.1 Amplification of the genomes of a phytoreovirus, a mycovirus and the 16 BTV vaccine strains.

A. Lane 1: Lambda DNA digested with Pst I

Lane 2: dsRNA extracted from *Tobacco leaf enation virus*

Lane 3: Amplified cDNA of the complete genome of *Tobacco leaf enation virus* (11 segments)

B. Lane 1: Lambda DNA digested with Pst I

Lane 2: dsRNA extracted from a mycovirus – two co-migrating segments (isolated from the phytopathogenic fungi *Botrytis cinerae*)

Lane 3: Amplified cDNA of the complete genome of the mycovirus (the cDNA of the two segments co-migrate – Also note that the amplified cDNA in both figures run slightly different than dsRNA)

C. Amplification of the complete genomes of all 16 BTV vaccine strains (serotypes loaded in each lane are indicated)

D. Amplified cDNA of 15 South African BTV vaccine strains separated by PAGE (serotypes loaded in each lane are indicated)

6.1.2 Amplification of large genes and from small amounts of dsRNA

The new dsRNA cloning method developed here overcomes two major hurdles that have long hampered research on dsRNA viruses. The first is the ability to amplify and clone full-length large (> 2.5 kb) dsRNA segments and the second, to do so from very small amounts (< 1 nanogram) of starting material. The result that all three genome segments of bacteriophage phi12, sizes 2.3; 4.1 and 6.8 kb respectively (Chapters 2 and 5), could be cloned proved the method's usefulness for cloning large dsRNA segments. The 6.8 kb large phi12 segment is the largest segment amplified and cloned so far. Another distinct advantage of the method is that it does not rely on the genes being 5'-capped. The dsRNA from phi12 is not 5'-capped as it originates from a prokaryote and the virus itself does not encode any capping enzymes as those of eukaryotic dsRNA viruses (Paul Gottlieb - personal communication). The only other method for cloning large dsRNA segments as full-length PCR amplicons depends on the 5' end of the RNA being capped and on the availability of relatively large amounts of dsRNA (Attoui *et al.*, 2000a). With the method developed in this study it was possible to amplify a complete dsRNA genome from as little as one nanogram of starting material (rotavirus group C - Chapter 2) compared to the 200 ng needed for the best other method (Attoui *et al.*, 2000a).

6.1.3 Time considerations

Currently the cloning of full-length amplicons of the genome of any member of the family *Reoviridae* takes only 4 - 5 working days. Enough dsRNA can be obtained from a single 75 cm³ flask of infected cells (Chapter 2) obviating the need for large amounts of cell-culture. The ability to process whole dsRNA genomes in single reactions for oligo-ligation, reverse transcription and PCR is a huge improvement. It significantly reduces the time, labour and costs of dsRNA cloning. Previously, in some cases processing single segments in multiple reactions for each segment resulted in 27 single reactions to amplify a complete genome (Chapter 2) as opposed to three using the new method. The fact that the method is sequence-independent is another major advantage from the point of view of saving time as well as making it widely applicable to known and unknown dsRNA species.

6.2 Applications of the optimised dsRNA cloning method

6.2.1 Sequence determination of dsRNA genomes, phylogenetic data and molecular epidemiology

The first obvious application of the cloning method was to determine the complete nucleotide sequence of viral dsRNA genomes and/or individual dsRNA segments. One of the greatest benefits of the method is that the cloned cDNA contain the entire segment including the 5' and 3' non-translated regions (NTRs).

A major contribution of this study was the cloning of the first full set of AHSV VP2 genes of the nine serotypes (Chapter 3) and the complete sequencing of the VP2 genes of the five serotypes (namely, 1, 2, 5, 7 and 8) which had not been done before. This allowed me to perform the first phylogenetic analysis on a full set of AHSV VP2 deduced amino acid sequences. The results revealed the same groupings of serotypes than that obtained with serology (Chapter 3). From the sequence analysis it was possible to identify putative serotype-specific regions and epitopes (Chapter 3) that may turn out to be useful in the development of recombinant vaccines and molecular diagnostic procedures. Currently the VP2 sequence information is used for establishing molecular epidemiological studies of AHSV field isolates (Koekemoer *et al.*, 2003).

More importantly, the sequencing of the nine AHSV VP2 genes was the start of a sequence databank for the reference and local field isolates for AHSV, since it is becoming clear that comprehensive information about the source and lineage of isolates are of importance. This will complement an orbivirus sequence databank that exists already at Pirbright (http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/orbivirus-accession-numbers.htm). The first practical use of the OVI databank was the phylogenetic characterization of AHSV7 isolates of the 1998/1999 season following an AHS outbreak in the western Cape. The molecular epidemiological study demonstrated that the outbreak could have been caused by a virus introduced from the Kwazulu Natal province (Koekemoer *et al.*, 2003). To extend the sequence databanks for the orbiviruses at OVI the VP2 and VP5 genes of all 24 serotypes of BTV has also been cloned and partially sequenced recently (Figure 6.1 C and 6.2).

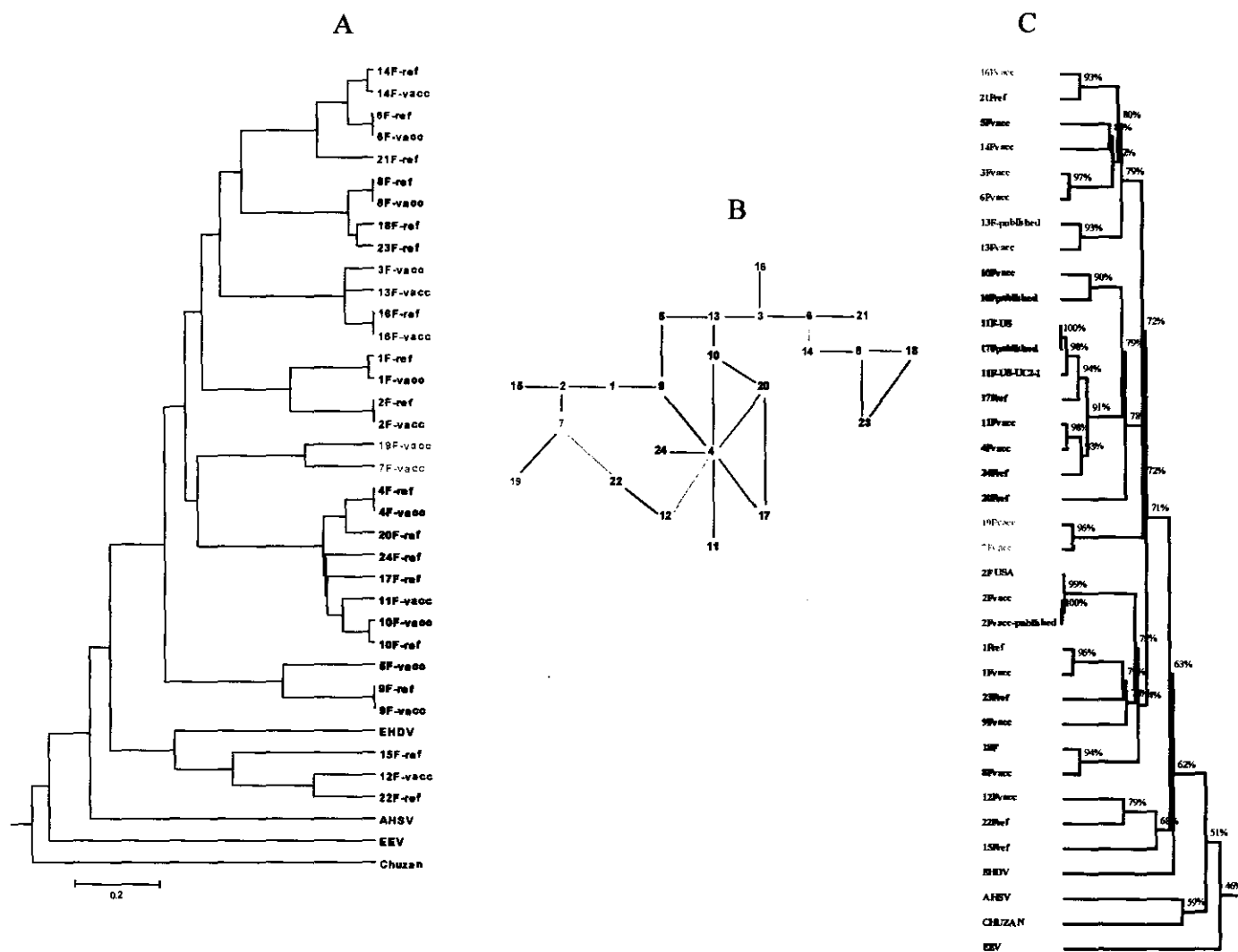


Figure 6.2 Comparison of the phylogenetic and serological analysis of VP2 and VP5 of all 24 BTV serotypes.

A. Dendrogram displaying the phylogenetic analysis of partial amino acid sequences of VP2 of all 24 BTV serotypes.

B. Dendrogram of the serological cross-reaction of all 24 BTV serotypes (adapted from B. J. Erasmus, *Bluetongue virus, in Virus Infections of Ruminants*, Z. Dinter and B. Morein (Eds), Elsevier Science Publishers, Amsterdam, Vol. 3, 227-237,(1990).

C. Dendrogram displaying the phylogenetic analysis of partial nucleotide sequences of the VP5 segment of all 24 BTV serotypes.

The databank at OVI will be developed specifically for African and South-African isolates of AHSV, BTV and EEV. In addition, the cloning of the full-length genomes of EEV serotypes Bryanston and Kyalami as full-length amplicons, has led to the sequencing of the complete genome of EEV (results to be published elsewhere). The cloning of the complete genome of phi12 has led to the sequencing of the complete genome and the elucidation of the gene assignments of the open reading frames (Chapter 5). The genome of phi12 which was said to be different from those of other phage isolates could be compared to the sequences of the genomes of other isolates (Chapter 5). This, together with the AHSV VP2 data and the range of viruses whose genomes were amplified thus far clearly shows that the new cloning method opens up the field of sequencing dsRNA genomes.

However, it is important to keep in mind that the new dsRNA cloning method uses PCR amplification and, thus, clones represent only one gene from an enormous pool of viruses. With the mutation rate of RNA viruses estimated to be 10^{-3} to 10^{-5} it is expected that the sequences of the same genes within a pool of viruses may differ.

For more accurate sequence determination from pools of viruses and getting an idea of the sequence variation, researchers can design oligonucleotide primers from nucleotide sequences of the terminal ends of the cloned genes and then directly sequence genes amplified by RT-PCR with specific primers and direct sequencing from these PCR products. However, in our hands recloning and sequencing of the same VP2-genes from different pools of viruses (different preparations of reference isolates) cloned with the newly described cloning method did not show any significant sequence variation (results not shown). Viruses from field isolates are, however, under immunological pressure and we have indeed found sequence variation from AHSV VP2 segments sequenced directly from RT-PCR products (JJO Koekemoer - personal communication).

Recently I cloned the entire BTV1 (reference strain) genome from cDNA without PCR amplification. This clearly highlighted another important factor to consider. The nucleotide sequences of several genes obtained from the BTV1 reference strain by direct cDNA cloning (data not shown) differed significantly from nucleotide sequences of the same reference strain determined by other researchers (Wade-Evans, 1988 and 1992). The only difference between the viruses used for cloning was their cell culture passage history as well as the cells they were propagated on. This shows that it is very important to state exactly how virus

preparations were done and report the cell culture passage history when submitting or publishing sequences.

6.2.2 Development of recombinant subunit vaccines

The main reason for the development of an efficient dsRNA cloning method at the OVI was to clone the VP2 genes from all nine AHSV reference serotypes for the development of a complete repertoire of recombinant subunit vaccines. It is important to keep in mind that horses need to be immune to all nine serotypes to be fully protected against the disease. However, the current live attenuated vaccine produced by Onderstepoort Biological Products (OBP) contains only 7 serotypes (1, 2, 3, 4, 6, 7 and 8). There are no vaccine strains for serotypes 5 and 9 (Van Dijk, 1998; Scanlen *et al.*, 2002). The original AHSV5 vaccine strain was discontinued when it proved to be not fully attenuated for foals after the neurotropic AHSV1 and AHSV6 vaccine strains were replaced with cell culture attenuated strains (Van der Meyden *et al.*, 1992; van Dijk., 1998). Attempts to develop a new fully attenuated serotype 5 vaccine strain has thus far not been successful. Serotype 9 has never been included in the OBP vaccine, since at the time of developing the vaccine (1970s) AHSV9 was not a problem in SA and serotype 6 provides some cross-protection. Another reason for developing recombinant subunit vaccine is that it will enable regulatory authorities and the horse industry to differentiate between vaccinated and naturally infected horses. This ability will significantly simplify the international movement of and trade in horses. Proof of concept for the efficiency of VP2-based AHSV vaccines exist. Baculovirus-expressed VP2 of AHSV serotypes 4 and 5 have already been shown to induce a fully protective immune response in horses (Roy *et al.*, 1996; Scanlen *et al.*, 2002).

In Chapter 3 the expression of VP2 proteins from 6 of the AHSV serotypes are shown. Previously three other serotypes have been expressed at OVI namely serotypes 3, 5 and 9. Therefore, the dsRNA cloning method developed in this study has enabled me to complete a full range of VP2 genes and expressed proteins for development of a complete set of recombinant vaccines and diagnostic reagents. The vaccine potential of the recombinant VP2s of all nine AHSV serotypes is now being evaluated.

6.2.3 Development of reagents and methods for molecular diagnostics

The first two important facts to establish in outbreaks of *Orbivirus* diseases are, which virus is involved, and which serotype of the particular virus. In the 20 years preceding this study it was shown repeatedly that the nucleotide sequences of some orbivirus genes were highly conserved across serotypes and closely related within a serogroup (such as the genes encoding the core, namely VP1, 3, 4, 6 and 7, and the non-structural protein NS1). The genes encoding the outer capsid protein VP2 varied substantially across and even within serotypes (Bremer *et al.*, 1990, Koekemoer *et al.*, 2003 and this study). These findings have led to the development of molecular diagnostic techniques for AHSV and BTV. For AHSV and BTV serogroup-specific NS1-gene based RT-PCRs have been developed (Bremer *et al.*, 1990; Wade -Evans *et al.*, 1990; Wilson *et al.*, 1993). These group-specific PCR methods are very useful in viral diagnosis and are among the OIE's prescribed diagnostic procedures for the respective viruses. However, the development of serotype-specific molecular diagnostic techniques for these viruses such as RT-PCRs have been hampered by the unavailability of cDNA and/or nucleotide sequences of the VP2-genes for all the serotypes.

Two different molecular diagnostic methods were developed based on the full set of cloned AHSV VP2 genes and their complete sequence data produced in this study. The first method was a hybridization procedure using the complete set of VP2-genes as serotype-specific probes (Chapter 4). The main advantage of this method was that it reduced the time for serotyping isolates from the 2-3 weeks needed for serology to 4-5 days. The second new and even quicker molecular method for AHSV serotyping which has recently been developed is based on the complete set of VP2 sequences that became available as a result of this study. It is a reverse line blot hybridization procedure. Primers for the RT-PCR of the first 550bp of the VP2 genes of all nine serotypes have been developed that makes it possible to amplify the first 550bp of the VP2 gene of any AHSV isolate in a single one-step RT-PCR. This PCR product can be labeled directly by incorporating DIG-labelled nucleotides in the PCR. The labeled PCR product is then used as a probe against the cDNAs of the nine different serotypes of AHSV. This method is able to serotype field isolates of AHSV from samples like spleen, lung or blood within 24 hours (J. J. O. Koekemoer - personal communication).

6.2.4 Reverse genetics

It is anticipated that the new dsRNA cloning method developed in this study will be instrumental in the development of reverse genetic systems for members of the family *Reoviridae*. Reverse genetics of viruses involves a system whereby replicating virus is rescued from its genetic material or genetic material and its protein constituents. Such a system allows researchers to mutate the genetic material of viruses and therefore study each of the factors involved in viral replication through mutation. For single-stranded RNA viruses, systems have been developed for the rescue of virus from the cDNAs representing the complete genomes of these viruses such as influenza A (Fodor *et al.*, 1999; Neumann *et al.*, 1999), rabies virus (Schnell *et al.*, 1994), vesicular stomatitis virus (Lawson *et al.*, 1995; Whelan *et al.*, 1995) and Newcastle disease virus (Peeters *et al.*, 1999) to name but a few.

To date however, reverse genetic systems have only been developed for three types of dsRNA viruses, namely for the birnaviruses (Yao and Vakharia, 1998; Boot *et al.*, 1999), the cystovirus bacteriophage phi6 (Olkonen *et al.*, 1990) and reovirus (Roner and Joklik, 2001). Purified dsRNA from the *Reoviridae* is not infectious (Mertens *et al.*, 2000). In fact, true rescue of dsRNA viruses have only been achieved for viruses/bacteriophages with 2 or 3 dsRNA segments.

The very first dsRNA reverse genetic system developed was that of bacteriophage phi6 (Olkonen *et al.*, 1990). Recently, bacteriophage phi6 has also been rescued by self-assembly of the virus from its purified protein and RNA constituents (Poranen *et al.*, 2001). The two birnaviruses, namely, infectious pancreatic necrosis virus (IPNV) and infectious bursal disease virus (IBDV) have both been rescued from the cDNA of their two dsRNA segments. IBDV was first rescued using infectious *in vitro* produced ssRNA (Mundt and Vakharia, 1996) and later from cDNA expressed *in vivo* from plasmids (Boot *et al.*, 1999). IPNV has also been rescued by *in vivo* expression of mRNA from cDNA (Yao and Vakharia, 1998). Reovirus was first rescued from mRNA derived from transcription of cores (therefore not recombinantly) as well as its *in vitro* translated protein constituents (Roner and Joklik, 1990). Both these elements were introduced at the same time into cells followed by infection with a temperature mutant 'helper' virus. This was recently followed by rescue of recombinant virus using the same procedure but replacing one of the mRNA segments with a recombinantly synthesized mRNA containing the gene encoding chloramphenicol transferase or CAT

(Roner and Joklik, 2001). The reovirus system, although said to be efficient, is difficult and messy and still does not allow rescue of virus completely from cDNA without "helper virus". It would be very useful to have a robust system for the pathogenic viruses of the family *Reoviridae*. To date no robust system similar to those of the ssRNA viruses (Reviewed by Neumann *et al.*, 2002) has been successful for the reverse genetics of the *Reoviridae* from cloned cDNA.

The failure to rescue members of the *Reoviridae* from cDNA is most likely due to the fact that they contain complex genomes with many segments (see Table 1) as well as the fact that historically cDNAs of the complete genomes of these viruses are often mixture from various isolates of the same virus or even viruses from different cell culture passages (see 6.2.1). These genes may not be compatible to rescue infectious virus. With the new dsRNA cloning method developed in this study a complete set of full-length genes from a homogenous set of viruses can be cloned. However, PCR mutations may hamper reverse genetics studies.

Subsequent to the work presented in this thesis, both these obstacles were largely overcome by cloning the complete genome of the BTV serotype 1 reference strain directly from cDNA (data not shown). cDNA was prepared using the same oligo-ligation method described in this study up to the cDNA annealing step. Annealed cDNA were restriction digested with enzymes for which sites exist in the ligated oligonucleotide sequences and cloned into the same restriction sites of pUC18 and pGem3Z vectors. Copious amounts (>3 ug) of total dsRNA were, however, needed. This method does still not exclude the possibility of mutations in the cDNA since reverse transcriptase enzymes have relatively high mutations rates (see Chapter 1). The use of these cloned cDNAs to rescue BTV is currently under investigation.

6.3 Important aspects of the optimised dsRNA cloning method

The most critical factors affecting the efficiency of the dsRNA cloning method developed in this study are as follows:

6.3.1 The quality of dsRNA

The quality of the dsRNA depends on the sample from which it is extracted as well as the method used for extraction. It was found that extraction from old clinical samples (like rotavirus stool samples that were stored properly for years) does not necessarily have an adverse effect on the quality of the dsRNA. Small amounts of contaminating single-stranded RNA also do not seem to affect the success of the method. A factor that was found to adversely affect cloning of full-length amplicons was the presence of degraded dsRNA. Even in cases where small amounts of degraded dsRNA were present (*Botrytis cinerae* mycovirus dsRNA) large smears of amplification products were present (data not shown). These degraded dsRNAs are often present after the large amounts of single-stranded RNAs are removed from total RNA by digestion with RNaseA at high salt concentration. This problem can be overcome by separating dsRNA segments on agarose gels and extracting individual segments prior to oligo-ligation. Despite these precautions, in some cases genes were cloned that missed 5-100 bp at their terminal ends (phytoreovirus and mycovirus - results not shown).

6.3.2 Oligonucleotide preparations

High quality oligonucleotides are needed to ensure successful dsRNA cloning. Only oligonucleotides prepared by certain manufacturers worked well in the current procedure. The oligonucleotides used for the ligation step were somewhat more tolerant of variation in quality. The reason for the difference in quality from different manufacturers is not clear but is probably related to the purification procedures used and possibly to the presence of primers with wrong nucleotide sequences. Currently oligonucleotides of only two suppliers have been found to be consistently reliable, namely those of Invitrogen (formerly Gibco) and Tib Molbiol.

6.3.3 Buffers and enzymes for oligo-ligation and cDNA synthesis

The buffers for oligo-ligation should preferably be made fresh and it is not advised to use the buffers supplied by the manufacturers. Furthermore, it is crucial that 10% DMSO is used in the ligation reaction, since this significantly increased ligation efficiency. It is also not recommended that old enzymes are used since their activity can no longer be guaranteed.

Both the ligation buffer and T4 RNA ligase could, however, be stored at -70°C for at least 6 months without loss of more than 20 % of its activity.

Buffers used for cDNA synthesis should also be made fresh and should not contain DTT (like the buffers supplied with enzymes), since DTT may lead to precipitates upon addition to samples containing unreduced MMOH. The presence of β -mercaptoethanol in the cDNA buffer (see final method) is, therefore, of great importance to reduce MMOH. It is important to note that the optimised buffer used in this method contains different concentrations of KCl and MgCl₂ than the commercial buffers.

6.3.4 The use of MMOH for dsRNA denaturation

The use of Methylmercury (II) hydroxide for denaturation of ligated dsRNA is strongly recommended. It has been reported (Wilson and Chase, 1993) that MMOH is at least ten times more efficient in denaturing dsRNA than DMSO and heat. Denaturation of dsRNA should, where possible, be ascertained by agarose gel electrophoresis prior to cDNA synthesis as this step is crucial to the success of the method. In some countries, however, the use of MMOH is not allowed. In these cases it is recommended that large dsRNA segments are separated and purified. The use of DMSO and heat did not result in amplification of the larger segments when it was attempted to amplify the whole genome (results not shown). Also, with the use of DMSO and heat more PCR cycles should be performed to obtain enough amplicons for cloning (3-5 cycles more).

Another factor that did not influence the method of Attoui *et al.* (2000a) was, however, crucial to the success of the current method. This is the cDNA annealing step after denaturation of excess RNA. This step was, however, shortened significantly compared to that of Vreede *et al.* (1998) - See final method in Figure 6.3.

6.4 Limitations and suggestions

While the cloning method has proven very useful thus far in cloning dsRNA genomes, there are currently still a few limitations to the procedure.

The first is the size limit. While the genome of phi12 (segments 2.3, 4.2 and 6.8 kb) could be successfully amplified (Chapter 2), the genome of a chimeric phi6 genome segment (Kindly supplied by Dr L Mindich) with a size of almost 14 kb could not be amplified. This limitation is thought to be brought about by the fact that the AMV reverse transcription enzyme that was used for reverse transcription (Chapter 2) has a limit of 5 kb (Promega). In the mean time new AMV-based reverse transcriptase enzymes are now on the market that are said to be able to produce cDNAs of up to 14 kb (Invitrogen, Sigma, Roche). Since the DNA polymerase used currently (Takara Ex *Taq*) can efficiently amplify segments of up to 20 kb, the use of the new RTs should theoretically make it possible to amplify these very large dsRNA segments.

The second limitation to the method is the presence of PCR mutations present in cloned cDNA molecules. The error rate of the enzyme reverse transcriptase is said to be 10^{-4} (Ji *et al.*, 1992). The error rate of the polymerases used in PCR differs substantially and varies between 2×10^{-4} to 6×10^{-6} according to manufacturers. The enzyme used to generate the results presented in this study, Takara Ex *Taq*, has an error rate roughly 4 times less than that of *Taq* polymerase (Takara Shimadzu corporation). Despite the lower amount of PCR cycles used in this study for the cloning of some genomes (Chapter 2) it was found that some cloned cDNAs segments contained PCR mutations. Premature stop codons were detected either by sequencing or by expression of truncated proteins (results not shown). The incidence of this was, however, very low.

I strongly suggest that the enzyme used for PCR amplification should have the lowest error rate possible. Since the completion of the results presented in this thesis, Expand Hi-Fi (Roche) has become my DNA polymerase of choice for PCR to amplify whole genomes, since it gives similar yields of whole genome amplicons and has a significantly lower error-rate than Takara Ex *Taq*. Recent studies also suggested that this enzyme gave very low error rates when used in conjunction with AMV or MuMLV RT enzymes (Malet *et al.*, 2003). They have also shown that, in general, error rates are sometimes even lower than those predicted by enzyme manufacturers.

Also it is suggested that where possible nucleotide sequence determination should be done directly from cDNA. This is possible in cases where copious amounts of separated amplified cDNA segments are available (Dr Shujin Rao, Pirbright - personal communication.)

Comparison of sequence data from amplified cDNA with that of cloned cDNA should be done to make absolutely sure that no mutations are present in cloned cDNAs.

6.5 Final method for the amplification and cloning of complete viral dsRNA genomes

This section describes the final method for dsRNA cloning as I currently use it. It is described in a practical laboratory protocol format.

MATERIALS

The chemicals and primers are from the following manufacturers:

Water

- HPLC grade from BDH: no 152736D. This is used this to prepare all RNase free stock solutions. Note that DEPC treated water is not used.

Enzymes

- T4 RNA Ligase (Amersham E2050Y, 600U, 40U/microliter)
- AMV Reverse transcriptase (Promega M5108 or alternatively Invitrogen's Cloned AMV RT 12328-019 - very good RT enzyme but very expensive)
- Polymerase for PCR: Takara Ex *Taq* (TaKaRa -RR001A - Use the supplied dNTPs for the cDNA synthesis as well) Alternatively, if copious amounts of dsRNA are available try proofreading enzymes like Roche's Expand and use it in the Takara buffer.

Plastic ware

- Tubes for purification of dsRNA: Safelock tubes from Eppendorf (Note that once again no DEPC was used neither sterilizing by autoclaving - use as is, they work well). Alternatively use DNase RNase free tubes from Eppendorf called Eppendorf Biopur
- PCR tubes are DNase and RNase free from Abgene AB-0620 (here you can substitute with whatever you use)

Chemicals

Most Chemicals are from SIGMA .

- HEPES Sigma Cat No H-0891
- DMSO Sigma Cat No D-8418
- MgCl₂ Sigma Cat No M-9272
- 1M MMOH Alfa Aesar (see their website)
- 2-Mercaptoethanol Sigma Cat No M-3148
- KCl Sigma Cat No P-9541
- Trizma Pre-Set Crystals pH8.3 Sigma Cat No T8943
- DTT Roche Cat No 197 777
- ATP Roche Cat No 519 979

Primers (for best results only use the suppliers mentioned)

- PC3 (ligation primer) 5' PO₄-GGATCCCGGGAATTCGG(A)₁₇-NH₂ 3'(HPLC purified) from MWG or Tib Molbiol (Invitrogen does not do the 3' amino)
- OligodT17 (the success of the method depends mostly on this primer) 5'-TTTTTTTTTTTTTTTTTT-3' from Invitrogen or Tib Molbiol- preferably from Invitrogen - HPLC purified. You can have it 5' phosphorylated if you want to clone cDNA directly without PCR amplification.
- PC2 (PCR primer) 5' P-CCGAATTCCTCCGGGATCC-3' (HPLC purified from Invitrogen or Tib Molbiol - preferably from Invitrogen - The 5' end is phosphorylated for better cloning of PCR products (then you can dephosphorylate your TA cloning vector.)

Kits

- Qiaquick Gel Extraction Kit (50) Cat No 28704 or 28604 (minElute for limited starting material)

METHOD

dsRNA extraction

Infect cell cultures and incubate until CPE is well advanced (>70%). DsRNA is usually extracted from one infected 75 cm² flask of BHK-cells for BTV, AHSV and EEV. For rotavirus use as much stool as possible (Do not centrifuge the stool and use the supernatant, but use it as is.)

We extract dsRNA using the commercial reagents Trizol (Gibco-BRL Life Technologies) or TRI-Reagent (Molecular Research Centre), which is basically the acid-phenol/guanidinium thiocyanate procedure (skip the EtOH wash). Dissolve RNA pellet in 120 µl of RNase free water or EB buffer from the Qiagen kit. This is followed by precipitation with 2 M LiCl to remove single-stranded RNA (if necessary) as follows: Add 8 M LiCl to a final concentration of 2 M (40 µl LiCl to 120 µl total RNA). Leave overnight at 4°C or if you are in a hurry put on water/ice slurry for 2 hours. Centrifuge at 14 000-16 000 × g for 30 minutes. The dsRNA is purified from the supernatant using a column from a Gel Extraction Kit (Qiagen). Alternatively, adjust the LiCl concentration in the supernatant to 4 M leave at 4 °C to precipitate the dsRNA and collect the dsRNA precipitate by centrifugation, wash with 70% EtOH and dissolve in RNase-free water or buffer EB from Qiagen kits.

Oligo-ligation

Primer PC3 (5' PO₄-GGATCCCGGGAATTCGG(A)₁₇-NH₂ 3') at a final concentration of 10 nanograms per microliter, is ligated to 1-1000 nanograms (or more if you want to) of purified dsRNA in 50 mM Hepes, 18 mM MgCl₂, 3 mM DTT, 1 mM ATP, 10 µg/ml BSA , 10 % DMSO and 25-40 U of T4 RNA ligase at 17 or 37 °C for 16 hours in a final volume of 30 µl. It should be noted that for optimal results the buffer is freshly prepared for every reaction.

purified dsRNA	22 μ l
10 \times ligation buffer (made fresh)	3 μ l
DMSO	3 μ l
primer PC3 (1 μ g/ μ l)	1 μ l
T4 RNA ligase (40 U/ μ l)	<u>1μl</u>
Total	30 μ l

Incubate at 17°C for 16 hours or at 37°C for two hours

cDNA synthesis

Ligated dsRNAs are purified over a Qiagen gel extraction column as per instructions for purification of dsDNA fragments. The purified ligated dsRNA is denatured by addition of 1 M MMOH (Alpha Aesar) to a final concentration of 50 mM in a final volume of 6 μ l and incubation for at least 30 minutes. cDNA of the complete genome is synthesized in a reaction containing 50 mM Tris (pH8.3), 10 mM MgCl₂, 70 mM KCl, 0.5 mM of each dNTP (from Takara kit), 50 mM β - mercaptoethanol, 500 nanograms of Oligo dT (17) and 5 Units of AMV Reverse Transcriptase in a final volume of 30 μ l (make buffer fresh or at least use fresh β - mercaptoethanol). Making master mixes for multiple samples works well (see Figure 6.1 C). The reaction is incubated at 42°C for 45 minutes followed by incubation at 50 °C for 15 minutes. The reaction is stopped by the addition of EDTA to a final concentration of 10 mM. Residual RNA is removed by addition of NaOH to a final concentration of 100 mM and incubating the reaction at 65 °C for 30 minutes. Tris (pH7.5-8.0) is then added to a final concentration of 100mM and HCl also to a final concentration of 100 mM. The reaction is incubated at 65 °C for one hour (longer incubation was not found to be necessary) to anneal the cDNA. The overhangs of the cDNA are filled in during the PCR reaction by incubating at 72°C for 5 minutes before the actual PCR cycling starts.

PCR amplification

To amplify the cDNA of the whole genome, 5 μ l of cDNA (without prior purification) is amplified in a 50 μ l PCR reaction containing 1 \times Takara Ex *Taq* buffer, 200 nM dNTPs, 5

Units of Takara Ex *Taq* polymerase and 100 picomoles of primer PC2 (5' P-CCGAATTCCCGGGATCC-3'). The reaction is incubated at 72 °C for 2-5 minutes (during which time the partial overhangs of the cDNAs are filled in) followed by incubation at 94 °C for 2 minutes. This is followed by 20-30 cycles of 94 °C for 30 seconds, 67 °C (depending on the T_m of the primer sequence) for 30 seconds and 72 °C for 4-7 minutes (depending on the size of the largest genome segment - allow 1minute per 1kb for largest segment). The annealing temperature of 67 °C may be lowered to 60 °C in the case of difficult templates but non-specific amplicons can then be expected. Therefore, the pattern of the PCR-amplicons should be compared with the pattern of the original dsRNA using AGE. The quality and quantity of the PCR amplicons are viewed after separation on a 1% agarose gel in TBE buffer stained with ethidium bromide.

Cloning of amplified cDNA products

Separate amplicons on a 1% TAE agarose gel and cut out desired bands. It is preferable to use Sybergold staining and visualise the amplicons on a DarkReader rather than using ethidium bromide and UV. Clone PCR products into a T/A cloning vector. Use blunt ended vectors for PCR products amplified with proofreading enzymes. Vectors may be dephosphorylated since all PCR products are phosphorylated (PCR primers are 5' phosphorylated). Use blue/white screening with negative and positive controls. Do not discard blue or light-blue colonies. Our experience is that cloned segments of up to 3.6 kb (e.g. rotavirus VP1) can give rise to blue colonies.

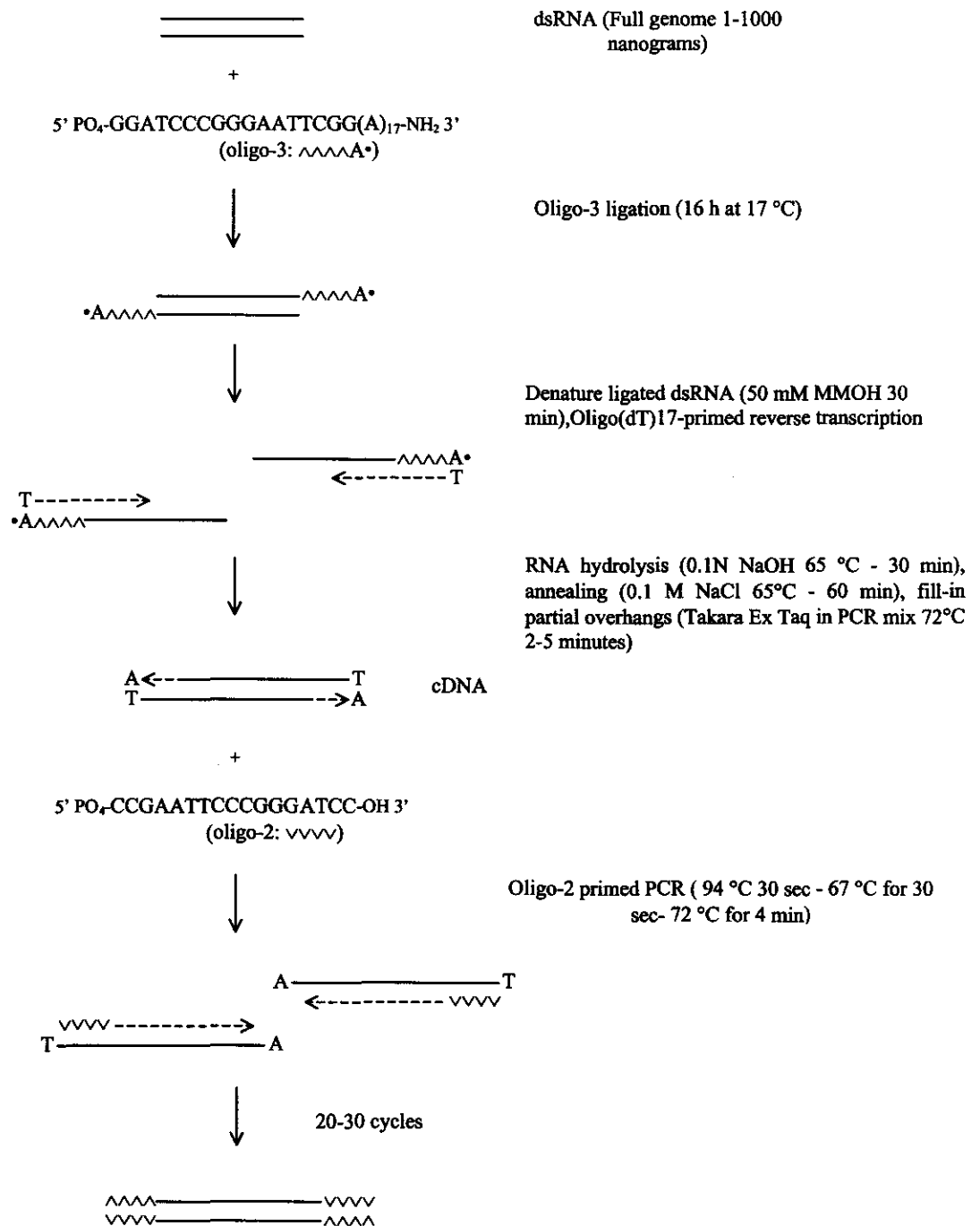


Figure 6.3 Diagram showing the final method for amplification of complete dsRNA genomes.

CHAPTER 7

Concluding summary

This study was initiated to address the problem of the lack of a procedure to clone large genes (>3.0 kb) of dsRNA viruses as complete full-length cDNAs. The problem specifically bottlenecked the recombinant subunit vaccine development projects for AHS and BT at the Onderstepoort Veterinary Institute. In addition, it prevented comprehensive molecular studies of all the members of the family *Reoviridae* not only in South Africa but also worldwide. The outcome of this study was that a sequence-independent single-primer amplification method for cloning large dsRNA genes was perfected and shown to be fast, robust, simple, efficient and suitable for routine use.

The most significant contribution to dsRNA cloning of this method is that it allows amplification of complete genome sets in single (one tube) reactions for oligo-ligation, reverse transcription and PCR amplification. This makes the method the most efficient and user friendly method of all published cloning and sequencing methods to date. Full-length genes and/or complete genomes can be cloned within 4-5 working days. The method can be used to clone dsRNA genomes from both prokaryotic and eukaryotic viruses since it does not rely on the 5' termini of the dsRNAs being capped as that of Attoui *et al.* (2000a). In this study the complete dsRNA genomes from various sources including mammalian, plant, fungal and bacterial viruses were amplified and cloned without any prior knowledge of the genome sequence. This is the first record of a method that allows the amplification of complete dsRNA genomes of many different dsRNA viruses, each in a single PCR reaction.

In all, nine complete genome sets of members of the family *Reoviridae* (size range 0.8 to 4.5 kb) were amplified of which eight were cloned, namely those of AHSV1, AHSV2, BTV1, EEV Bryanston, EEV Kyalami, a human group A rotavirus, a human group C rotavirus, reovirus Dearing and a *Tobacco leaf enation phytoreovirus*. The group A rotavirus genome was cloned from dsRNA extracted directly from a stool sample, demonstrating that the method can be used for non-cultivated clinical virus samples. It was shown that the method is highly sensitive, so much so that the complete group C rotavirus genome could be amplified in a single PCR reaction and cloned from less than 1 nanogram of dsRNA. The maximum

size of dsRNA genes that could be cloned was tested. The genome of bacteriophage ϕ 12, a cystovirus, of which all three genome segments are large, viz. 2.3, 4.1 and 6.8 kb respectively was also amplified in a single PCR and cloned. The 6.8 kb gene is the largest dsRNA gene that has been amplified and cloned as a full length segment by any method. A chimeric cystovirus dsRNA segment of approximately 14 kb could not be cloned. The complete set of outer capsid VP2 genes of all 9 AHSV and 24 BTV serotypes have been cloned. These provide the basis for the future development of complete repertoires of recombinant subunit vaccines and serotyping diagnostic methods and reagents for these serious orbivirus diseases.

The successful cloning of large (>2.5 kb) dsRNA genes finally perfects the original sequence-independent amplification and cloning method and reaches the two ultimate goals for dsRNA cloning formulated by Lambden *et al.* (1992). The first goal, that of being able to clone non-cultivable human rotavirus genomes directly from small clinical samples, was met by cloning of two complete rotavirus genomes directly from stool samples, one from an uncultured human group A rotavirus and another from 1 ng starting material of a group C rotavirus. The second goal, namely that the method should be applicable without prior knowledge of any sequence information and generate full-length cDNA clones of each genome segment, allowing unequivocal delineation of the 5'- and 3'-terminal gene sequences, was achieved by cloning two complete EEV genome sets.

A drawback of the PCR amplification stage of the procedure is the possibility of introducing point mutations due to the error rates of the DNA polymerases used in PCR amplification. To avoid this problem, experiments were done during this study to directly clone the cDNA without PCR amplification. Cloning of cDNA was feasible when relatively large amounts (>3 ug) of total dsRNA of complete genomes were available. This is the first report of cloning of a complete dsRNA genome as full-length genes using an oligo-ligation method without prior PCR amplification. This enables researchers to have specific restriction sites present flanking either end of the full-length cDNAs. This is a very important step for cloning cDNA without PCR amplification to be used for functional and expression studies, the development of recombinant vaccines and, ultimately, for the anticipated development of the first reverse genetics system for the *Reoviridae*.

Cloned viral dsRNA genomes could be used for the determination of the nucleotide sequence of dsRNA genomes and individual genes. This sequence information was suitable for and allowed phylogenetic studies. The phylogenetic studies of AHSV and BTV VP2 amino acid sequences showed the same grouping as serology. This again re-confirmed the fact that VP2 is the main determinant of the serotype in these viruses. Furthermore, the sequences allows development of sequence databanks for the dsRNA viruses and molecular epidemiological studies. We developed a method that overcomes the tedious procedures of typing AHS isolates serologically. The cloned AHSV VP2 genes were used to produce DIG-labeled probes to hybridize serotype specifically to corresponding denatured dsRNA. This method allowed serotyping of field isolates of AHSV within 4 days as opposed to the 2 weeks using traditional serological procedures.

Historically larger genes often had to be pieced together from smaller clones, especially in cases where full-length large genes were needed for expression purposes. Here the full-length cloned cDNAs were shown to have full open reading frames and allowed recombinant protein expression. This in turn has allowed structure and functional studies of viral proteins, specifically the production of EEV CLPs. The limitation of not having a complete repertoire of all nine AHSV VP2 genes was overcome by the cloning, sequencing and expression of the VP2 genes of all 9 AHSV serotypes. This is the first time that a full set of VP2 genes from all the serotypes of an important orbivirus has been cloned, sequenced and expressed. It has allowed the development of recombinant vaccines for all 9 serotypes of AHSV which are currently under investigation. This sets the scene for development of complete repertoires of vaccines for all dsRNA viruses of veterinary importance at the OVI.

While some limits are still evident such as the possibility of PCR errors, as well as the inability to amplify and clone very long dsRNA segments (>10 kb), it is estimated that most of the limits of the current method will soon be overcome by the introduction of new and more efficient enzymes for reverse transcription and PCR. Already we have been able to clone a complete genome of BTV directly from cDNA without PCR amplification. It is just a matter of time before new RT enzymes will have the ability to transcribe very large RNA templates. Already AMV based enzymes are available that is said to be able to amplify 14 kb (Roche). This will overcome the two major restrictions that the cloning method currently has.

In conclusion, all of the aims of this study have been met and some aims have even been surpassed. This study has made a significant contribution to enable dsRNA molecular biology to deliver on many of the promises it has to offer as far as dsRNA virus structure, function, molecular diagnostics, epidemiology and recombinant vaccines are concerned.

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