Development of a single real-time RT-PCR method for the group-specific identification of African horsesickness virus and bluetongue virus

By

Lesego Gladys Modibedi

Submitted in partial fulfillment of the requirements for the degree Magister
Scientiae in the Faculty of Veterinary Science
Department of Veterinary Tropical Diseases
University of Pretoria

Supervisor: Dr J.J.O. Koekemoer

DECEMBER 2008

DECLARATION

I hereby declare that the work in this dissertation is my own and has not previously been submitted for a degree at any other university.

ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the generosity of the following individuals:

Dr. Otto Koekemoer for all the guidance, his critical but constructive valuable advice and support throughout the period of this study. With his enthusiasm, inspiration and great effort at explaining things clearly and simply made this an exciting project to work on.

Ms Isabelle Wright for helping with cell culture techniques and for generously providing the virus samples.

Dr. Christiaan Potgieter for providing the BTV field samples.

The R & T manager at OVI, **Dr. T Musoke** for making available the resources needed to do this work.
I wish to thank the staff at the Biochemistry Division, my student colleagues in the DST programme and my friends at OVI for all the support and caring they provided.

I wish to thank my entire extended family, my siblings: Lerato, Kgololosego and Kgopolo, my son, Letlhogonolo and my niece Lesedi for providing a loving environment for me.

To my partner Lesetja, I thank you for your support and all the sound advice.

Lastly and most importantly, I would like to thank my parents; who raised me, supported me, unconditionally loved me and passed on the values that continue to sustain me. **"Ke a leboga Batlagadi".**

Exodus 15:2

The Lord is my strength and song, He has become my salvation; He is my God, and I will praise Him.

SUMMARY

African horsesickness is an infectious but non-contagious disease caused by an orbivirus belonging to the *Reoviridae* family. The disease is classified as notifiable by the OIE because of the potential severe economic consequences that can result from outbreaks. Bluetongue, an arthropod-transmitted disease of wild and domestic ruminants, is caused by the bluetongue virus, which is the prototype species of the genus *Orbivirus*. Bluetongue is also a notifiable disease because it can spread very rapidly in naïve populations of susceptible livestock. Strict restrictions have been issued for the trade in animals and animal products from infected areas.

In the present study, a duplex one-step real-time RT-PCR using the fluorogenic dye SYBR® Green I was developed for the specific detection and identification of AHSV and BTV in one reaction, using melting temperatures (T_m) to discriminate between the viruses. Two primers pairs were designed to bind to areas of homology within genome segment 7 (VP7) of AHSV and genome segment 5 (NS1) of BTV respectively. The duplex real-time RT-PCR based test utilizes single tube RT-PCR amplification in which AHSV and BTV primers were used simultaneously.

The RT-PCR primers amplified 232 bp of genome segment 7 from all nine serotypes of AHSV and 79 bp of genome segment 5 from all 22 BTV serotypes that were tested. When both viruses were present, two melting peaks were simultaneously generated at 76.30 °C and 80.04 °C representative of BTV and AHSV amplification products respectively. Serogroup-specific products were amplified from dsRNA of field isolates of AHSV and BTV. dsRNA from EHDV and EEV failed to demonstrate either the 232 bp specific AHSV PCR product or the 79 bp specific BTV product.

These results indicate that the duplex real-time RT-PCR could be a useful technique for detection of AHSV and BTV from isolated viral dsRNA.

LIST OF ABBREVIATIONS

AGID	Agar gel immunodiffusion
AHS	African horsesickness
AMV	Avian Myeloblastosis Virus
βME	Beta-mercapto-ethanol
BT	Bluetongue
BTV	Bluetongue virus
CsCl	Cesium chloride
cDNA	Complementary deoxyribonucleic acid
CP	Crossing points
CPE	Cytopathic effect
Ct	Threshold cycle
^{0}C	degrees Celsius
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphates
dsRNA	double-stranded Ribonucleic acid
EEV	Equine encephalosis virus
EHDV	Epizootic haemorrhagic disease virus
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's minimum essential medium
KCl	Potassium chloride
LiCl	Lithium chloride
µl	microlitre

MGB	Minor groove binder
MgCl	Magnesium chloride
ml	millilitre
mM	millimolar
MMOH	Methyl Mercury (II) hydroxide
mRNA	Messenger RNA
Nt	Nucleotide
NTC	No template control
NS	Non-structural
OIE	Office International des epizooties
OVI	Onderstepoort Veterinary Institute
PAGE	Polyacrylamide gel electrophoresis
Pfu	Plaque forming units
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase Polymerase chain reaction
ssRNA	Single-stranded ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
TE	Tris-EDTA
T_m	Melting temperature
U	Unit
µg/ml	microgram per milliliter
UV	Ultra violet
VP	Viral protein
£	Sterling
g/cm^3	grams per cubic centimeter

TABLE OF CONTENTS

CHAPTER 1

1. *LITERATURE REVIEW*

1.1. INTRODUCTION

African horsesickness (AHS) is a disease that has originally been described in Africa. In Southern Africa it was recognized subsequent to the introduction of horses by the first settlers in the Cape of Good Hope in 1652. The first major outbreak occurred in 1719 when over 1700 animals died and over the next 200 years major and several lesser outbreaks have been recorded in Southern Africa (Henning, 1956). Travelers such as Livingstone, Anderssen, Burchell and Scherzer were unable to use their horses due to severe AHS (Flemming, 1878).

The methods that have been prescribed for international trade (OIE Manual of Standards for Diagnostic Tests and Vaccines, 2004), such as complement fixation (McIntosh, 1956) rely upon virus isolation procedures followed by virus neutralization tests to serotype the virus using serotype-specific antisera. This can take about two to three weeks before completion.

Bluetongue is a disease of sheep and is characterized by fever and lesions of the mouth and feet. The name bluetongue was derived from the bluish discoloration of the lining of the lips, the tongue and the gums. It has been recognized since the introduction of merino sheep into the Cape colony in the late 18th century. The first incidence of bluetongue in the temperate areas of South Africa was reported by Spreull (1902) and it was described as "malarial catarrhal fever of sheep". Later a full description of the disease was given by Spreull where he indicated that bluetongue is not contagious and was caused by a filterable agent (Spreull, 1905).

Bluetongue is usually not a fatal disease, but the infection has an economic impact due to production losses and restrictions placed on the export of animals or animal products from BTV endemic countries (MacLachlan *et al*., 1989). Conventional diagnostic assays that are used to identify the virus depend on virus isolation, followed by serial passage in cell culture to confirm the presence of the virus by the appearance of typical cytopathic effects. Routine serotyping is carried out by virus neutralization tests. The disadvantage of these assays is that they are cumbersome and may need to be repeated due to inconclusive results.

Agar gel immunodiffusion which detects anti-BTV antibodies and competitive enzyme linked immunosorbent assays (Afshar *et al*., 1989; Lunt *et al*., 1988) can also be used for laboratory confirmation of BTV. RT-PCR is also used for identification of viral nucleic acid in blood and other tissues of infected animals. Although an RT-PCR is available, it is a nested PCR (Katz *et al*., 1993) which means two amplification steps are needed, and it relies on gel based identification of the PCR products. It is very sensitive and specific but is labour intensive and due to the nested step, also prone to contamination.

1.2. ORBIVIRUS CLASSIFICATION

Orbiviruses constitute a genus within the *Reoviridae* family that includes vertebrate, arthropod, and plant pathogens. There are nine genera in the *Reoviridae* family which include *Orthoreovirus*, *Rotavirus*, *Coltivirus*, *Aquareovirus*, *Cypovirus*, *Fijivirus*, *Phytoreovirus* and *Oryzavirus*. The *Orbivirus* genus contains 20 confirmed serogroups, based on their serologic cross-reactivities. Several unclassified dsRNA viruses have also been isolated (Mertens *et al.,* 2000). Nine serotypes of African horsesickness virus (AHSV 1-9) (Howell, 1962; McIntosh, 1958) and 24 serotypes of bluetongue virus (BTV1-24) have been identified (Howell, 1969).

1.3. THE VIRUS

1.3.1. PHYSICAL ASPECTS OF ORBIVIRUSES

Orbiviruses are non-enveloped viruses with a genome consisting of ten double-stranded RNA segments (Verwoerd, 1970) each encoding at least one viral protein (seven structural and three or four non-structural) (Reviewed by Roy, 1989). The virion has a spherical appearance with an icosahedral capsid and a diameter of about 80 nanometers. The buoyant density of the virion is 1.36 g/cm^3 in CsCl. The virus can exhibit a marked decrease in infectivity around pH 6.5-10.2, and remains relatively stable around pH 8-9. At pH values less than 5.0 both virion and core are disrupted and the outer coat proteins may be lost. The virus has been shown to be fairly resistant to treatment with solvents or detergents (Reviewed by Mertens *et al*., 2000).

1.3.2. GENOME STRUCTURE, ORGANIZATION AND CODING ASSIGNMENTS

The ten BTV and AHSV dsRNA segments can be separated by PAGE. These segments are numbered 1 to 10 in order of migration and may also be referred to as large, medium and small segments (L1 to L3, M4 to M6, and S7 to S10) (Verwoerd *et al*., 1979). Each of the genome segments codes for seven structural proteins (VP1-VP7) and four non-structural (NS1-NS3/A) proteins (Verwoerd *et al*., 1970; Roy *et al*., 1990; Roy, 1992).The structural proteins are numbered in order of their decreasing sizes on SDS-PAGE. VP2 and VP5 are located on the outer capsid (Bremer et al., 1976; van Dijk & Huismans, 1982; Roy et al., 1994), and they determine the antigenic variability of the virus and the serotype (Verwoerd *et a*l., 1972; Huismans & van Dijk, 1990; Grimes *et al*., 1998).The two major proteins VP3 and VP7 and the three minor proteins VP1, VP4 and VP6 make up the inner shell or the core. VP7 is the most abundant protein in the core and it is highly conserved among the various BTV serotypes (Gumm & Newman, 1982; Huismans & van Dijk, 1990; Mertens *et al.,* 2000). Sequencing of cloned copies of genome segment 7 of various orbiviruses has shown that there is close similarity between BTV, AHSV and EHDV (Huismans & Erasmus, 1981; Wilson *et al*., 2000).

NS1 is present in the cytoplasm where it forms tubules which are characteristic of orbivirus replication (Nel *et al.,* 1991). NS2 is associated with the outer capsid and has the ability to bind single-stranded RNA; it also plays a role in virus replication (Huismans *et al*., 1987a; Van Staden *et al*., 1991; Kar *et al*., 2007). NS3/3a are both found in glycosylated and non-glycosylated forms in infected cells and are responsible for virus release from infected cells (Bansai *et al.,* 1998; Hyatt *et al.*,1993).

1.4. EPIDEMIOLOGY AND DISTRIBUTION

1.4.1. AFRICAN HORSESICKNESS

Du Toit (1944) discovered that both African horsesickness and bluetongue are transmitted by *Culicoides* midges when he injected emulsifications made from wild caught insects into animals. This finding was later confirmed by Wetzel and colleagues (1970) when they demonstrated the transmission of the virus by *Culicoides* from an infected insect to a susceptible horse.

Culicoides imicola has been considered to be the major species that is involved in transmission of AHSV in the field (Meiswinkel, 1998; Meiswinkel *et al*., 1994).

Because of this, AHS has a seasonal occurrence, and its prevalence is influenced by climatic conditions and other conditions which favour the breeding of *Culicoides* species. It is most prevalent in warm coastal regions or low lying, moist inland areas during the second half of summer (Coetzer & Guthrie, 2004).

African horsesickness is endemic in eastern, central and most parts of Southern Africa and occurs regularly throughout sub-Saharan Africa (Theiler, 1921; Barnard, 1998). There have been frequent reports of the disease in North African countries from where it has occasionally extended into countries of the Middle East and Spain. Outside Africa, AHS outbreaks have been experienced in countries such as Spain (1987-1990) and Portugal (1989) and it seemed as if the virus had overwintered four times in the process (Rodriguez *et al.*, 1993; Mellor & Hamblin, 2004).

1.4.2. BLUETONGUE

Bluetongue occurs throughout temperate and tropical regions of the world (Gibbs & Greiner, 1994; Mellor, 1990). The distribution of BTV is restricted to areas where competent *Culicoides* vectors are present and where climate conditions are favourable for adult vector activity (Mellor & Boorman, 1995; MacLachlan, 2004).

A variety of *Culicoides* species which include *Culicoides imicola*, *Culicoides variipennis*, *Culicoides insignis* and *Culicoides wadai* have been proven to be vectors of BTV in different parts of the world such as Africa, the Middle East, North America and Australia (Reviewed by Verwoerd & Erasmus, 2004). Prior to 1998, Europe had suffered BT epizootics but the disease was not endemic to the region. Recently, new outbreaks have occurred each year from 1998 to 2008 in southern and central Europe, involving five different serotypes (Mertens & Mellor, 2003; Purse *et al.,* 2005). Serotype 8 was identified during the European outbreak in 2006 infecting ruminants in a wide area across the country. During this period up to 10 %

of cattle in affected areas succumbed to the disease and the fatalities reported for sheep were between 30 and 50% (www.promedmail.org. accessed 20-08-2007).

1.5. CLINICAL SIGNS

1.5.1. AFRICAN HORSESICKNESS (AHS)

There are four recognized clinical forms of African horsesickness; the acute or pulmonary form, the subacute or cardiac form, the mixed form and the febrile form which is also known as “horsesickness fever” that is found in enzootic areas only (Rodriguez *et al.*, 1993; Theiler, 1921). The pulmonary form occurs most commonly when AHSV infects fully susceptible horses. Fever may be the only sign, following the incubation period and the rectal temperatures may reach a maximum of 41° C or even higher. Sometimes an apparently healthy horse may suddenly become listless, dyspnoeic and die shortly thereafter. Fever subsides gradually in recovering horses (Coetzer & Guthrie, 2004).

The mixed form is the most common form of AHS and is a combination of the cardiac and pulmonary forms of disease. This form of AHS is rarely diagnosed as such clinically. The diagnosis can only be made during necropsy when lesions of both the pulmonary and cardiac forms are observed. The mortality rate is approximately 70% and death usually follows three to six days after the onset of fever (Coetzer & Guthrie, 2004).The cardiac or sub-acute form of the disease is characterized by fever, subcutaneous oedematous swelling of the head and neck, and a time course of several weeks. Some animals may repeatedly lie down or appear restless when standing, and death usually occurs within four to eight days from the onset of fever. The mortality rate can exceed 95% (Coetzer & Guthrie, 2004).

1.5.2. BLUETONGUE (BT)

The clinical signs of bluetongue vary in different ruminant species and breeds of sheep. The severity of the disease is affected by environmental conditions and many infections in sheep are clinically inapparent. The course of the disease in sheep can vary from peracute to chronic, with a mortality rate of between 2 and 30 % (Verwoerd & Erasmus, 2004).

Cattle, game, goats and most wild ruminant species such as the American white-tailed deer, have an asymptomatic or subclinical bluetongue infection. In sheep the signs of bluetongue are reflected as congestion, oedema and haemorrhage as a consequence of virus-mediated vascular injury. When sheep die as a result of acute BT disease, the lungs may show interalveolar hyperaemia, severe alveolar oedema and the bronchial tree may be filled with froth. BTV infected sheep in endemic areas develop mild disease (MacLachlan, 2004; Erasmus, 1975).

1.6. LABORATORY CONFIRMATION OF CLINICAL DIAGNOSES

Most of the approved tests for orbivirus identification and serotyping require virus isolation from unclotted whole blood, spleen, lymph node or lung samples. For trade purposes, the OIE has prescribed procedures to achieve identification and serotyping and they are given in the Manual of Standards for Diagnostic tests and Vaccines (2004).

1.6.1. AFRICAN HORSESICKNESS (AHS)

Clinical signs and lesions in association with previous epidemiological information are often sufficient to allow clinical diagnoses to be made. However, AHS must be confirmed by isolation and identification of the virus because most of the clinical signs and macroscopic lesions are not characteristic of the disease (Mellor & Hamblin, 2004). The virus can be isolated either by direct inoculation onto cell culture, or by intracerebral inoculation of infant mice. The presence of the virus can be detected by monitoring mice for neurological signs. Virus neutralization which is type-specific for the 9 AHS serotypes is used for typing virus isolates (Howell, 1962). These methods require the use of laboratory animals for virus isolation and the production of antisera.

Several serological tests may be used for identification of virus isolates, these include complement fixation (McIntosh, 1956), ELISA (Laviada *et al.*, 1992; Hamblin *et al.*, 1991) and the agar gel immunodiffusion (Afshar *et al.,* 1989). Complement fixation can be used to distinguish between the 9 AHSV and other causes of disease in horses. It has been extensively used and has been approved for international trade by the OIE (Manual of Standards for diagnostic Tests and Vaccines, 2004). Even though it is frequently used for demonstration of group-specific antibodies against AHS virus, it has shortcomings such as the anti-complementary effect of some sera. An indirect ELISA and competitive ELISA using either AHS virus antigen or a recombinant protein have been validated. These procedures have been found to be sensitive enough to detect virus antigens from infected spleen samples, leading to minimal usage of complement fixation (Hamblin *et al.,* 1991; Wade-Evans *et al.*, 1993).

1.6.2. BLUETONGUE (BT)

Rapid and reliable confirmation of bluetongue virus and serotype differentiation is essential at the beginning of an outbreak to allow for early selection of the correct vaccine for the identified serotype. The attenuated live-virus vaccine that has been used to control BTV in endemic areas of South Africa comprises of three bottles containing five serotypes each, which have to be administered separately at three-week intervals. Traditionally, laboratory diagnosis of BTV has relied upon the detection of BTV antigen by virus isolation in embryonated chicken eggs followed by cell culture and virus neutralization tests. These techniques can take several weeks to confirm initial clinical diagnosis and they are labour intensive (Howell & Verwoerd, 1971; Erasmus, 1975).

Techniques such as complement fixation which detect group-specific antigens are often used to identify BTV. Even though it can not distinguish between different serotypes of BTV, it is able to distinguish the different orbiviruses. The agar gel immunodiffusion test has replaced complement fixation because it is simple to perform and it is easy to generate the antigen that is used. It has been endorsed as one of the standard testing procedures for international trade. However, this test has been shown to lack specificity in that it detects antibodies to other orbiviruses (EHD serogroup) (Afshar *et al.,* 1989; Reddington *et al.*, 1991).

Competitive ELISA which measures BTV-specific antibodies can be used to overcome the problem of cross reactivity with other orbiviruses (Afshar *et al.*, 1989). An indirect-ELISA which can be used to distinguish between natural infection and animals vaccinated with inactivated virus, based on the detection of NS3 has been reported (Laviada *et al.*, 1995). The applicability of this test is limited by the fact that inactivated vaccines are currently not in use.

1.7. NUCLEIC ACID BASED DIAGNOSTIC TESTS

1.7.1. POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (Saiki *et al.*, 1985; Mullis & Faloona, 1987) is an *in vitro* technique that allows amplification of a specific deoxyribonucleic acid region lying between two regions of known nucleotide sequences. It has had a great impact in recent years as a key development in molecular biology and follows the same principle as semi-conservative replication of DNA. One of the reasons for the success of the PCR is the simplicity of the reaction and relative ease of the practical manipulation steps.

The PCR consists of three distinct steps which include denaturation, annealing and extension. These steps are repeated 30-40 times or cycles. During denaturation, the double-stranded template DNA is denatured by heating the reaction to above 90 °C. The second step is annealing, during which the primers bind to their complementary bases on the single-stranded DNA at a temperature of 55 °C – 65 °C. The third step, extension, is carried out by a thermostable DNA polymerase. The most commonly used enzyme is Taq DNA polymerase. The DNA polymerase binds to the hybridized primer and begins to add complementary nucleotides forming a new strand. The result is a new double-stranded DNA molecule consisting of one of the original strands and one newly synthesized strand. As the system is taken through successive cycles of denaturation, annealing and extension, all the new strands will act as templates and there will be an exponential increase in the amount of DNA produced.

Kawasaki & co-workers (1988) reported on a procedure whereby RNA is converted into cDNA which can then be used as a template in PCR. The process is termed reverse transcription PCR. RT and PCR can make use of a single primer set to amplify specific fragments because the reverse transcription reaction is, as with PCR, initiated by the annealing of an oligonucleotide primer to a target sequence.

To render a better diagnostic service by reducing the time between suspicion of a disease and providing diagnostic results, molecular biology techniques like RT-PCR are being used. RT-PCR has proved to be the most powerful tool in the field of diagnostic medicine and has been successfully used to identify several infectious diseases of veterinary importance, including African horsesickness (Zientara *et al*., 1994; Zientara *et al*., 1995; Bremer & Viljoen, 1998; Sailleau *et al*., 2000, Koekemoer & van Dijk, 2004) and bluetongue (Wade-Evans *et al*., 1990; Akita *et al*., 1992; Billinis *et al*., 2001).

The sensitivities of these different methods were found to be between 1pg and 1fg or 10^2 molecules of virus per reaction and 4.1 $TCID_{50}$ /ml for AHSV (Zientara *et al*., 1994; Stone-Marschat *et al*., 1994; Koekemoer & van Dijk, 2004; Aradaib *et al*., 2006). BTV-specific methods detected as low as 5 $TCID_{50}$ (Billinis *et al*., 2001). Some of these assays were performed directly from clinical samples (Koekemoer & van Dijk, 2004) or dsRNA was directly extracted from infected cells (Aradaib *et al*., 2006).

These RT-PCR assays are based on the principle that the presence of a particular virus is indicated by the amplification product obtained after using a target specific primer set during the RT-PCR. The techniques have been shown to be very rapid, but there are risks involved in relying entirely on the presence of an RT-PCR amplification product to indicate the presence of a particular virus or serotype in a test sample. False negative results can occur as a result of sequence variations in primer binding sites and the amplification of non-specific products can be incorrectly interpreted as an indication of a specific virus.

Currently none of the published serogroup-specific RT-PCRs for AHSV have been approved for use as international trade tests. Routine diagnosis of BTV is based primarily on RT-PCR methods that detect viral dsRNA. The world animal health organization (Office International des Epizooties, OIE) has approved one nested PCR (Katz *et al.*, 1993) that amplifies part of genome segment 5 (NS1) of BTV for use as an international trade test.

Even though RT-PCR based assays can be reliably used to detect viral nucleic acid without virus isolation, they require post-PCR handling steps. These methods limit the number of samples that can be tested in a day.

1.7.2. GROUP-SPECIFIC AND SEROTYPE SPECIFIC TARGETS FOR DETECTION OF AHSV AND BTV NUCLEIC ACID

Detection of the viral genome by RT-PCR is a convenient and effective method and several genome segments have been used in group specific or serotype specific PCR based methods, some of which have been acknowledged by the OIE. VP2 is a variable protein located in the outer capsids of the virion and it determines the antigenic variability of the virus and serotype (Verwoerd *et al.*, 1972; Huismans *et al.*, 1987b; Scanlen *et al.,* 2002; Sailleau *et al.*, 2000). Several serotype-specific RT-PCR assays have been reported for segment 2 of BTV (Mertens *et al.*, 2007) and AHSV (Koekemoer & van Dijk, 2004; Sailleau *et al.*, 2000). In this study the genome segment encoding NS1 was chosen for the group-specific real-time PCR for BTV because it is the most targeted gene for group specific nucleic acid based tests (Katz *et al.*, 1993; Toussaint *et al.*, 2007). Sequence analysis of genome segments encoding NS1 revealed that it has a 97% nucleic acid identity (Roy & Gorman, 1990); therefore it is highly conserved among the BTV serotypes.

The genome segment encoding VP7 was chosen as a target sequence for primers that can be used for AHSV group-specific real-time PCR. VP7 is the major immunodominant serogroup antigen (Huismans & van Dijk, 1990) and has been reported to be highly conserved among the AHSV serotypes (Bremer *et al.*, 1990). Various RT-PCRs have been developed to detect VP7 of BTV (Anthony *et al.*, 2007) and AHSV (Zientara *et al.*, 1993; Zientara *et al.*, 1995).

1.7.3. REAL-TIME PCR

The principle of real-time PCR is based on the detection and quantification of a fluorescent reporter signal during the PCR amplification. Combination of thermal cycling, fluorescence detection and software analysis on one instrument has made the detection of fluorescence during each cycle possible (Wittwer *et al.*, 1997). As no post-PCR steps are required, the possibility of cross-contamination by PCR products is minimized and this advantage makes real-time PCR a method of choice for diagnostic applications. The target genes can be quickly identified independently and multiple PCR reactions can be carried out in one tube by employing melting curve analysis. Real-time PCR is faster, is more sensitive and has a wider dynamic range and reproducibility compared to conventional PCR (Schröter *et al.*, 2001; Reviewed by Gunson *et al.*, 2006).

Numerous chemistries such as dual labelled probes (Hydrolysis and Hybridization probes) and dsDNA intercalating dyes (SYBR® Green I) are available to carry out real-time PCR (Wittwer *et al.,* 1997). SYBR® Green I assays represent an economical approach which may be suitable for large-scale molecular diagnosis and is the most commonly used intercalating dye in both commercial and in-house master mixes. When SYBR® Green I is used in a real-time system, the accumulation of amplicons in the reaction can be monitored over time. After PCR amplification, the decrease in fluorescence resulting from the release of SYBR® Green I during DNA melting point analysis is continuously monitored by slowly increasing the temperature (Wittwer *et al.,* 1997). The melting temperature (T_m) of specific amplicons and unique shape of the melting peak can be used to differentiate the target amplicons (Ririe *et al.*, 1997). Crossing points (CP) or threshold cycle (Ct) is also used to characterise individual reactions at a point where fluorescence first rises above defined background fluorescence and the lower the Ct the more abundant the initial target.

Multiplex PCR is the amplification of several targets simultaneously within a single reaction tube using several primer pairs, saving considerable time. Multiplex SYBR Green-I based real-time PCR methods have been described for quantification of Japanese encephalitis virus (Santosh *et al.*, 2007), detection of enteric viruses (Beuret, 2004), detection and typing of Avian influenza viruses (Ong *et al.*, 2007) and detection of tetracycline efflux genes of Gram-negative bacteria (Fan *et al.,* 2007). These methods could rapidly identify the investigated organisms within hours and they were also found to be sensitive with detection limits as low as 10^3 CFU per reaction (Fan *et al.,* 2007) and were 10-100 fold more sensitive when compared to conventional RT-PCR (Ong *et al.*, 2007).

1.8. AIM OF THE STUDY

Prevention, control and surveillance of AHS and BT require reliable and accurate diagnostic procedures. One of the crucial parameters for identification of AHS and BT is to minimize the manual input because there are no post-PCR analysis steps. The results can be obtained in a shorter time than conventional PCR because it has shorter cycling times.

The aim of the study was to use sequence data and modern molecular techniques to design a real-time RT-PCR that can detect two serogroups of orbiviruses in a single, one-step test. This will make it possible to use a single protocol and one set of reagents to specifically detect two different but related viruses in a routine diagnostic setup. Although it is not likely that clinical samples suspected to contain bluetongue might contain AHSV and vice versa, either or both of the viruses might be present in the midge vector. In addition to the advantage of having one test that can be used to detect two orbiviruses, it will be useful as a screening test when entomological surveys are carried out. The assay can also be used during epidemiological studies and it will also help to monitor BT and AHS infection in endemic and non-endemic countries when exporting livestock free of these diseases.

1.9. OBJECTIVES OF THE STUDY

The main aim of this project was to develop a group-specific duplex Real-time PCR for AHSV and BTV. The following objectives were carried out in order to achieve the main aim:

- Analyzed all available sequence information from AHSV and BTV for identification of potential areas on the genome that can be targeted for group-specific RT-PCR amplification of all AHSV serotypes 1-9 and all the BTV serotypes that are prevalent in South Africa (BTV 1-19 & 22-24).
- Sequenced specific regions of genome segment 7 of AHSV 1-9 and genome segment 5 of 19 BTV serotypes (2-9, 11-19 & 22-24) using primers that have been reported for AHSV (Zientara *et al.,* 1995) and BTV (Katz *et al.*, 1993) RT-PCRs.
- Designed a set of primers based on the analyzed genomic sequences.
- Investigated the specificity of the RT-PCR primers with dsRNA prepared from the 9 AHSV and 22 BTV reference serotypes and related orbiviruses.
- Tested the sensitivity of the assay by using a dilution series of genomic dsRNA.
- Converted PCRs into a real-time format using DNA binding dye (SYBR® Green I).
- Tested the two sets of PCR primers for use in combination as a duplex reaction.
- Investigated the specificity of the RT-PCR primers using field isolated AHS and BT viruses.

CHAPTER 2

2. *MATERIALS AND METHODS*

2.1. INTRODUCTION

This chapter describes all the materials and methods that were used to carry out the experimental work described in chapters 3, 4 & 5 for development of primers and a method for real-time RT-PCR based assay targeting parts of AHSV genome segment 7 and BTV genome segment 5.

2.1.1. VIRUSES AND CELLS

AHSV 1-9 and BTV 1-19& 22-24 reference strains, which were in a freeze dried form, were obtained from the OIE Reference Center for AHS and BT at the Onderstepoort Veterinary Institute. Tables 2.1 and 2.2 list the names and year of isolation of the AHSV and BTV reference strains that were included in this study.

Table 2.1

AHSV reference strains

Isolate	Serotype	Year of isolation
AHSV 29	1	1962
AHSV 82	2	1961
AHSV 13	3	1963
AHSV 32	4	1962
AHSV 30	5	1962
AHSV 2	6	1975
AHSV 31	7	1962
AHSV 10	8	1962
AHSV 90	9	1961

AHSV information was previously reported by Potgieter & co-workers (2003). The reference strains were obtained from I. Wright at the OIE reference centre for Bluetongue and African horsesickness at the Onderstepoort Veterinary Institute, South Africa.

Table 2.2

BTV reference strains used in the study

Isolate	Serotype	Year of isolation
BT 494	1	1989
BT 513	2	Unknown
BT 535	3	1986
BT 922	4	1986
BT 545	5	1986
BT 5011	6	Unknown
BT 8011	7	1979
BT 585	8	1985
BT 460	9	1979
BT 1926	10	Unknown
BT 1891	11	Unknown
BT 635	12	Unknown
BT 1894	13	Unknown
BT(Unknown)	14	Unknown
BT (Unknown)	15	Unknown
BT 7766	16	Unknown
BT 037	17	1979
BT(Unknown)	18	1962
BT 143	19	1976
BT81/184	22	1979
BT 68/5	23	Unknown
BT 24	24	1985

List of BTV reference serotypes used to validate the real-time RT-PCR; all viruses were received from the OIE reference center for AHS and BTV at Onderstepoort Veterinary Institute.

2.1.2. CELL CULTURE AND VIRUS PROPAGATION

Vero cells were grown in 175 cm^2 cell culture flasks. EMEM supplemented with 8% foetal bovine serum, 1% of L-Glutamine , 1% 100x Pen/Strep/ Fungizone , (10,000 U/mL Penicillin G, 10,000 µg/mL Streptomycin, 25 µg/mL Amphotericin B) and 1% non-essential amino acids was used. The cells were incubated at 37 ° C until a confluent monolayer was formed. Virus stocks of all the reference strains that were used in this study were prepared according to the following procedure: freeze dried virus was suspended in 1 ml of EMEM supplemented with 1% L-Glutamine and 1% Pen/Strep. Five ml of EMEM was added to four 175 cm^2 cell culture flasks and 0.25 ml of the suspended virus was added to each flask and this was found to be adequate for infecting with a sufficiently high multiplicity of infection. The flasks were then incubated for 30 minutes in a 5 % carbon dioxide incubator. After every 10 minutes the flasks were swirled such that the virus containing medium could be spread throughout the flask. At the end of incubation about 10 ml medium was added to each flask. Individual flasks infected with the different viruses were kept at 37 °C for 24-48 hours until CPE was observed in 80-100% of the cell monolayer. The cells together with the medium were harvested and stored at 4 °C.

To prepare infected cells for dsRNA isolation, Vero cells that reached confluency were infected with the virus working solution by removing half of the medium in each flask. 1ml of the untitred virus working solution was then added to each flask and it was incubated for 2-3 days in a 5 % carbon dioxide incubator until CPE was seen in 100% of the cell monolayer. Ten 175 cm^2 flasks were used per dsRNA preparation.

2.1.3. dsRNA EXTRACTION FROM INFECTED CELL CULTURES

Cells were harvested by decanting the medium and cells into a 50 ml tube and collected by centrifuging at 4 °C for 10 minutes at 3000 rpm. Total RNA was extracted using a commercial acid/ phenol guanidinium based reagent (TRI-reagent, Molecular Research Center, Inc.) and this method is based on that of Chomczynski and Sacchi (1987). This consists of using 1 ml of Tri Reagent per 75 cm^2 flask. 0.2 ml of Chloroform per 1 ml of Tri Reagent used was then added and the sample was centrifuged at 12 000 x g for 15 minutes. Thereafter the aqueous phase which contains the RNA was transferred into a new tube. 0.5 ml Isopropanol per 1 ml of Tri reagent used was added to precipitate the RNA. The sample was then centrifuged at 12 000 x g for 10 minutes at 4 °C. The RNA pellet was resuspended in 200 µl sterile TE buffer per flask, and incubated for 30 minutes at 30 °C. Lithium chloride was added to a final concentration of 2M (Diaz-Ruiz & Kaper, 1978) and kept overnight at 4 °C. The sample was centrifuged at 12000 x g in a benchtop centrifuge at 4 °C for 1 hour to precipitate single stranded RNA (ssRNA) (Diaz-Ruiz & Kaper, 1978). The supernatant was aspirated and the concentration of the LiCl in the supernatant was increased to 4M, thereafter it was put at 4 °C overnight. To precipitate dsRNA the sample was centrifuged at 4 °C for 1 hour. The resulting dsRNA was washed with 75 % ethanol and the pellet was air dried. Then the pellet was resuspended in 100 µl TE buffer. Three µl of the sample was run on a 1% agarose gel.

2.1.4. RT-PCR

Viral dsRNA samples were denatured with 20 mM MMOH (Wade-Evans *et al.*, 1990) for 15 min at room temperature, followed by addition of 0.2 M βME to reduce MMOH. The RT-reactions contained 2 µl of 2.5 mM dNTP-mix, 10 pmol of each of the forward (AHSV S7/01 & BTV NS1/A) and reverse primers (AHSV S7/02 & BTV NS1/B) (see

Tables 3.1 & 4.1), 4 µl cDNA buffer (50 mM Tris-HCl, pH 8.3, 100 mM KCl, 5 mM $MgCl_2$), 5 units of AMV reverse transcriptase (Promega) and water to a final volume of 20 µl. Reverse transcription was carried out at 42 °C for 45 minutes. 5 µl of this cDNA mixture was used for PCR amplification in a total volume of 30 µl. The PCR mixture contained 5 µl of a 2.5 mM dNTP-mix, 10 pmol of each of the forward and reverse primers, 5 U of Ex-Taq polymerase (Takara), 3 µl of the supplied 10x buffer and water to a final volume of 30 µl. Thermal cycling conditions were as follows: initial denaturing of the cDNA was carried out for 3 minutes at 95 °C, followed by thirty cycles of 95 °C for 30s, 55 °C for 30s and 72 °C for 90s, and a final incubation at 72 °C for 10 min. After electrophoresis on a 1.2% TBE agarose gel stained with ethidium bromide, amplicons of the expected size were excised from the gel and purified using a MinElute gel-extraction kit (Qiagen) according to the manufacturer's instructions.10-15 µl of buffer was used to elute the DNA from the column.

2.1.5. SEQUENCING OF PCR PRODUCTS

The purified DNA was used for sequencing of the amplification product in both directions using the original PCR primers. Sequencing was done on a 3100 Genetic Analyzer sequencer using the ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit v3.0 (Perkin-Elmer Applied Biosystems). Raw sequence data for each AHSV and BTV isolate was assembled using Staden Package's GAP 4 (Bonfield *et al.*, 1995).

2.2. REAL-TIME RT-PCR

The RNA Master SYBR® Green I Kit (Roche Diagnostics) which is a “Hot Start” reaction mix was used for all the real-time PCR reactions. This kit is adapted for one-step RT-PCR by making use of Tth DNA polymerase together with aptamers. Tth DNA polymerase is a thermostable enzyme that exhibits both reverse transcriptase and DNA polymerase activity, allowing both reverse transcription and amplification to be combined in a single reaction tube. The assay was performed in a volume of 20 µl containing 3.25 mM of $Mn(OA_c)_2$, 0.2 µM of each primer (AHSV forward & reverse primer pair (Table 3.1) and BTV forward & reverse primer pair (Table 4.1)), 7.5 µl of RNA Master SYBR® Green I and 9.2 µl of water. 1 µl of dsRNA was added to make up 20 µl. Amplification was carried out in a LightCycler 1.5 and the thermocycling programme was as described in Table 2.3.

2.3. DUPLEX ONE- STEP real-time RT-PCR

The one-step RT-PCR kit (Roche) was used to reverse transcribe the dsRNA then amplify cDNA in a duplex RT-PCR containing both primer pairs (AHSV primer set and BTV primer set). The reaction mix contained 7.2 µl PCR grade H_2O, 3.25 mM Mn $(OA_c)_2$, 0.2 µM of each primer (two forward and two reverse) and 7.5 µl of the LightCycler® RNA Master SYBR® Green I. Three capillary tubes were used in this experiment, whereby capillary tube 1 contained 3 µl of AHSV dsRNA, 3µl of BTV dsRNA was added to capillary tube 2, and in the third tube there was a mixture of AHSV and BTV (1.5 µl of AHSV dsRNA and 1.5 µl of BTV dsRNA) which were then added to 17 µl of the reaction mix. Results were analyzed with melting curve analysis and Tm values were assigned from a plot generated by the Roche Light cycler instrument to verify the specificities of the amplicons. The products were analyzed on a 2% agarose gel.

Table 2.3

Real-time PCR programme and PCR conditions

Reverse Transcription

Target (° C)	Hold (hh:mm:ss)	Slope (° C/s)	Sec Target (° C)	Step size (° C)	Step delay (cycles)	Acquisition mode
61	00:20:00	20	0	0	0	None

Pre-PCR denaturing

<table>
<tr><th colspan="3">Programme name</th><th colspan="4">Denaturation</th></tr>
<tr><th colspan="3">Cycles</th><td colspan="2">1</td><th>Analysis mode</th><th>None</th></tr>
<tr><th>Target (° C)</th><th>Hold (hh:mm:ss)</th><th>Slope (° C/s)</th><th>Sec Target (° C)</th><th>Step size (° C)</th><th>Step delay (cycles)</th><th>Acquisition mode</th></tr>
<tr><td>95</td><td>00:00:30</td><td>20</td><td>0</td><td>0</td><td>0</td><td>None</td></tr>
</table>

Table 2.3 (cont.)

PCR

Programme name			PCR			
Cycles			30		Analysis mode	Quantification
Target (° C)	Hold (hh:mm:ss)	Slope (° C/s)	Sec Target (° C)	Step size (° C)	Step delay (cycles)	Acquisition mode
95	00:00:05	20	0	0	0	None
55	00:00:15	20	0	0	0	None
72	00:00:10	20	0	0	0	Single

Table 2.3(cont.)

Melting curve analysis

Programme name			Melt			
Cycles			1		Analysis mode	Melting curves
Target (° C)	**Hold (hh:mm:ss)**	**Slope (° C/s)**	**Sec Target (° C)**	**Step size (° C)**	**Step delay (cycles)**	**Acquisition mode**
95	00:00:00	20	0	0	0	None
65	00:00:15	20	0	0	0	None
95	00:00:00	0.2	0	0	0	Continuous

CHAPTER 3

3. AHSV GROUP-SPECIFIC REAL-TIME RT-PCR

3.1. INTRODUCTION

In this study a real-time RT-PCR was developed for the group-specific detection of AHSV from dsRNA preparations. As discussed in Section 1.7.1.1. it was decided to select genome segment 7 for this purpose. Both sequence data available in the GenBank database and data obtained from nucleotide sequencing of genome segment 7 were used to design a pair of RT-PCR primers. These primers were tested for their ability to amplify cDNA from the nine reference strains of AHSV. Once the group-specificity of the primers were established, they were used in a real-time RT-PCR that made use of SYBR Green I as a reporter of cDNA amplification. Finally the sensitivity of the method was determined by making use of a dilution series of dsRNA from AHSV 1.

3.2. SPECIFIC OBJECTIVES

- ✓ To gather data by searches of the GenBank database for AHSV genome segment 7 nucleotide sequences
- ✓ To make use of a set of published RT-PCR primers (Zientara *et al.*, 1995) to amplify cDNA from reference strains of AHSV 1-9 for sequencing.
- ✓ To align the sequences obtained from above approach to each other and identify potential sites for the design of group-specific primers.
- ✓ To test the selected primers with dsRNA from the nine AHSV reference strain viruses.

- ✓ To use the same primers and templates in a one-step SYSBR Green I real-time RT-PCR.
- ✓ To determine the minimum amount of dsRNA that can be detected in the real-time RT-PCR format.

3.3. RESULTS

3.3.1. DATABASE SEARCHES

Seven entries with full-length AHSV genome segment 7 sequence data could be retrieved from the GenBank database. The accession numbers and serotypes are:NC-006011(AHSV 4), S 69829(AHSV 9), D 12533(AHSV 4), AF 021238(AHSV 6), U 90337(AHSV 9), A 27209(AHSV 4) and X 56676(AHSV 4).

3.3.2. NUCLEOTIDE SEQUENCING OF GENOME SEGMENT 7

The primers that were used for partial amplification of genome segment 7 (Zientara *et al.*, 1995) amplified the region between nucleotides 1-1179. Amplification products could be obtained from dsRNA of the reference strains of serotypes 1-9. After sequencing of these PCR products as described in Section 2.2.2 using the same primers that were used for the RT-PCR, usable sequence data was obtained from the AHSV 2 and AHSV 5 reference strain viruses.

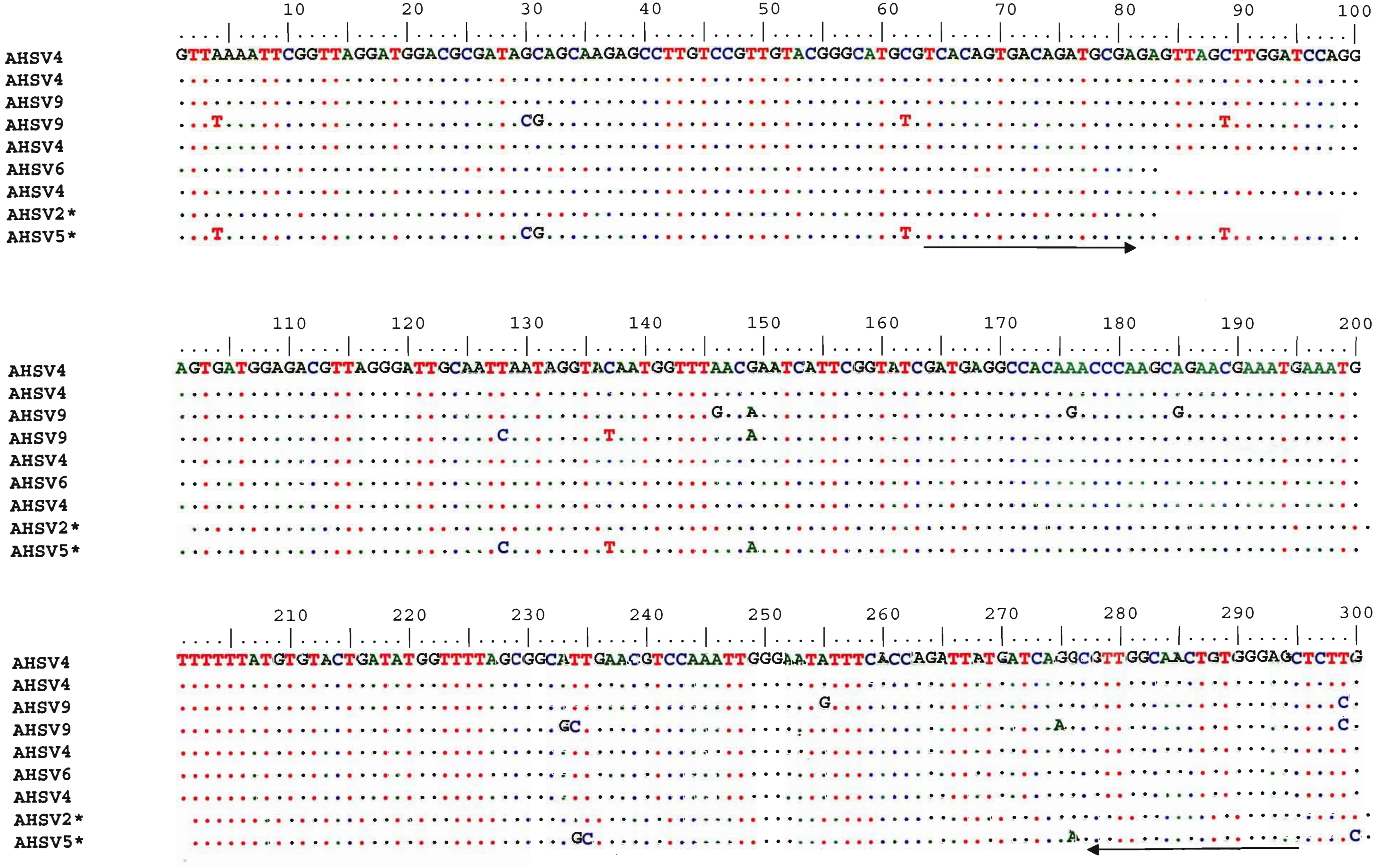
3.3.3. SEQUENCE ANALYSIS

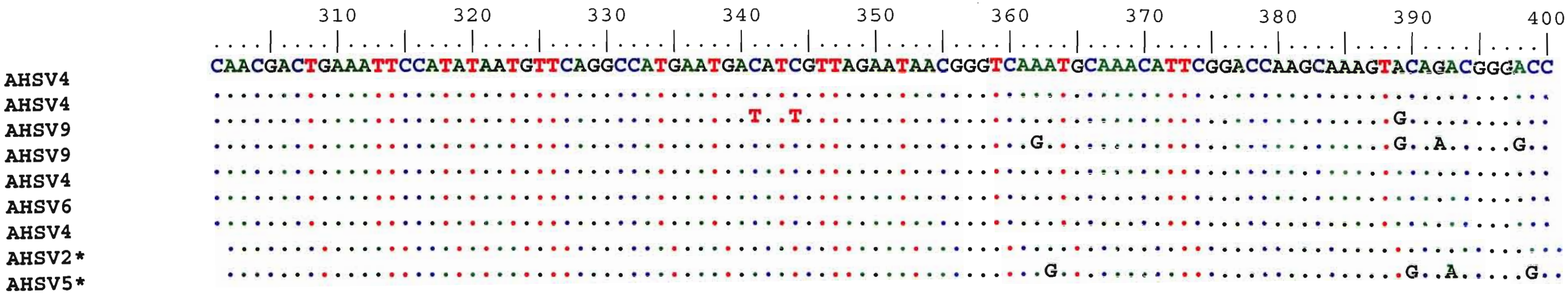
The nucleotide sequences were aligned to each other using a computer program, Clustal X (Thompson *et al.*, 1997). A total of 9 sequences covering 5 serotypes were aligned. The alignments can be seen in Figure 3.1 and the part of genome segment 7 that is represented in the alignment is from nucleotide 1 to 400. The two areas that were selected for the design of the forward and reverse primers are indicated on the alignment. These primers would result in the amplification of a 232bp product.

Table 3.1

Table of Primers for AHSV group-specific amplification.

Primer	Primer orientation	Primer binding site	Size of amplicon (bp)	Primer sequence	Reference
AHSV S7/01	Forward	1-20	1178	GTTAAAATTCGGTTAGGATG	Zientara *et al.*, (1995).
AHSV S7/02	Reverse	1179-1159	1178	GTAAGTGTATTCGGTATTGA	Zientara *et al.*, (1995).
AHSV S7/03	Forward	63-80	232	GTCACAGTGACAGATGCG	This study.
AHSV S7/04	Reverse	295-278	232	CTCCCACAGTTGCCAAC	This study.


10 20 30 40 50 60 70 80 90 100
AHSV4
GTTAAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCATGCGTCACAGTGACAGATGCGAGAGTTAGCTTGGATCCAGG
AHSV4
AHSV9
AHSV9
AHSV4
AHSV6
AHSV4
AHSV2*
AHSV5*
110 120 130 140 150 160 170 180 190 200
AGTGATGGAGACGTTAGGGATTGCAATTAATAGGTACAATGGTTTAACGAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAAATG
210 220 230 240 250 260 270 280 290 300
TTTTTTATGTGTACTGATATGGTTTTAGCGGCATTGAACGTCCAAATTGGGAATATTTCACCAGATTATGATCAGGCGTTGGCAACTGTGGGAGCTCTTG



```
                  310       320       330       340       350       360       370       380       390       400
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AHSV4     CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTCAAATGCAAACATTCGGACCAAGCAAAGTACAGACGGGACC
AHSV4     ....................................................................................................
AHSV9     ........................................T..T............................................G...........
AHSV9     .............................................................G..........................G..A.....G..
AHSV4     ....................................................................................................
AHSV6     ....................................................................................................
AHSV4     ....................................................................................................
AHSV2*     ....................................................................................................
AHSV5*     .............................................................G..........................G..A.....G..
```

Figure 3.1: Sequence alignment of the real-time RT-PCR primer footprints for genome segment 7 of AHSV serotypes 2, 4, 5, 6 & 9. * Indicates new sequences that were generated from reference strain viruses. All other sequences are from GenBank. RT-PCR primers were identified within nucleotides 63-295. Arrows indicate the primer binding regions.

3.3.4. GROUP-SPECIFIC RT-PCR

dsRNA preparations from the 9 reference strains of AHSV were denatured and used as templates for RT-PCR as described earlier (Section 2.1.4) using the forward primer AHSV S7/03 and reverse primer AHSV S7/04 (Table 3.1). Amplification products that corresponded to the predicted size of 232bp were obtained from dsRNA prepared from all the serotypes of AHSV (Fig.3.2).

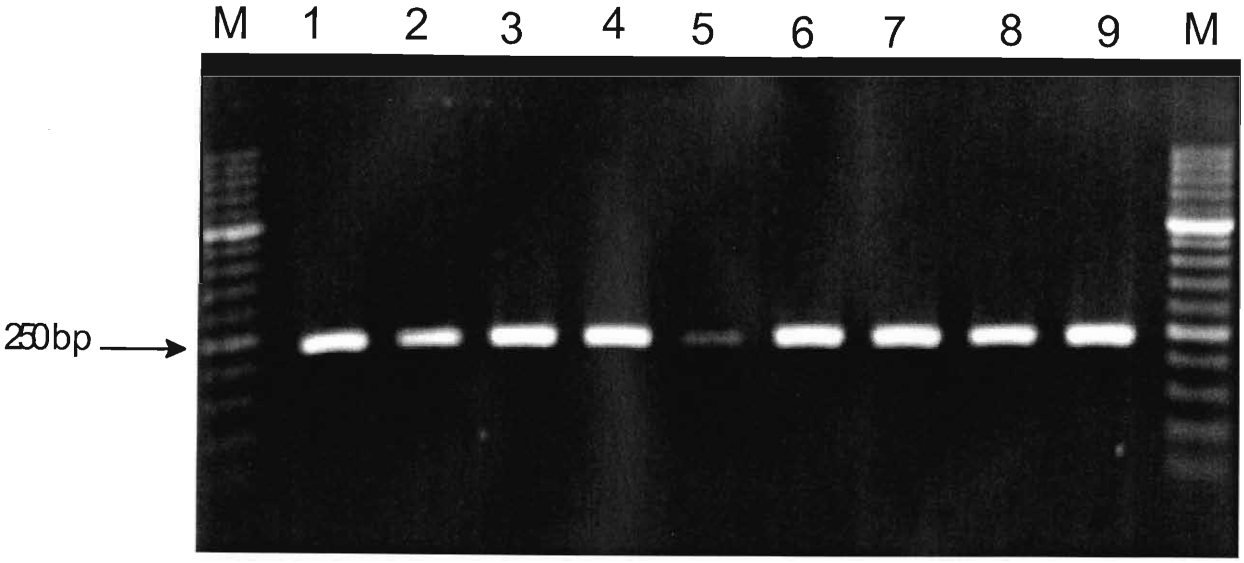


Figure 3.2: Detection of the 232 bp AHSV specific RT-PCR products from dsRNA of AHSV 1-9 on ethidium-bromide stained agarose gel. All the reactions were carried out using one group-specific primer set and identical reaction conditions. Lane M: DNA Molecular weight marker XIII, 50- 750 bp ladder (Roche diagnostics); Lanes 1-9: AHSV 1-9.

3.3.5. REAL-TIME RT-PCR AND MELTING CURVE ANALYSIS

The same primer set that was found to be suitable for the amplification of part of genome segment 7 of all the reference strains of AHSV using a conventional RT-PCR approach was used in a one-step real-time RT-PCR protocol. The Lightcycler® SYBR® Green I kit was used as described in Section 2.2. The amount of dsRNA that was used corresponds to 170 – 195 ng per serotype. Amplification was measured as an increase in fluorescence at 530 nm which results from the interaction of the SYBR Green I dye and the newly synthesized cDNA. Fluorescence increase was measured for each reaction and is represented graphically as amplification curves (Fig. 3.3) and as CP-values (Table 3.2). The CP-values were determined by the LightCycler analysis software and represents a fraction of the PCR cycle at which the fluorescence increases above a threshold value and indicates the start of amplification.

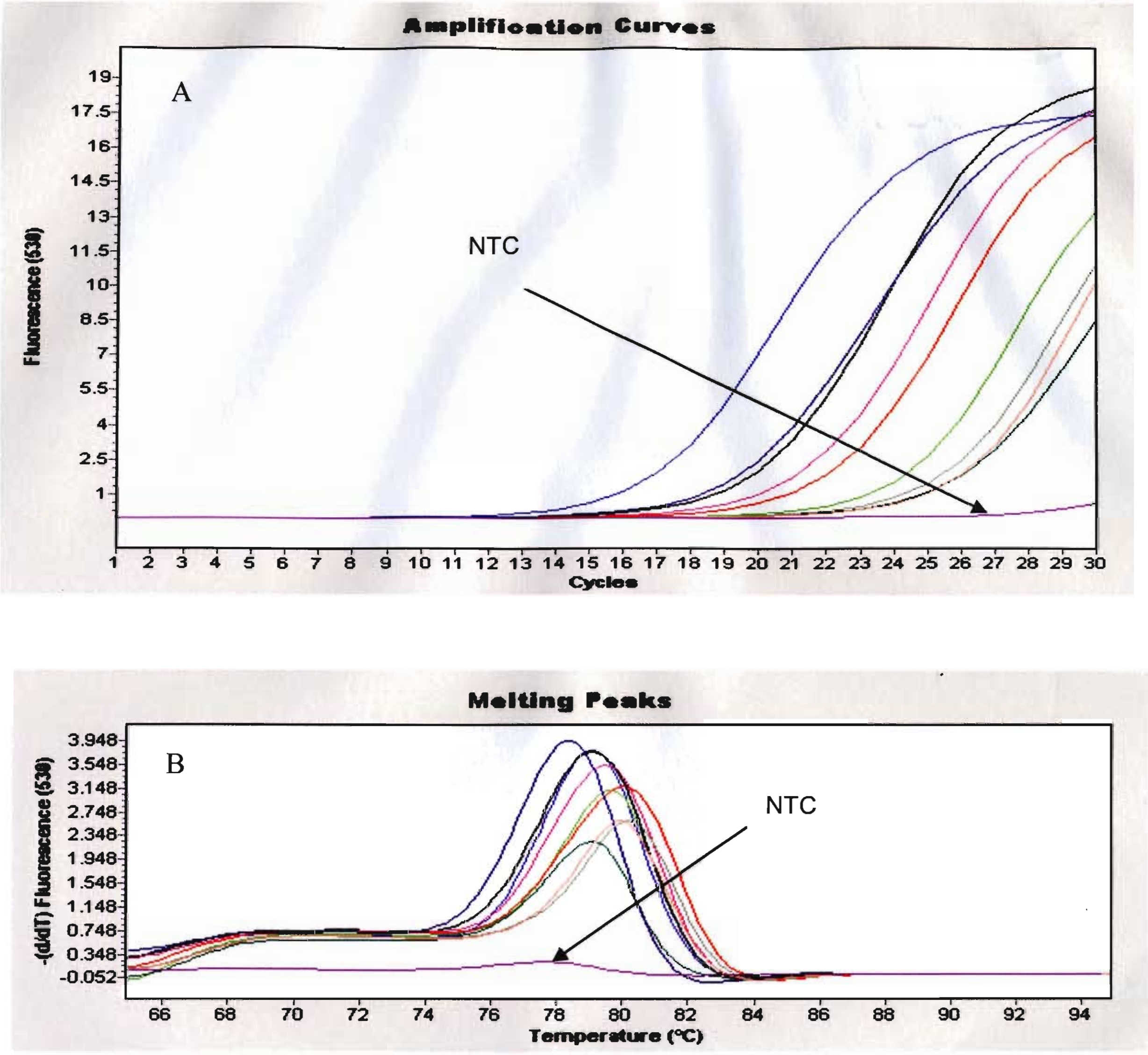


Figure 3.3: AHSV group-specific SYBR Green I-based real-time RT-PCR showing (A) the amplification plot of AHSV 1-9 and (B) melting curves obtained when the 232 bp of segment 7 from the nine AHSV serotypes were amplified. Specific melting temperatures ranged between 78.48 °C and 80.36 °C. Melting curve analysis was performed for verification of amplification products by means of their specific melting temperatures. Detailed amplification and melting curve data for each of the viruses are given on Table 3.2 (* NTC= no template control).

Table 3.2

Measured melting temperature and CP-values of the PCR products for group-specific real-time PCR of AHSV 1-9

Sample name	Tm(°C)	Cp	Year of isolation
AHSV 1	79.22	16.03	1962
AHSV 2	79.74	24.46	1961
AHSV 3	80.18	21.04	1963
AHSV 4	79.26	19.14	1962
AHSV 5	79.61	20.43	1962
AHSV 6	79.23	>25.00	1975
AHSV 7	78.48	18.68	1962
AHSV 8	80.36	>25.00	1962
AHSV 9	80.08	>25.00	1961
NTC	78.15	>25.00	n/a

In order to identify the amplification products as AHSV-specific, a melting curve analysis was carried out after the completion of the PCR. This identifies the temperature at which the dsDNA amplification products in the reaction melt at a peak rate. The result is expressed as a peak T_m value and is dependant on the length and GC-content of the dsDNA molecule that is being melted.

The peak T_m values for each of the nine amplification products were measured and are given in Table 3.2. The values ranged from 78.48 °C for serotype 7 and 80.36 °C for serotype 8. Although the NTC could clearly be distinguished from the AHSV samples based on the shape of the amplification curves (Fig. 3.1), a peak T_m value of 78.15 °C was reported.

3.3.6. SENSITIVITY

Different amounts of dsRNA from the reference strain of AHSV 1 were used to determine the limit of sensitivity of the real-time RT-PCR. The total amount of dsRNA in the samples ranged from 173.8 ng to 173.8 fg in a tenfold dilution series (Table 3.3). The detection limit was found to be 173.8 pg for total RNA (Fig. 3.4).

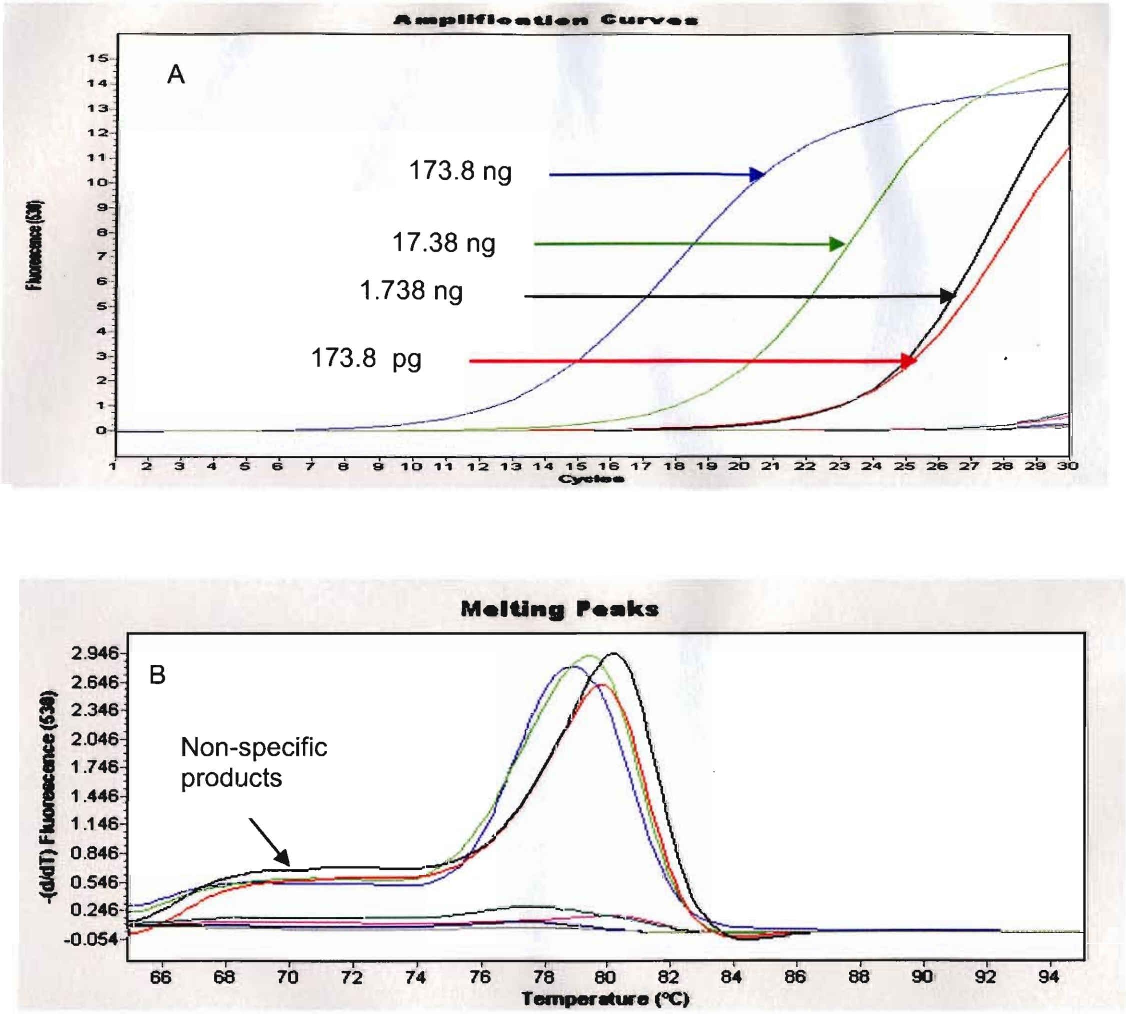


Figure 3.4: A) Amplification curves of decreasing concentration of AHSV dsRNA in serial 10-fold dilutions from 173.8 ng to 173.8 fg. The detection limit for the assay was 173.8 pg. (B) The graph shows the melting peak analysis at various template dilutions with melting peak temperatures varying between 78.45 °C and 80.70 °C.

Table 3.3

Measured melting temperatures and CP-values of the PCR fragments for dilution series of AHSV 1

Sample name	Tm(°C)	Cp
173.8 ng	79.05	12.85
17.38 ng	79.33	18.63
1.738 ng	79.87	>25.00
173.8 pg	80.25	24.71
17.38 pg	80.7	>25.00
1.738 pg	79.19	>25.00
173.8 fg	78.45	>25.00
NTC	78.99	>25.00

3.4. DISCUSSION

The work that is described in this chapter had as its main aim the design of a real-time RT-PCR method that can be used to detect all nine serotypes of the AHSV. This was done by analysing genome segment 7 sequence data that were obtained from public databases and generated as part of this study from South African reference isolates of the viruses. AHSV genome segment 7 was chosen as a target gene for the RT-PCR because it is conserved among the nine serotypes and amino acid homology of 100% has been reported for VP7 of AHSV6, AHSV 9 and AHSV 4 (Williams *et al.,* 1998).

Although RT-PCR amplification products could be obtained from all dsRNA preparations, usable data was only generated from the serotype 2 and 5 cDNA sequencing reactions. This was probably due to the amount of cDNA that was sequenced not being sufficient or it was possibly contaminated by non-specific amplification products. To determine the conserved region, the VP7 genes (segment 7) of AHSV 2 and 5 obtained after sequencing were aligned with AHSV 4, 6 and 9 sequences published on GenBank. The sequence alignments revealed an identity of 96.44% between the 1179 nucleotide sequences of genome segment that were aligned. This is close to what has been reported in the literature by Williams & co-workers (1998).

The RT-PCR primers were selected in two areas of homology such that the size of the amplification product would be 232 bp (Fig 3.2). The primers were tested in RT-PCR amplification and the results showed that the primers amplified cDNA from dsRNA of all nine AHSV serotypes. All the amplification products were of the predicted size and it can therefore be concluded that the primer binding sites were conserved in all nine the reference serotypes. This is important as sequence data were not obtained for all the serotypes.

It was then decided that this primer set (AHSV S7/03 and AHSV S7/04, Table 3.1) would be tested in a real time format. The format that was selected was a SYBR green based real-time PCR method. This technology was selected as it is relatively cheap when compared to probe based systems but can still be used to distinguish amplification products with different peak T_ms (Ririe *et al.*, 1997).

Increase in fluorescence was measured as cDNA amplification took place and the CP-values gave an indication of the relative amounts of starting material (denatured RNA) that was present at the beginning of the reaction. The CP-values varied between 12.85 and 25.00 for the nine serotypes. Although all care was taken to keep the amounts of dsRNA in the different reactions at the same level, there were still variations that are reflected in the CP values. CP-values are often used together with standard curves to carry out accurate quantification of the amount of starting material (Lekanne-Deprez *et al.,* 2002; Santhosh, *et al.*, 2007; Ong *et al.*, 2007).

A post PCR melting curve analysis (Wittwer *et al.,* 1997) was incorporated into the method to make detection of the amplification product more specific. As SYBR Green fluorescence will take place in the presence of any dsDNA, non-specific amplification products would also contribute to the levels of fluorescence that are observed during the real-time PCR. During a melting curve analysis dsDNA products with different T_ms can be distinguished (Ririe *et al.*, 1997). The objective was to design the real-time RT-PCR such that the AHSV amplification product could be distinguished from non-specific dsDNA products, such as primer-dimers, or from BTV-specific amplification product during the next phase of the research. The genome segment 7 nucleotide sequence alignments showed a high level of homology but several variable sites occurred within the area of cDNA amplification. These sequence variations lead to peak T_m values that ranged from 78.48 °C and 80.36 °C. This level of variation is the same as what have been described for other group-specific BTV real-time RT-PCR tests (Jiménez-Clavero *et al.*, 2006).

The sensitivity of the method was tested with a dilution series of dsRNA from AHSV 1 and the minimum amount of dsRNA needed was determined to be 173.8 pg. This sensitivity is lower than that observed with most AHSV specific conventional RT-PCRs, which range between 1pg and 0.1 fg or 10^2 molecules of virus per reaction (Koekemoer & van Dijk, 2004; Aradaib *et al.,* 2006; Stone-Marschat *et al.*, 1994; Zientara *et al.*, 1994). The low sensitivity could be ascribed to two main factors. Firstly, SYBR green detection is not as sensitive as probe based methods, especially when comparing it to TaqMan® probes (Rodriguez-Sanchez *et al.*, 2008). These probes can detect 1.2 $TCID_{50}$/ml of AHSV dsRNA in a group-specific manner. The problem with using probes is that they are very sensitive to nucleotide substitutions in the target are of the probe. In this respect the SYBR Green based method has an advantage as one set of reagents, as demonstrated with the results from this work, can be used to identify cDNA amplification products with a number of sequence variations.

The second reason for decreased sensitivity is that the endpoint of detection could not be reached. At dilutions lower than 173.8 pg of AHSV dsRNA the peak melting temperature as determined by fluorescence measurements cannot be distinguished from that of non-specific amplification products in the reaction (Fig 3.4 B and Table 3.3). It is therefore possible that AHSV-specific cDNA amplification takes place at lower concentrations of the AHSV dsRNA, but using this technique it is not possible to demonstrate this. One possible way of addressing this is through the incorporation of an unlabelled oligonucleotide that is complementary to a specific region on genome segment 7 into the reaction. If this target area is homologous in all serotypes of AHSV it will result in a unique melting curve that would be distinguishable from the melting curves that result from non-specific products

(Seipp *et al.,* 2007). This would not be as expensive as incorporating a labelled oligonucleotide probe but it will increase the specificity of the melting curve analysis, possibly leading to increased sensitivity.

In summary, a new serogroup-specific RT-PCR test was designed using sequence data of genome segment 7 of AHSV. The test was shown to be able to detect all nine serotypes of AHSV and the primers that were designed could be used in a real-time PCR format using SYBR Green as a reporter of cDNA amplification. The plan was to use this test during the next phase of the study together with a similar group-specific test for BTV to develop a multiplex real-time RT-PCR that can detect both viruses in one reaction.

CHAPTER 4

4. BTV REAL-TIME RT-PCR

4.1. INTRODUCTION

The aim of the work described in this chapter was to design a group-specific real-time RT-PCR method for the detection of BTV serotypes that are prevalent in South Africa. These include serotypes 1-9 and 22-24. The strategy that was followed to design and test the primers and convert the method to a real-time format was similar to that which was used for the development of an AHSV group-specific RT-PCR (Chapter 3). In this case genome segment 5 (NS1) was targeted as more sequence information was available for it and because this genome segment has been shown to be highly conserved (Roy and Gorman, 1980). The specificity and sensitivity of the method was again tested using dsRNA prepared from reference strain viruses.

4.2. SPECIFIC OBJECTIVES

- ✓ To amplify cDNA from reference strains of BTV 1-19 & 22-24 using a set of RT-PCR primers that have been reported by Katz and co-workers (1993) and sequence the resulting PCR products.
- ✓ To align the sequences obtained and identify potential primer target regions.
- ✓ To test the specificity of the selected primers with dsRNA from BTV 1-19 & 22-24 and ensure that they are not identical to those of AHSV.
- ✓ To use the same primers and templates in a one-step real-time PCR making use of SYBR Green I dye.
- ✓ To determine the sensitivity of the real-time RT-PCR method

4.3. RESULTS

4.3.1. NS1 SEQUENCE ANALYSIS AND PRIMER DESIGN

dsRNA prepared from BTV reference strains were partially amplified with primers that have been reported by Katz & co-workers (1993). Amplification products were obtained from dsRNA of the reference strains of serotypes 2-8, 11-16, 18, & 22-23. RT-PCR amplicons which were excised from 1.2% TBE agarose gel stained with ethidium bromide were purified with the MinElute gel extraction kit (Qiagen). These PCR products were sequenced as described in Section 2.2.2 using NS1 gene specific primers (Katz *et al.*, 1993). Reactions were analysed on a 3100 genetic analyzer (Applied Biosystems).

The newly obtained nucleotide sequence data covering position 1-100 of genome segment 5 representative of 16 serotypes were assembled with Clustal X (Thompson *et al.*, 1997). The alignments are shown on Fig. 4.1. Potential primer target regions were selected between nucleotides 16 and 95 within genome segment 5. The areas where the forward and reverse primers were selected are indicated on Fig.4.1.The reverse primer sequence displayed sequence heterogeneity at two sites, position 84 (T/C mismatch) and at nucleotide 90 (A/G mismatch). At these positions degenerate bases were utilized to allow for base-pairing with either of the nucleotides found at these sites.

Table 4.1

Primers for group-specific amplification of segment 5 (NS1) from BTV dsRNA

Primer	**Primer orientation**	**Primer binding site**	**Size of amplicon (bp)**	**Primer sequence**	**Reference**
BTV NS1/A	Forward	11-31	273	GTTCTCTAGTTGGCAACCACC	Katz *et al.*, (1993)
BTVNS1/B	Reverse	284-265	273	AAGCCAGACTGTTTCCCGAT	Katz *et al.*, (1993)
BTV NS1/01	Forward	16-34	79	CAACCACCAAACATGGAGC	This study.
BTV NS1/02	Reverse	75-95	79	GCCAA**R**AAAGT**Y**CTCGTGGC	This study.

The locations of the primer binding sites are listed above.

Degeneracies:**R**= A & G; **Y**= C & T.

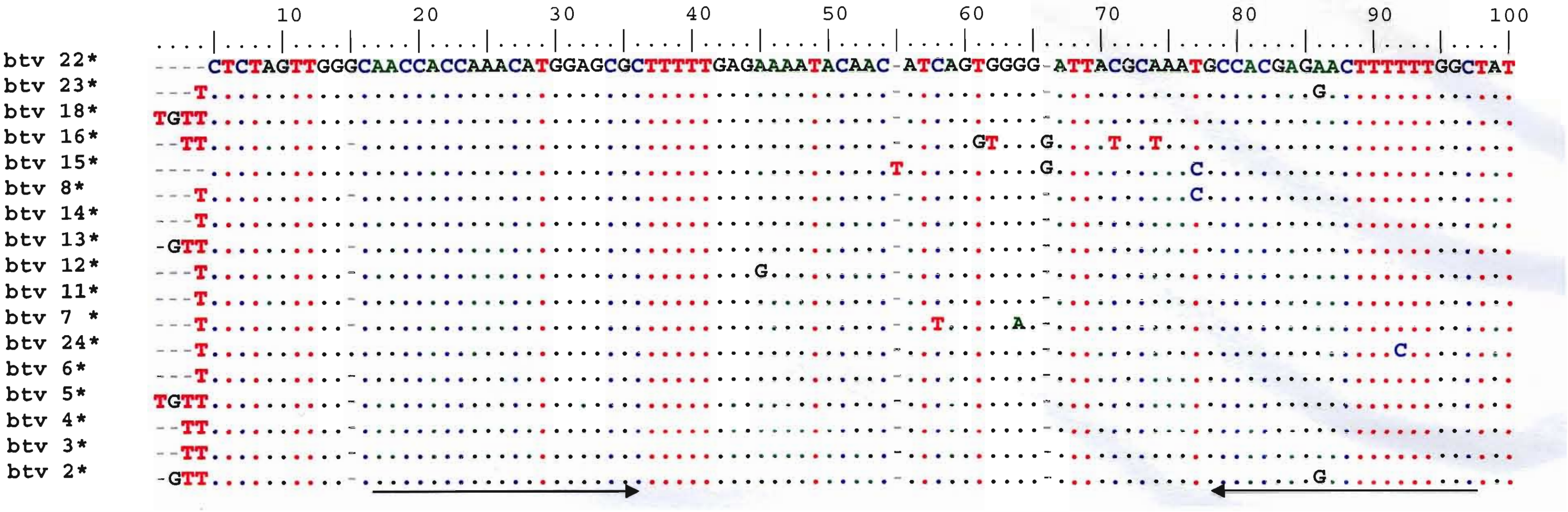


Figure 4.1: Sequence alignment of nucleotides 16-95 of genome segment 5 of BTV reference strains. Forward and reverse primers are both indicated by arrows. * Indicates new sequences that were generated from reference strain viruses.

4.3.2. GROUP-SPECIFIC BTV RT-PCR ASSAY

Double-stranded RNA preparations of the 22 BTV serotypes were extracted from Vero cell-cultures (2.1.3) infected with the virus reference strains. dsRNA was denatured with MMOH and subjected to RT-PCR (2.1.4) using the forward primer BTV NS1/01 and the reverse primer BTV NS1/02 (Table 4.1). Amplicons were analyzed by 2% TBE agarose gel electrophoresis. The specific 79 bp PCR products were obtained from all BTV dsRNA of all reference serotypes (Fig. 4.2).

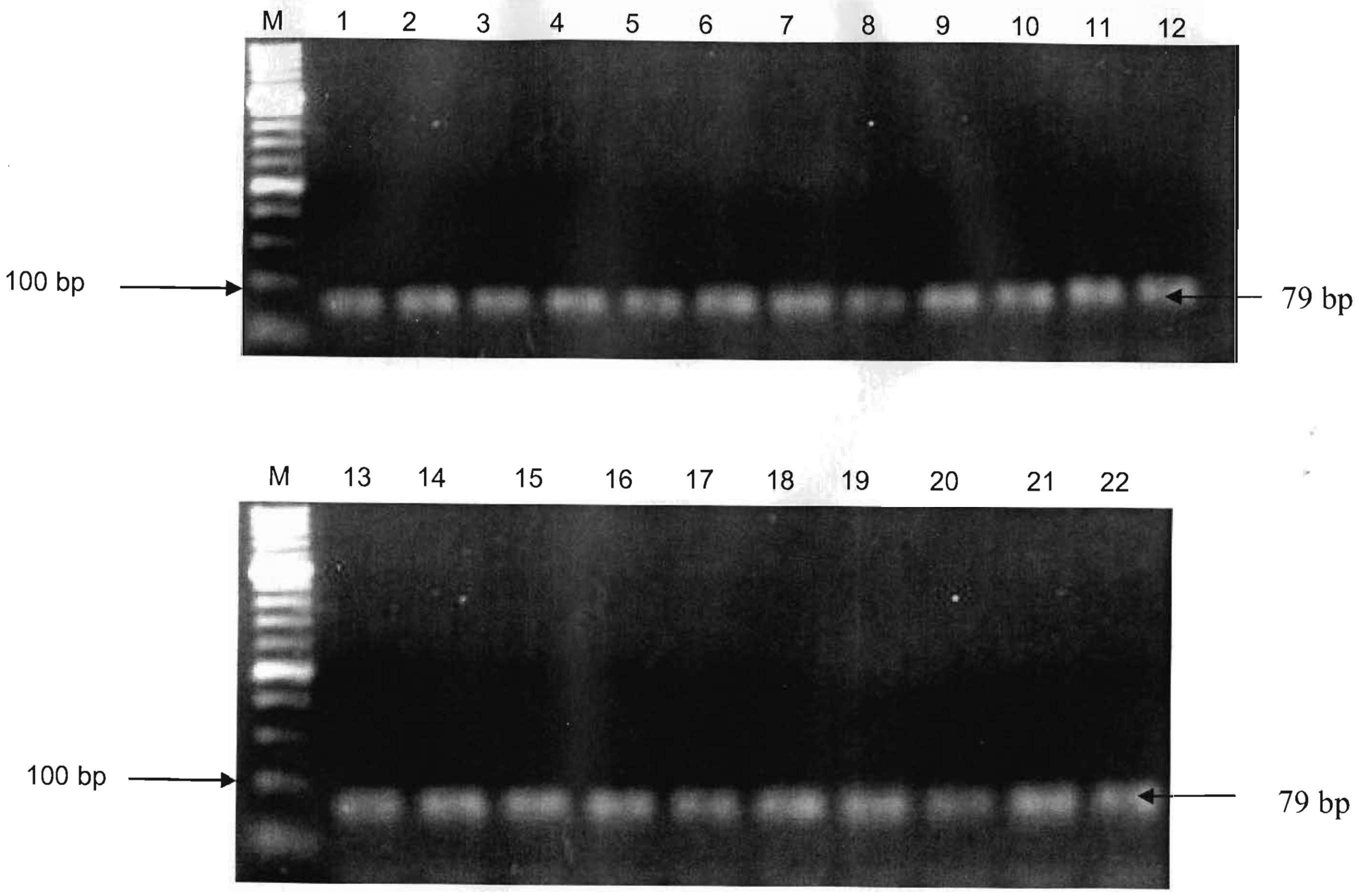


Figure 4.2: Visualization of the 79 bp BTV PCR specific product on an ethidium bromide -stained agarose gel from dsRNA of 22 BTV reference isolates. Lane M: Molecular weight marker XIII (Roche), 50-750 bp; Lanes 1-22: BTV serotype 1-19 & 22-24.

4.3.3. REAL-TIME PCR AND MELTING CURVE ANALYSIS

After investigating the ability of the BTV primers to amplify part of genome segment 5 from all the BTV serotypes, they were used in a one-step real-time format (2.2) using SYBR® Green I as a reporter dye. The amount of template dsRNA that was used was about 14 - 18ng per serotype. It was also investigated whether peak melting temperatures were sufficiently different from that of the AHSV amplicons to make it possible to distinguish BTV from AHSV. This was done using the same reaction conditions as that which were used for RT-PCR and melting curve based detection of AHSV.

Amplification was observed for all 22 serotypes of BTV in the 530 nm channel (Fig. 4.3). Amplification of the different serotypes started from cycle 15 to cycle 19. After the thirty cycles of PCR were completed, melting curve analysis revealed that the melting peak temperatures varied between 75.64 °C in the case of serotype 19 and 77.38 °C for serotype 8. Table 4.2 below shows the rest of the melting peak temperatures and CP-values. The NTC had a peak T_m value of 73.44 ° C, but after the melting curve analysis it was clearly distinguishable from the BTV samples (Fig.4.3).

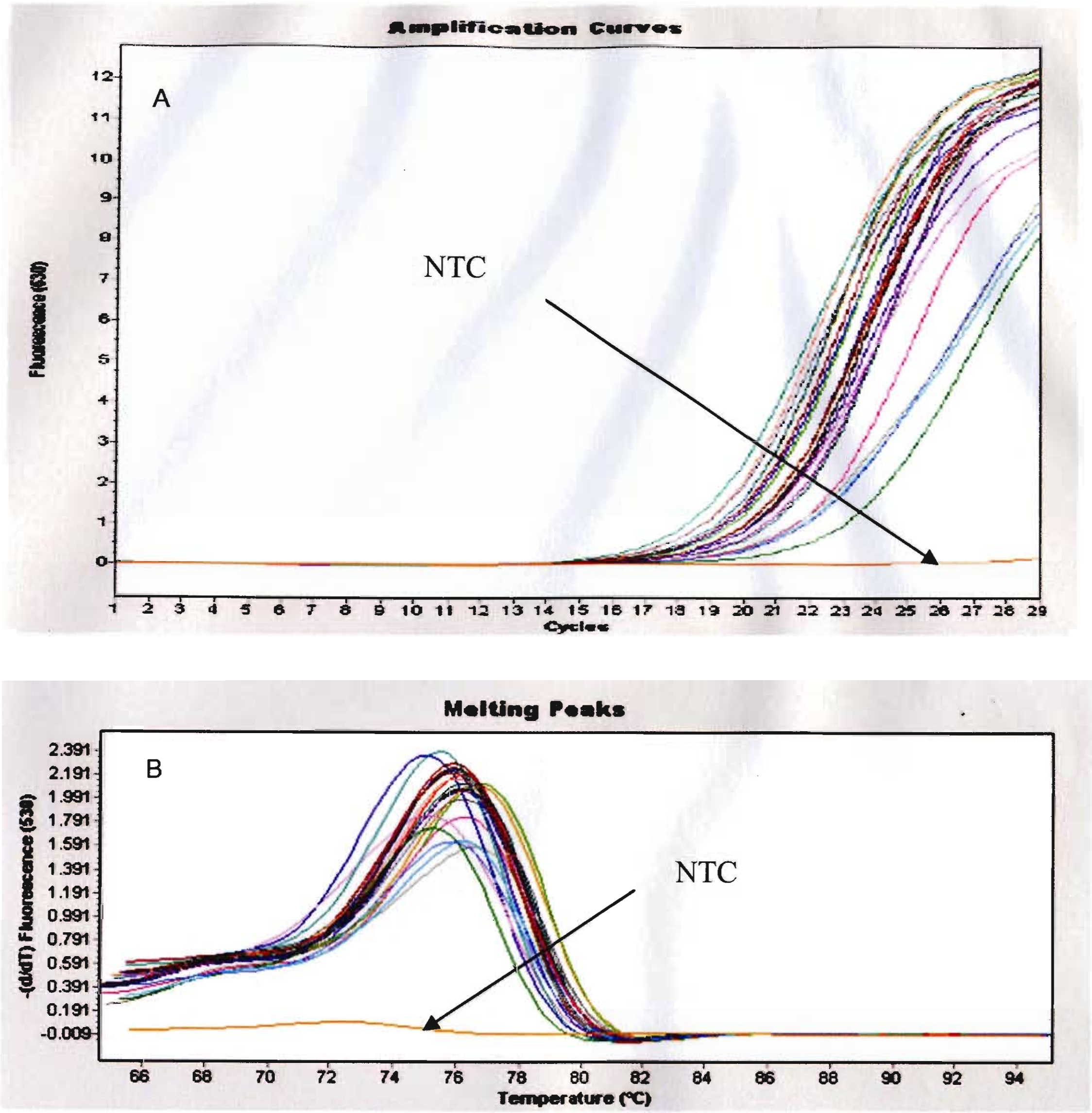


Figure 4.3: BTV group-specific Real-time PCR using SYBR® Green I kit. (A) Amplification plot for simultaneous amplification of BTV 1-19 & 22 -24. (B) Melting peak temperatures of BTV 1-19 & 22-24. Melting temperatures varied between 75.64 °C and 77.38 °C. Melting curve was used to analyze the melting temperatures of the different amplicons. Specific data for each serotype are indicated in Table 4.2.

Table 4.2

Cp-values and Measured T_m of the PCR fragments for BTV serotypes 1-19 & 22-24

Sample name	Tm(° C)	Cp	Year of isolation	Sample name	Tm(° C)	Cp	Year of isolation
BTV 1	76.6	18.73	1989	BTV 14	76.87	18.1	Unknown
BTV 2	77.28	18.96	Unknown	BTV 15	76.24	19.55	Unknown
BTV 3	76.69	19.63	1986	BTV 16	75.94	23.64	Unknown
BTV 4	76.72	19.56	1986	BTV 17	76.24	19.53	1979
BTV 5	76.77	20.67	1986	BTV 18	76.4	19.42	1962
BTV 6	76.78	20.05	Unknown	BTV 19	75.98	17.7	1976
BTV 7	75.64	19.48	1979	BTV 22	76.44	19.38	1979
BTV 8	77.38	21.32	1985	BTV 23	76.84	21.16	Unknown
BTV 9	76.57	18.09	1979	BTV 24	76.51	21.26	1985
BTV 10	76.69	19.92	Unknown	NTC	73.44	>24.00	N/A
BTV 11	77.13	18.57	Unknown				
BTV 12	76.47	18.56	Unknown				
BTV 13	76.43	18.72	Unknown				

4.3.4. SENSITIVITY OF BTV real-time RT-PCR ASSAY

The sensitivity of the assay was determined by using different starting amounts of BTV 7 dsRNA. 14.38 ng of total dsRNA was used in the following dilution series: 14.38 ng, 1.44 ng, 143.8 pg, 14.38 pg, 1.438 pg, 143.8 fg and 14.38 fg. As shown in Fig. 4.4.A and B, the results indicated that the real-time RT-PCR was able to detect up to 14.38 pg dsRNA. At dilutions containing less than 14.38 pg total dsRNA the melting peaks could not be distinguished from that of the NTC.

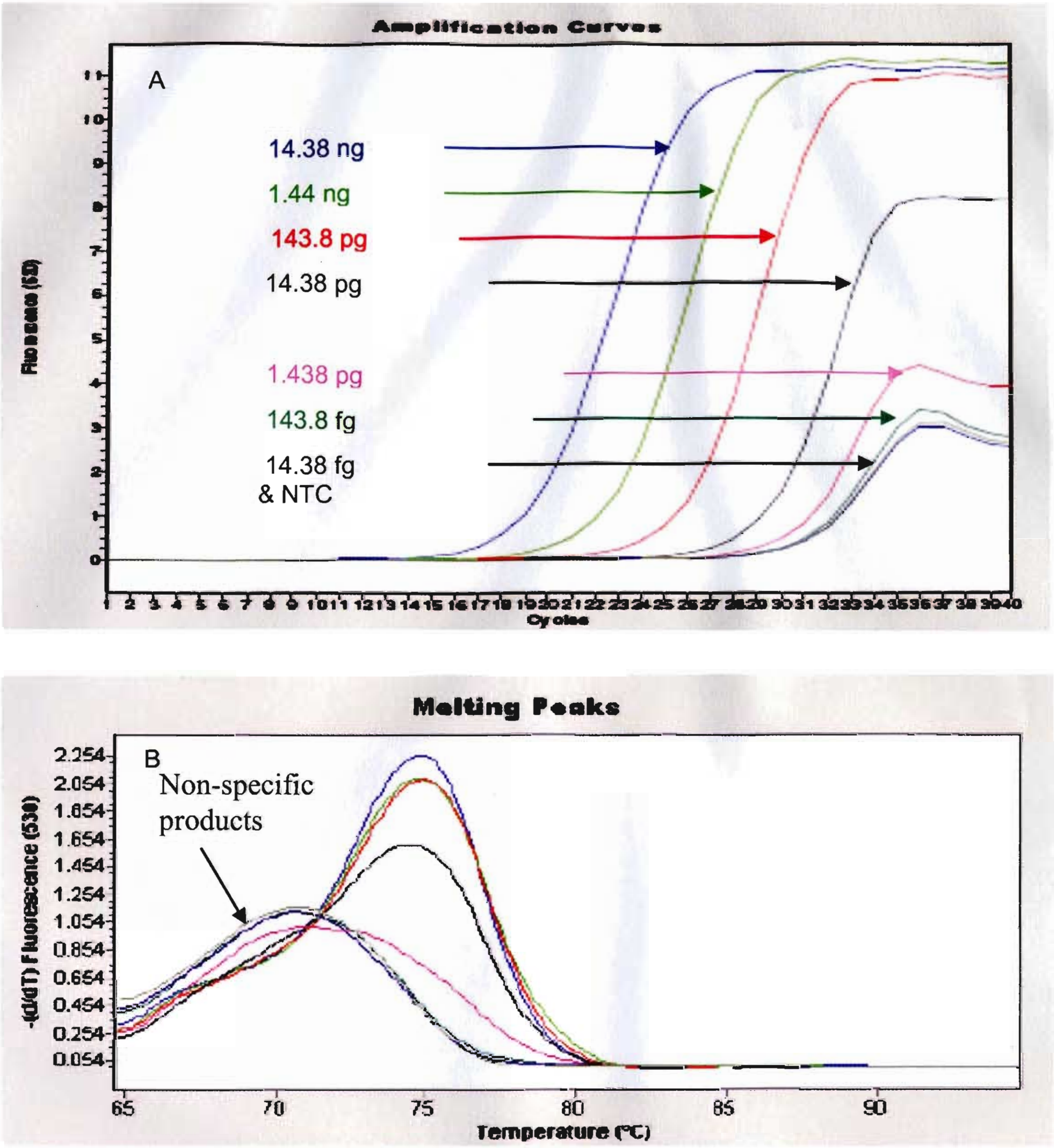


Figure 4.4: Sensitivity of BTV RT-PCR amplification determined with 10-fold serial dilution of dsRNA from 14.38 ng to 14.38 fg. (A) Amplification plot for BTV dsRNA by real-time RT-PCR based on SYBR Green I chemistry. (B) Melting peak temperature profiles of the different template dilutions with temperatures varying between 75.49 °C and 77.50 °C.

Table 4.3

CP- values and measured melting temperatures (T_m) of the PCR fragments for dilution series of BTV 7

Sample	Tm(° C)	Cp
14.38 ng	75.49	18.3
1.44 ng	75.65	21.7
143.8 pg	75.76	24.85
14.38 pg	75.7	28
1.438 pg	71.26	28.87
143.8 fg	71.20*	29.47
14.38 fg	71.25*	29.51
NTC	71.10*	29.53

* Melting temperatures were manually determined because they were not detected by the software.

4.4. DISCUSSION

The objective of this chapter was to design a specific and rapid one step RT-PCR based method for detection of all 22 reference serotypes of BTV that occur in South Africa. This was done to provide a method that could be developed and applied to decrease the time needed for the completion of molecular BTV diagnostics. For this purpose a set of primers previously published by Katz & co-workers (1993) were used to sequence BTV genome segment 5, because this genome segment has been shown to contain highly homologous regions that are useful for designing consensus primers that are specific for all the serotypes (Hwang *et al.*, 1993).

To determine homologous regions, sequence data obtained from 17 BTV reference isolates (Fig 4.1) were aligned. This sequence information was compared to genome segment 5 sequences found on GenBank. All the sequenced products that were analyzed showed high similarity with the published sequences of BTV segment 5. The newly obtained sequences had a nucleotide identity of 86.90%, which is comparable to that found by Monaco & co-workers (2006).

A region between nucleotides 16 and 95 was selected to design the RT-PCR primers (Fig.4.1).The area targeted by the forward primer showed 100% identity between all the sequences analyzed whilst the area where the reverse primers was designed displayed two substitutions. A degenerate primer was designed to ensure amplification of the target sequence. The ability of the primers to identify BTV was shown and all the amplification products were of the predicted size indicating that the primers were designed in conserved areas of genome segment 5.

The conventional RT-PCR assay that we developed was converted into a real-time, one-step format to avoid the post-PCR analysis steps and to reduce the risk of contamination. A SYBR Green I based method was used as described in Chapter 3. Specific amplification was obtained from all the BTV isolates. The CP-values that were observed varied between 17.7 and 24. Different isolates of the same serogroup may have slightly different sequences of the amplified fragments, contributing to variations in melting temperatures (Ririe *et al.,* 1997). This was observed in this study when the specific melting temperatures for BTV isolates varied between 75.64 °C and 77.38 °C. These Tm values are less than those of AHSV (78.48 °C - 80.18 °C) (Chapter 3); therefore a melting curve analysis can be easily used to distinguish BTV from AHSV.

A ten-fold serial dilution of BTV 7 was used to determine the detection limit of the method. The viral RNA was detected down to a concentration of 14.38 pg; below this level the test could no longer distinguish BTV amplification products from non-specific products. This analytical sensitivity of the amplified product derived from 14.38 pg of target sequence is higher than the sensitivity of real-time PCR for AHSV (3.2.3.2).This could be attributed to the fact that the endpoint of detection for AHSV could not be reached. Secondly the BTV amplicon (79 bp) is smaller than the AHSV (232 bp), therefore this could have resulted in increased primer binding efficiency for BTV.

This sensitivity is also lower than that observed with recently reported BTV real-time RT-PCRs which detected viral RNA down to a dilution of 10^{-5} (Jiménez-Clavero *et al.,* 2006, Shaw *et al.*, 2007) because probe based techniques are more sensitive than intercalating dye based ones. The melting temperatures that are lower (~71 °C) than those of the BTV-specific PCR products observed for dilutions lower than 14.38 pg were indicative of non-specific products. This could possibly be caused by the presence of primer-dimers.

In conclusion, sequence information of genome segment 5 of BTV was used to design a serogroup-specific RT-PCR test. The primers had the ability to detect all 22 BTV serotypes and it was possible to use the primers in a real-time format with SYBR Green I dye as a reporter. The BTV-specific primers were subsequently used with the AHSV group-specific primers (Chapter 3) in a duplex real-time RT-PCR to detect both viruses in one reaction under the same reaction conditions.

CHAPTER 5

5. *MULTIPLEX REAL-TIME PCR FOR AHSV AND BTV*

5.1. INTRODUCTION

The objective of the present work was to use real-time PCR methods developed in Chapters 3 & 4 in a duplex form. This was achieved by combining both AHSV and BTV primer pairs into one reaction to identify both viruses. These primers were used in a real-time RT-PCR format making use of SYBR Green I dye as a reporter of cDNA amplification. The specificity of the primers was tested by including dsRNA from field isolates of both AHSV and BTV, and from EEV and EHDV isolates. Both EEV and EHDV are arthropod-borne diseases with the aetiological agents belonging to the *Orbivirus* genus, family *Reoviridae.* EEV and EHDV have overlapping distribution with and cause similar diseases to AHSV and BTV respectively.

5.2. SPECIFIC OBJECTIVES

- ✓ To test the AHSV (Chapter 3) and BTV (Chapter 4) primers in a duplex one-step real-time RT-PCR containing dsRNA from both viruses.
- ✓ To investigate the specificity of the primers with dsRNA from other *Orbiviruses*.

5.3. RESULTS

5.3.1. AHSV AND BTV MULTIPLEX PCR

The specificity of the SYBR Green I real-time RT-PCR followed by a melting curve analysis in a multiplex format was determined. AHSV 1 and BTV 7 reference serotypes were used as templates in the real-time multiplex PCR. The Lightcycler® SYBR® Green I kit was used as detailed in Section 2.2. Three capillary tubes were used whereby capillary tubes 1 and 2 contained 193.5 ng of AHSV 1 and 150.8 ng of BTV 7, and the third capillary tube contained AHSV and BTV dsRNA. AHSV primers (S7/03 & S7/04) and BTV primers (NS1/01 & NS1/02) at a concentration of 10 pmol/µl each were pooled.

During the real-time PCR the increase in fluorescence was measured in the 530 nm channel for each reaction, and it is represented as amplification curves and CP-values. When in separate reactions, AHSV 1 and BTV 7 showed melting peaks at 79.36 °C and 75.27 °C respectively. However, when in duplex form, initial results of only one peak representing AHSV (79.38 °C) was observed, although a shoulder of BTV melt peak was visible (Fig. 5.1(A)). This AHSV melt peak overlapped with and concealed the melting peak of BTV. When the amplicons were separated by 2% agarose gel electrophoresis, two amplicons (lane 3) were visible representing 232 bp for AHSV and 79 bp for BTV (Fig. 5.1(B)). This shows that although the conditions were not suitable for both melting peaks to be demonstrated, the gel results showed that both products were amplified. Lanes 1 and 2 showed bands that corresponded with the expected molecular weight for both AHSV (232 bp) and BTV (79 bp) amplification products.

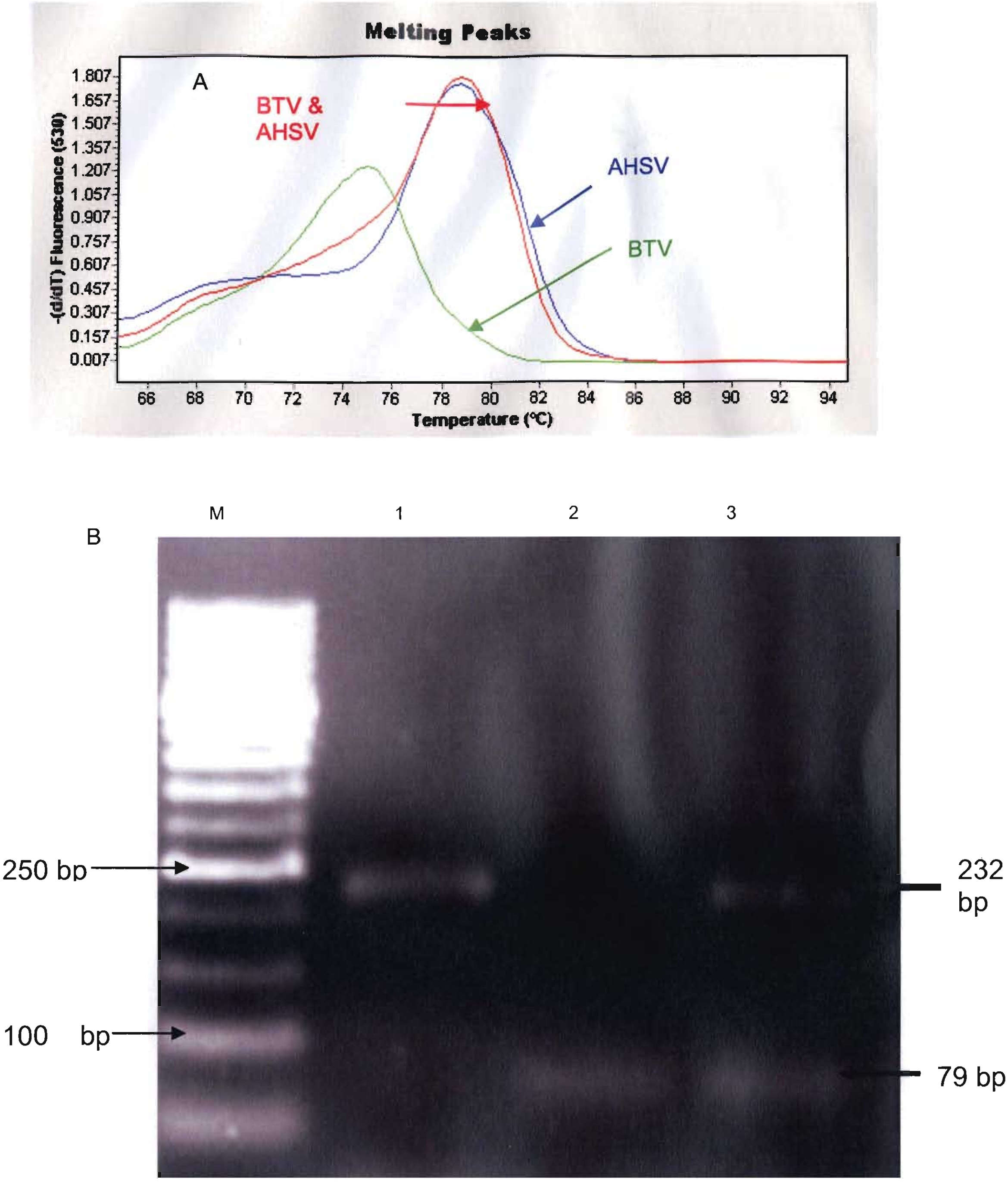


Figure 5.1. (A) Fluorescence melting curve of BTV and AHSV duplex real-time RT-PCR. The AHSV peak overshadows that of BTV when in duplex form (red plot).(B) Gel showing AHSV and BTV amplicons using AHSV 1 and BTV 7 reference strains. PCR products were run on a 2% agarose gel, stained with ethidium bromide. Lane1: molecular weight marker XIII, (Roche), 50-750 bp Lane 2: AHSV 1, Lane 3: BTV 7 and Lane 4: AHSV 1 and BTV 7.

5.3.2. EFFECT OF CONCENTRATION ON THE DUPLEX RT-PCR

This experiment was carried out to investigate whether template concentration affected peak resolution for BTV. Both AHSV 1 and BTV 7 reference serotypes were subjected to 10-fold serial dilutions and the following template concentrations were used in capillary tubes 1-4:

- Tube 1: AHSV 193.5 ng and BTV 150.8 ng ,
- Tube 2: AHSV 19.35 ng and BTV 15.08 ng,
- Tube 3: AHSV 1.935 ng and BTV 1.508 ng,
- Tube 4: AHSV 19.35 pg and BTV 15.08 pg.

The melting peaks of the first two tubes were the same as those obtained in Figure 5.1 and only the peak corresponding to AHSV was visible on the melting curve. In lanes 1 and 2 (tubes 1 and 2), bands corresponding to AHSV (232 bp) and BTV (79 bp) were observed after agarose gel electrophoresis, confirming that both serogroups amplified although the melting peak of BTV was not observed on the melting curve. Bands that are indicative of primer-dimers were also visualized below the 50 bp marker (Fig. 5.2). Lanes 3 and 4 (tubes 3 and 4) had no specific amplicons showing that there was not enough dsRNA templates to be amplified by the PCR.

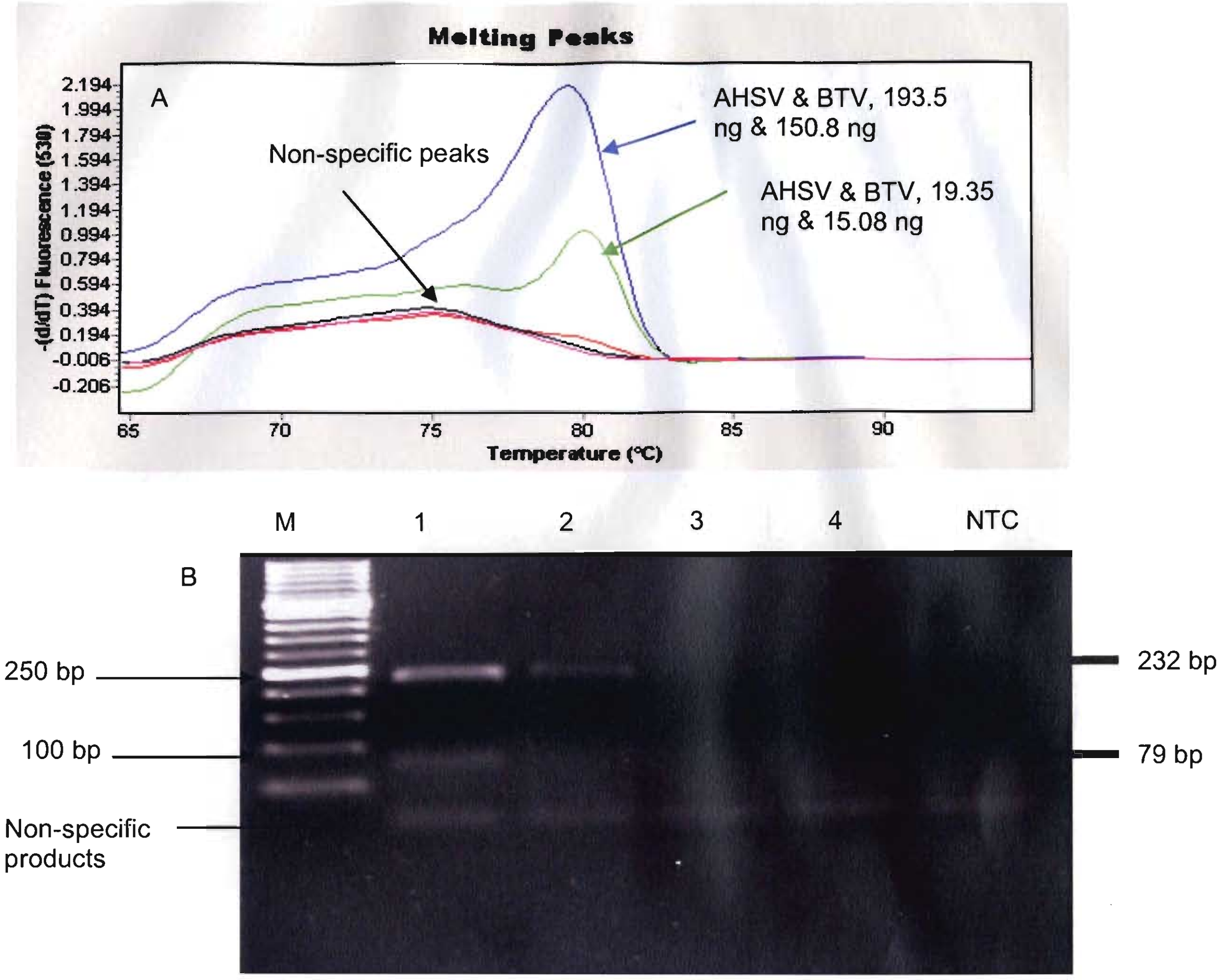


Figure 5.2: Multiplex real-time PCR determined with 10-fold serial dilutions of BTV and AHSV dsRNA. Amounts of dsRNA used for AHSV ranged from 193.5 ng to 19.35 pg and 150.8 ng to 15.08 pg for BTV. (A) Fluorescence melting curve for dsRNA of BTV 7 and AHSV 1 reference strains in SYBR Green I multiplex real-time PCR and (B) 2% Agarose gel electrophoresis of PCR products obtained by real-time RT-PCR. The bands present in Lanes 1-2 represent AHSV (232 bp) and BTV (79 bp) PCR products. Lanes 3-4 show the results of real-time RT-PCR when the templates were diluted to 10^{-3} and 10^{-4} respectively. Lane M: Molecular weight marker XIII, 50-750 bp (Roche). NTC- No template control.

5.3.3. EFFECT OF RAMP RATE ON DISCRIMINATION OF MELTING PEAKS

To investigate possible ways of resolving the AHSV and BTV melt peaks in the same test, the melting curve annealing ramp rate was changed from 20 ° C/s to 5 ° C/s during the melting curve analysis because a slow ramp rate can result in discrimination of the melting peaks and higher resolution of the amplicons. A NTC, whereby dsRNA was replaced with nuclease-free water was run alongside the samples to rule out cross contamination. Two peaks could be distinguished at 76.30 °C and 80.04 °C representative of BTV and AHSV respectively as shown by the red line in Fig 5.3A. These results were confirmed by loading the PCR products onto a 2% agarose gel. PCR product from AHSV 1 was loaded onto the first lane, in the second lane it was BTV 7, the mixture of BTV 7 and AHSV 1 was loaded onto lane 3 and the NTC was loaded in lane 4. All these amplicons showed results that correspond with either AHSV (232 bp) or BTV (79 bp).

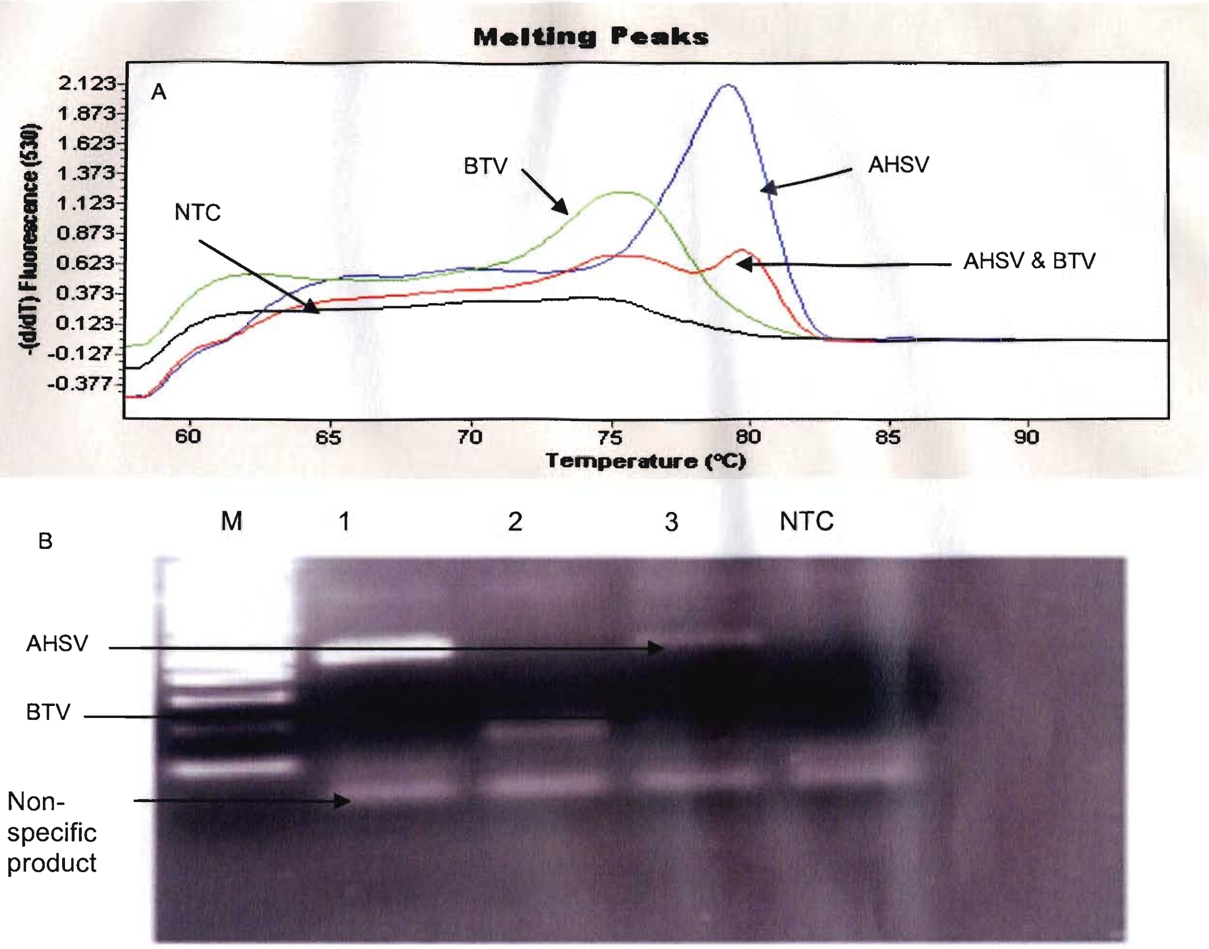


Figure 5.3: Real-time RT-PCR for both AHSV and BTV in a duplex format using peak T_m detection. (A) Melt peak analysis of duplex targets. The reaction tubes were subjected to a decreased melt rate (0.2 °C/ s). (B) Agarose gel electrophoresis (2% agarose / TBE with Ethidium bromide staining) confirming the presence of both amplicons (Lane 3). M:Molecular weight marker XIII,50-750 bp (Roche), Lane 1: AHSV 1, Lane 2: BTV 7, Lane 3: AHSV 1 & BTV 7, Lane 4: No template control.

5.3.4. TESTING OF real time-RT-PCR SPECIFICITY USING FIELD ISOLATES

A single nucleotide substitution in the region that is targeted by the primer can cause hybridization of the primers to be insufficient and inhibit detection of the samples by real-time PCR (Jiménez-Clavero *et al.*, 2006).These sequence variations can also lead to false negative results, therefore to rule out any possibility of false negatives BTV field isolates were used to investigate the specificity of the BTV real-time RT-PCR method. The assay was able to detect all BTV isolates (Fig. 5.4) with melting temperatures varying between 75.98 °C and 76.87 °C (Table 5.1).

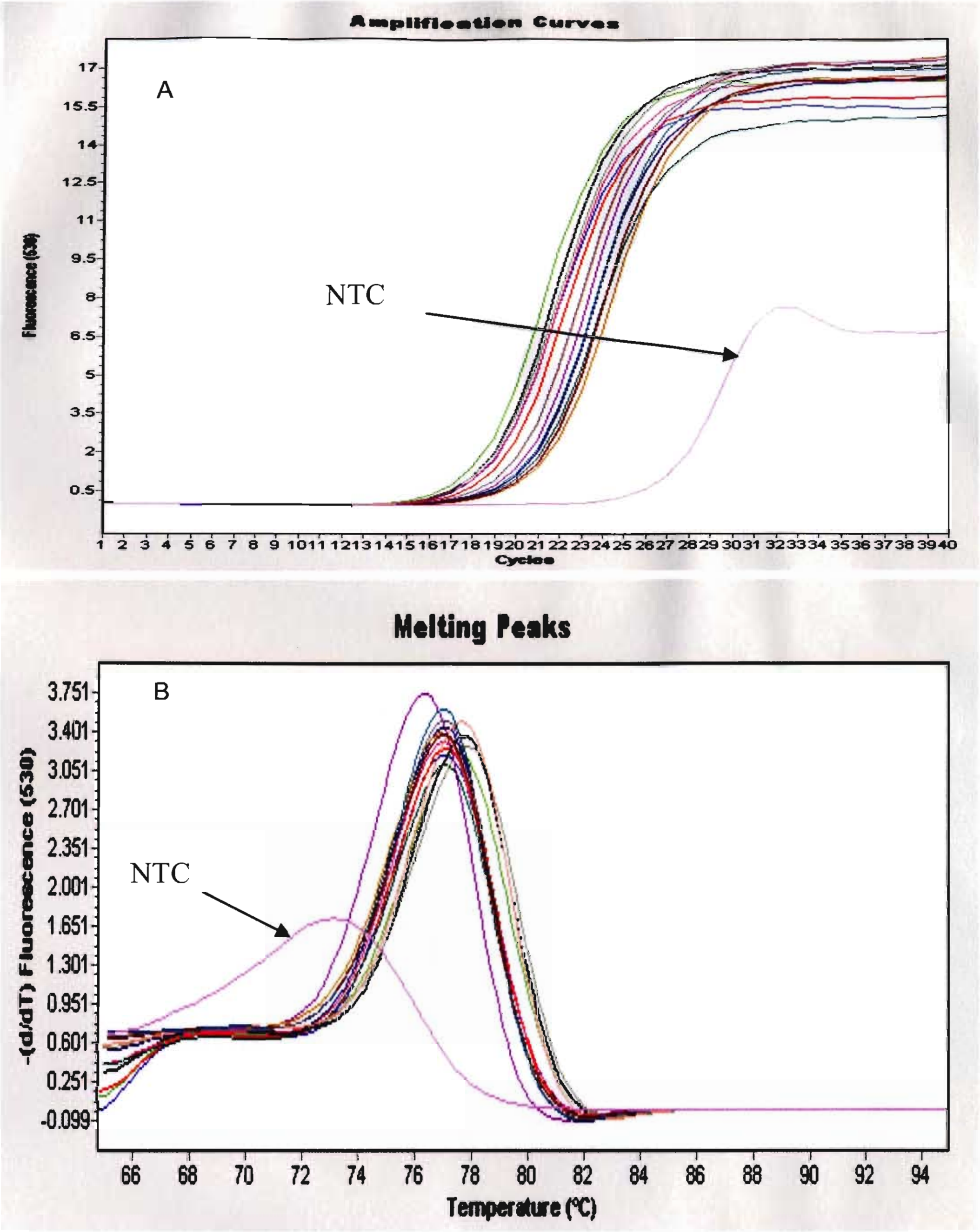


Figure 5.4: Results of BTV real-time RT-PCR using BTV field isolates. (a) Amplification plot and (b) Melting curve with melting temperatures ranging between 75.98 °C and 76.87 °C. BTV field isolates were used to investigate the specificity of the developed test.

Table 5.1

Cp-values and melting temperatures for BTV field isolates

Serotypes & sample no.s	Tm (°C)	Cp	Year of isolation
9(71)	77.27	17.65	1996
5(80)	77.73	17.03	1997
12(83)	77.3	18.04	1998
11(86)	77.89	17.54	1998
24(87)	77.2	17.76	1998
4(90)	77.31	19.53	1999
1(91)	77.19	19.43	1999
14(93)	78.09	17.68	1999
2(95)	77.83	18.74	2000
6(97)	76.54	19.09	2000
3(102)	77.16	20.11	2000
17(103)	77.19	19.44	2000
16(104)	77.23	19.82	2000
15(105)	77.24	18.7	2000
NTC	73.5	25.25	n/a

The specificity of the AHSV real-time RT- PCR primers was also investigated using 14 different AHSV field isolates (Table 5.2). Amplification was observed with all the AHSV field isolates (Fig 5.5) that were tested. Melting curve analysis of the amplicons allowed identification of the amplicons as AHSV specific. The melting temperatures for these isolates varied between 78.59 °C and 79.41 °C.

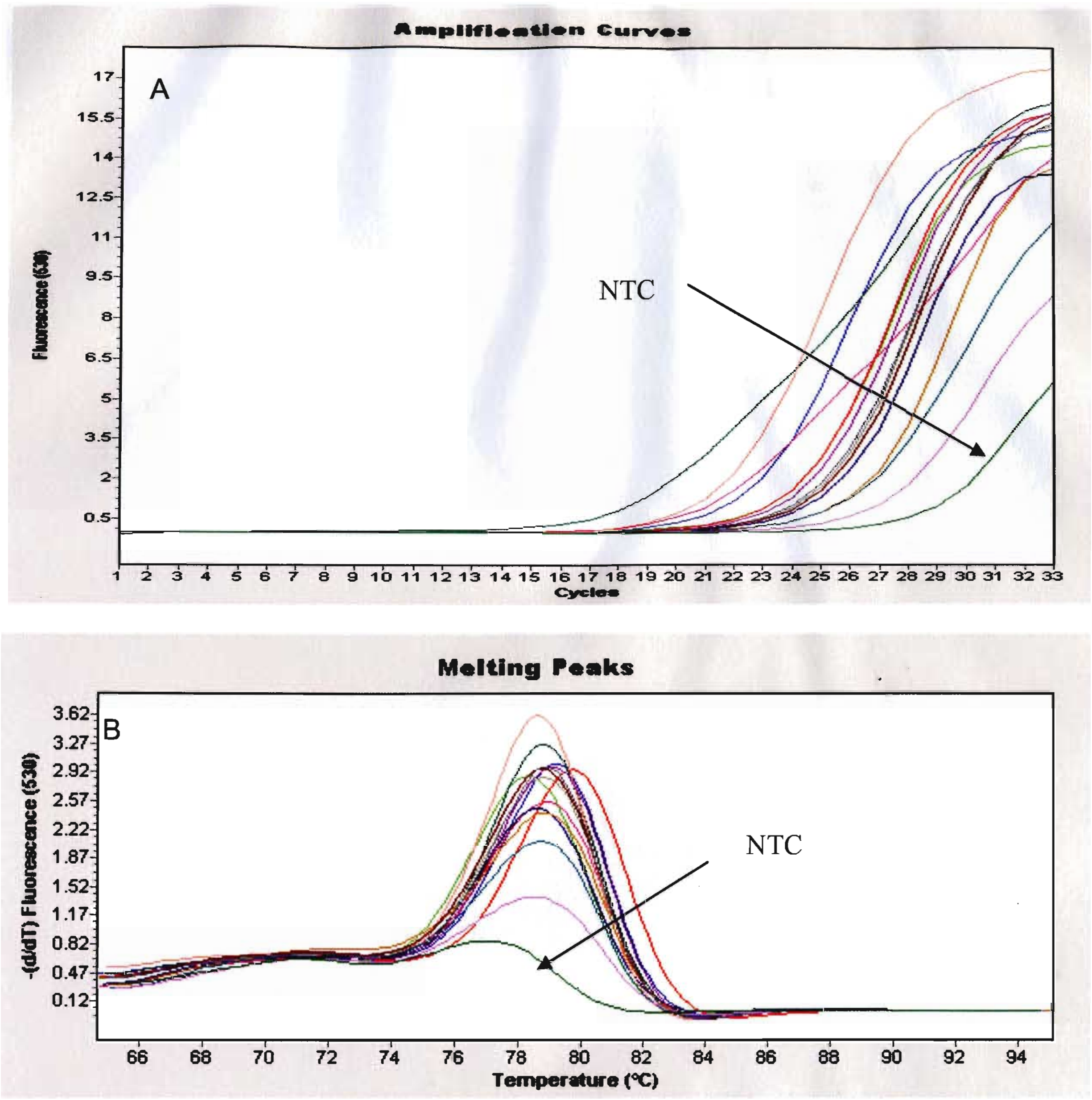


Figure 5.5: Specificity of AHSV real-time RT-PCR based on SYBR Green I chemistry showing (A) Amplification plot and (B) Melting curve with specific melting temperatures ranging from 78.59 °C - 79.41 °C using dsRNA from different field isolates of AHSV.

Table 5.2

Cp-values and melting temperatures of AHSV field isolates

Serotype	Tm (° C)	Cp	Year of isolation
1	79.41	21.63	1999
2	78.59	22.96	1997
3	79.97	23.07	2002
5	79.2	20.27	2006
6	79.01	17.68	1998
7	78.92	24.02	2004
8	79.1	23.83	2001
9	78.82	20.73	2001
1	79.32	23.42	2001
2	79.23	25.08	2001
3	79.12	25.32	2001
4	79.04	23.99	2001
5	79.14	23.74	2002
6	79.3	27.32	2001
NTC	78.54	>28.00	n/a

In order to discard the possibility of cross-reaction with EEV and EHDV, we investigated the group-specificity of the assay with samples containing dsRNA from isolates of EEV Kyalami and EHDV1.One hundred and ninety three ng of AHSV, 150.8 ng of BTV, 160 ng of EEV and 165.3 ng of EHDV dsRNAs were used as templates when investigating the specificity of the multiplex real-time RT-PCR. The AHSV 1 isolate, with a 232 bp amplified fragment, had a T_m of 79.30 °C and the BTV 7 specific RT-PCR produced a 79 bp fragment with T_m of 76.45 ° C. Melting curve analysis of EEV and EHDV did not give any discernable melting peaks (Fig. 5.6). Around cycle 33, amplification was observed for both EEV and EHDV samples indicative of non-specific amplification because they produced much lower fluorescence when compared to those of AHSV and BTV.

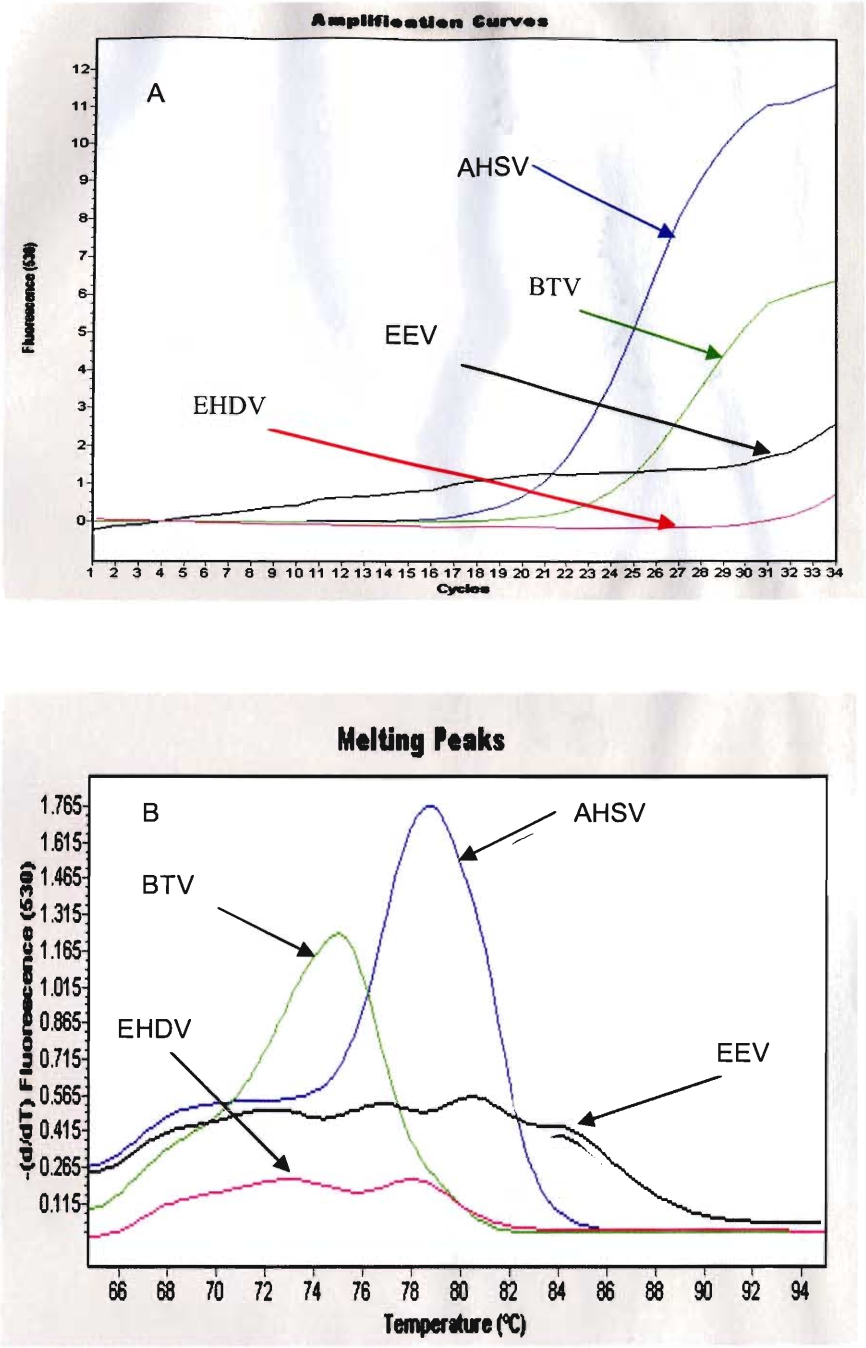


Figure 5.6: Specificity of the multiplex real-time RT-PCR assay. rRT-PCR was performed on RNA extracted from Vero cells infected with AHSV serotype 1 and BTV serotype 7 as well as EEV Kyalami and EHDV 1 respectively. (A) Amplification plot from the SYBR Green real-time RT-PCR showing detection of AHSV and BTV, with EEV and EHDV (represented with black and red plots) being detected towards the end of the reaction and (B) Melting curve with the specific T_ms for BTV at 76.45 °C and AHSV at 79.30 °C respectively.

5.4. DISCUSSION

This part of the study was carried out to determine if the two real-time RT-PCRs that can detect AHSV and BTV respectively, can also be used to detect both viruses in one test. This was accomplished by combining the reagents for both PCRs into one tube and using them in a reaction that contains dsRNA of both viruses as templates. It has been shown that AHSV and BTV amplification give distinct melting peaks (Chapters 3 & 4); therefore, it was expected to see both peaks as a result of both dsDNA amplification products being present at the time of the melting curve analysis.

When this experiment was performed for identification of both BTV and AHSV, only a clear peak corresponding to AHSV was observed initially (Fig.5.1 A), although it had a shoulder that could be interpreted as representative of BTV. Overshadowing of the BTV melting peak by that of AHSV was possibly due to a high ratio of AHSV amplicon to that of BTV, resulting in loss of sensitivity for the BTV amplicon. This was also shown in the gel electrophoresis as the band representative of AHSV was more intense than that of BTV (Fig.5.1 B).

It was decided to carry out experiments where different parameters were optimized in an effort to make the two melting peaks clearly discernable. A study by Varga & James (2006) showed that higher resolution of T_m is accomplished at a lower melt curve annealing ramp rate, producing more reliable detection of weak amplicons. A similar observation was made in this study. When the melt curve annealing ramp rate was changed from 20 °C/s to 5 °C/s, both AHSV and BTV could be demonstrated in one reaction (Fig.5.3). In this study the T_m associated with BTV were consistently lower and distinct from the T_m associated with AHSV isolates, facilitating easy identification of the two viruses.

When template concentrations of both AHSV and BTV were changed in equal ratios (5.3.2.), only one melting peak corresponding with the T_m of AHSV was observed. The melt peak for AHSV was higher than that of BTV. This is because more SYBR Green dye binds to the larger amplicon (AHSV, 232 bp) compared with the smaller amplicon (BTV, 79 bp), causing a bigger change in fluorescence. When PCR products were loaded onto a 2 % agarose gel, bands that correspond with the expected molecular weight (232 bp for AHSV and 79 bp for BTV) were revealed (Fig.5.2 B, Lanes 1 & 2).

These findings were followed up by investigations of the test specificity with field isolates of AHSV and BTV (5.3.4) to rule out any possibility of false negatives due to genetic variations among the strains (Monaco *et al.,* 2006; Bonneau *et al.*, 1999). The AHSV genome segment 7 group-specific primers and the BTV genome segment 5 group-specific primers could be used in real-time RT-PCR to detect dsRNA from their corresponding serogroups. These target sites in the genome were well conserved since there was successful amplification of dsRNA from field isolates. The melting temperatures of the reference and field isolates ranged between 78.48 °C and 80.18 °C for AHSV and 75.98 °C and 77.38 °C for BTV. These variations were small enough and avoided an overlap between AHSV and BTV.

The specificity of the primers for AHSV and BTV was also tested with EEV and EHDV, which are members of the family *Reoviridae* and are related to BTV and AHSV (Iwata *et al.*, 1992), to rule out any possibility of false positives. EEV and EHDV cause diseases that have similar symptoms as those of AHSV and BTV, and it is possible to get EEV positive samples that have to be tested for AHSV, and the same may apply to BTV and EHDV. Therefore, to rule out cross-reactions and to provide a molecular tool for differential diagnosis, EEV and EHDV were included in the test to investigate the group-specificity of the AHSV and BTV primers. No cross-reactions were observed with RNA from EEV or EHDV showing that the primers are indeed group-specific for dsRNA of AHSV or BTV. Towards the end of PCR (Cycles 30-34), non-specific amplification was observed on EEV and EHDV samples. This was due to detection of primer-dimers by SYBR Green dye because it binds to any dsDNA of any size. These products could, however, be easily distinguished from the AHSV or BTV amplicons by means of the melting curve analysis.

In conclusion, a duplex real-time RT-PCR test was developed by pooling the primers for AHSV and BTV identification in one reaction. This was achieved by using a slow ramp rate for the melting curve analysis, producing two peaks that can clearly be distinguished from each other. The specificity of both tests was also shown by making use of field isolates of AHSV and BTV and other closely related orbiviruses.

CHAPTER 6

6. GENERAL DISCUSSION AND CONCLUSION

The main aim of this work was to develop a one step duplex real-time RT-PCR making use of sequence data and modern molecular techniques for detection and identification of AHSV and BTV under the same reaction conditions. At a routine diagnostic laboratory like the OVI, diagnostic assays for both AHSV and BTV are carried out in the same laboratory by the same personnel, therefore it would be beneficial to have one set of reagents and one protocol such that the diagnostic services could be streamlined. Both AHSV and BTV are dsRNA viruses and all the pre-PCR steps (dsRNA isolation, denaturing and cDNA synthesis) are identical, but the primer sets that are used are different.

Real-time PCR has several advantages over conventional PCR which include rapidity and sensitivity. Real-time PCR is less likely to produce false positives as a result of contamination during sample preparation because post-amplification manipulation of the amplicon is not required. These assays are therefore described as closed systems. There are currently five main chemistries for detection of PCR product during real-time PCR, which can be classified into amplicon specific or non-specific methods of detection. These chemistries include DNA-binding fluorophores, linear oligoprobes, 5' Nuclease oligoprobes and hairpin oligoprobes (Mackay *et al*., 2002).

SYBR Green I has a high affinity for double-stranded DNA and has enhanced fluorescence upon DNA binding. It can be used as an alternative to other methods such as linear oligoprobes, 5' Nuclease oligoprobes and hairpin oligoprobes because it is less expensive.

This intercalating dye can be directly applied to any dsDNA without the need to design and synthesize fluorescently labelled target-specific probes. When using SYBR Green dye, the amplicons are detected by an increase in fluorescence and multiplexing is made feasible by the possibility of identifying the amplified products by their melting temperatures (Ririe *et al.*, 1997). SYBR Green I dye chemistry was chosen because SYBR Green I based real-time RT-PCR tests display a real advantage over probe based tests (Jimenez-Clavero *et al.*, 2006) because it is not influenced by genetic variation to the same extend as what probe based tests are. In other words, the variations within the amplicons would not interfere with the production of a fluorescence signal when using SYBR Green I dye.

The first goal of this study was to investigate the ability of the primer sets that have been designed to amplify genome segment 7 of any of the nine AHSV serotypes and genome segment 5 of the 22 BTV serotypes that occur in South Africa. It was also the objective of this study to determine the detection limit of the developed real-time RT-PCR method. These findings were followed up by investigations to develop a duplex method whereby both AHSV and BTV could be detected under the same reaction conditions.

Both primer sets that were designed for AHSV (Chapter 3) and BTV (Chapter 4) were combined in one reaction tube and the primer annealing temperatures were adjusted to ensure that BTV and AHSV products were amplified efficiently. This resulted in a duplex real-time RT-PCR for both AHSV and BTV (Chapter 5). Further, it was shown that a SYBR Green I-based melting curve determination multiplex method is usable for simultaneous identification of two products from the same reaction tube (Fig.5.3). It was also shown that when two peaks have relatively close melting temperatures, the amplicon with low concentration loses its sensitivity and becomes indistinguishable. At faster melt rate T_ms of all fragments are shifted higher, therefore resulting in the T_m of one fragment approaching that of the second and becoming indistinguishable. However, a slow melt rate resulted in improved discrimination of the melt peaks, giving an increased resolution of the peaks.

When the limits of detection for both AHSV and BTV were determined, they were found to be lower than the previously published values for conventional RT-PCRs (Stone-Marschat *et al.*, 1994; Zientara *et al.*, 1994; Koekemoer & van Dijk, 2004) and real-time RT-PCRs (Jiménez-Clavero *et al.*, 2006; Toussaint *et al.*, 2007). Even though there were amplifications from dsRNA diluted beyond 10^{-3} for both AHSV and BTV, the amplification of non-specific products interfered with the melting curve analysis (Figs 3.4 & 4.4). This had the effect that even though BTV or AHSV specific amplicons were present, they could not be detected because of the strong signals from the primer-dimers that formed in the reaction around cycles 30-35. This is one of the inherent drawbacks of using SYBR Green as an indicator of cDNA amplification.

To improve sensitivity a high resolution melting (HRM) analysis incorporating SYBR Green I (Pornprasert *et al.*, 2008) and an unlabelled probe can be used. HRM is a closed system that does not require a labelled probe. When using this technique, the accuracy of the melt curve is maximized by acquiring fluorescence data over small temperature increments. Amplicons containing different sequences can be discriminated based on the melt curve shape because it is the function of the DNA sequence being melted. These unlabelled probes are included in the PCR master mix which also contains asymmetric ratios of primers. Some of the benefits of HRM include high sensitivity, specificity and a decreased risk of PCR carryover contamination because it is a closed-tube system.

Several field isolates of both AHSV and BTV (5.3.4) were used as templates for the real-time RT-PCR to test the specificity of the new method because genetic variations among the strains, causing nucleotide substitutions can cause the primers not to bind to the specific target (Jiménez-Clavero *et al.*, 2006). It was shown that the primer sets are group-specific when 79 bp (nt 16-95) of genome segment 5 (BTV) (Fig.5.4) and 232 bp (nt 63-295) of genome segment 7 (AHSV) (Fig.5.5) could be successfully amplified and identified without any real-time signal from EEV and EHDV which are closely related to AHSV and BTV (Iwata *et al.,* 1992) (Fig.5.6).

Valuable information could be obtained from studies of the midge vectors which are known to transmit BTV and AHSV. Only one serogroup can be isolated from vector midges when both AHSV and BTV are expected (personal communication Dr Gert Venter, OVI).This is most likely due to the fact that the isolation procedure favours one of the viruses. The real-time RT-PCR described in this study has the ability to identify and detect both viruses; therefore, it could potentially be used to screen vector populations for presence of either or both viruses.

In conclusion, the duplex real-time RT-PCR assay described here using SYBR® Green I dye as a reporter, appears to be a promising tool for rapid, specific and accurate identification and detection of AHSV and BTV. The observed sensitivity was lower than the other real-time PCRs that have been reported for both BTV and AHSV, but it can be enhanced by using high resolution melt and unlabelled probes. This duplex real-time RT-PCR provides a valuable method that has been tested using AHSV serotypes 1-9 and the 22 BTV serotypes (1-19 & 22-24) that circulate in South Africa as well as a range of field isolates from both species. However, different aspects such as annealing temperature still need to be optimized for both targets to be efficiently amplified. The method must also be optimized to make it more sensitive and be able to work with less of the template. To increase efficiency of the RT-PCR and to avoid formation of primer-dimers, primers will have to be redesigned.

When low-copy number detection is needed, sequence-specific fluorescent probes can be used to provide additional sensitivity, but the design, synthesis and purification of these probes will require care.

The SYBR Green I real-time RT-PCR method developed and applied in this study represents a new analytical tool that allows large-scale diagnosis of AHSV and BTV because the assay can be completed within 24 hours. The assay can be applied as a rapid screening test for the purposes of certifying animals free of AHSV or BTV. This can be very useful in routine diagnostic laboratories where there are often large numbers of samples to be tested for the presence of either of these viruses. The greatest benefit of the method is that it is a first ever single test, with the same reagents and has the potential to be used for identification of two orbiviruses in clinical samples and insect vectors. This assay is much quicker to perform (lasts only about 2 hours) than virological tests which can take up to two weeks to obtain results, starting with virus isolation and ending with a description of the virus serotype.

CHAPTER 7

7. REFERENCE LIST

1. Afshar, A. Thomas, F.C. Wright, P.F. Shapiro, J.L. & Anderson, J. 1989. Comparison of competitive ELISA and AGID tests for detecting blue-tongue virus antibodies in cattle and sheep. *Veterinary Records*. 124: 136-141.

2. Akita, G.Y. Chinsangaram, J. Osburn, B.I. Lanconescu, M. & Kaufman, R. 1992. Detection of bluetongue virus serogroup by polymerase chain reaction. *Journal of Veterinary Diagnostic Investigations*. 4: 400-405.

3. Anthony, S. Jones, H. Darpel, K.E. Elliott, H. Maan, S. Samuel, A. Mellor, P.S. & Mertens, P.P.C. 2007. A duplex RT-PCR assay for detection of genome segment 7(VP7 gene) from 24 BTV serotypes. *Journal of Virological Methods*. Doi:10.1016/j.jviromet.2006.12.013.

4. Aradaib, I.E. Mohemmed, M.E.H. Sarr, J.A. Idris, S.H. Ali, N.O.M. Majid, A.A. & Karrar, A.E. 2006. A simple and rapid method for detection of African horsesickness virus serogroup in cell cultures using RT-PCR. *Veterinary Research Communications*. 30: 319-324.

5. Barnard, J.H. Epidemiology of African horsesickness and the role of the zebra in South Africa. *Archives of Virology. Supplementum*.14: 13-19.

6. Bansai, O.B. Stokes, A. Bansai, A. Bishop, D. & Roy, P. 1998. Membrane organization of bluetongue virus nonstructural glycoprotein NS3. *Journal of Virology*. 72: 3362-3369.

7. Beuret, C. 2004. Simultaneous detection of enteric viruses by multiplex real-time RT-PCR. *Journal of Virological Methods*. 115: 1-8.

8. Billinis, C. Koumbati, M. Spyrou, V. Nomikou, K. Mangana, O. Panagiotidis, C.A. & Papadopoulos, O. 2001. Bluetongue virus diagnosis of clinical cases by a duplex reverse transcription-PCR: a comparison with conventional methods. *Journal of Virological Methods*. 98: 77-89.

9. Bonfield, J.K. Smith, K.F. & Staden, R.A. 1995. A new DNA assembly program. *Nucleic Acids Research*. 24: 4992-4999.

10. Bosman, P. Bruckner, G.H. & Faul, A. 1995. African horse sickness surveillance systems and regionalization/zoning, the case of South Africa. *Revue Scientifique et Technique (International Office of Epizootics).* 14: 645-653.

11. Bonneau, K.R. Zhang, N. Zhu, J. Zhang, F. Li, Z. Zhang, K. Xiao, L. Xiang, W. & Machlachlan, N.J. 1999. Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States and the People's Republic of China. *Virus Research*. 61: 153-160.

12. Botha, W.J.S. Jaeger, H.G. Aitchison, H. & Stoltsz, W.H. 1996. Vaccines against viral diseases. Onderstepoort Biological Products. National Department of Agriculture. pp. 28-42.

13. Bremer, C.W. Huismans, H. & van Dijk, A.A. 1990. Characterization and cloning of the African horsesickness virus genome. Journal of General Virology. 71:793-799.

14. Bremer, C.W. & Viljoen, G.J. 1998. Detection of African horsesickness virus and discrimination between two equine orbivirus serogroups by reverse transcription polymerase chain reaction. *Onderstepoort Journal of Veterinary Research.* 65:1-8.

15. Bremer,C.W. 1976. A gel electrophoretic study of the protein and nucleic acid components of African horsesickness virus. *Onderstepoort Journal Of Veterinary Research.* 43: 193-199.

16. Chomczynski, P. & Sacchi, N. 1987. Single step method for RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry.* 162: 156-159.

17. Chomczynski, P. & Sacchi, N. 1987. Single step method for RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry.* 162: 156-159.

18. Chuma, T. Le Blois, H. Sánchez-Viscaino, J.M. Diaz-Laviada, M. & Roy,P. 1992. Expression of the major core antigen VP7 of African horsesickness virus by a

recombinant baculovirus and its use as a group-specific diagnostic reagent. *Journal of General Virology*.73: 925-931.

19. Coetzer, J.A.W. & Guthrie, A.J. 2004. *African horse sickness*. In: Coetzer, C.A.W., Tustin, R.C. (Eds.). Infectious diseases of Livestock, Vol 11, Oxford University Press, Cape Town, South Africa, pp1231-1246.

20. Diaz-Ruiz, J.R. & Kaper, J.M. 1978. Isolation of viral double-stranded RNAs using a LiCl fractionation procedure. *Preparative Biochemistry*.8:1-17.

21. Du Toit, R.M. 1944. The transmission of Bluetongue and Horsesickness by culicoides. *Onderstepoort Journal of Veterinary Science*. 19: 7-16.
22. Erasmus, B.J. 1975. Bluetongue in sheep and goats. *Australian Veterinary Journal*. 52: 196-198.

23. Fan, W. Hamilton, T. Webster-Sesay, S. Nikolich, M.P. & Lindler, L.E. 2007. Multiplex real-time SYBR Green I PCR assay for detection of tetracycline efflux genes of Gram-negative bacteria. *Molecular and Cellular Probes*. 21: 245-256.

24. Flemming, G. 1878. The horse-sickness in South Africa. *Veterinary Journal or Annals of Comparative Pathology*. VII: 100-104.

25. Gibbs, E.P. & Greiner, E.C. 1994. The epidemiology of bluetongue. *Comparative Immunology, Microbiololgy and Infectious diseases*. 17: 207-220.

26. Gorman, B.M. Taylor, J. & Walker, P.J. 1983. The Reoviridae. In: Joklik, W.K. (Ed.), Orbiviruses, Plenum, New York, pp 287-357.

27. Grimes, J.M. Burroughs, J.N. Gouet, P. Diprose, J.M. Malby, R. Zientara, S. Mertens, P.P.C. & Stuart, D.I. 1998. The atomic structure of the blue tongue virus core. *Nature*. 395: 470-478.

28. Gumm, I.D. & Newman, J.F. 1982. The preparation of purified bluetongue virus group antigen for use as a diagnostic reagent. *Archives of Virology*. 72:83-93.

29. Gunson, R.N. Collins, T.C. & Carman, W.F. 2006. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. *Journal of Clinical Virology*. 4:355-367.

30. Hamblin, C. Mertens, P.P.C. Mellor, P.S. Burroughs, J.N. & Crowther, J.R. 1991. A serogroup specific enzyme-linked immunosorbent assay for the detection and identification of African horse sickness viruses. *Journal of Virological Methods*. 31: 285-292.

31. Hernández, M. Rodriguez-Lazaro, D. Esteve, T. Prat, S. & Pla, M. 2003. Development of melting temperature-based SYBR Green I polymerase chain reaction methods for multiplex genetically modified organism detection. *Analytical Biochemistry*. 323: 164-170.

32. Henning, M.W. 1956. African horsesickness, Perdesiekte, Pestis Equorum. In: Animal Diseases of South Africa. Pretoria: Central News Agency Ltd.pp785-808.

33. Howell, P.G. 1962. The isolation and identification of further antigenic types of African horsesickness virus. *Onderstepoort Journal of Veterinary Research*. 29:139-149.

34. Howell, P. G. 1969. The antigenic classification of strains of bluetongue virus, their significance and use in prophylactic immunization, PhD Thesis, University of Pretoria, Pretoria.

35. Howell, P.G., Verwoerd, D.W. 1971. Bluetongue Virus. In: Gard, S., Hallaver, C., Meyer, K.F. (Eds.), Virology Monographs, vol.9. Springer Verlag, New York.pp.35-74.

36. Huismans, H. & van Dijk, A.A. 1990. Bluetongue virus structural components. *Current Topics in Microbiology and Immunology*: Bluetongue Viruses. 162: 21-41.

37. Huismans, H. van Dijk, A.A. & Els, H.J. 1987a. In Vitro phosphorylation and purification of a non-structural protein of bluetongue virus with affinity for single-stranded RNA. *Journal of Virology*. 61:3589-3595.

38. Huismans, H. Van der Walt, N.T. Cloete, M. Erasmus, B.J. 1987b. Isolation of a capsids protein of bluetongue virus that induces a protective immune response in sheep. *Virology*. 157: 172-179.

39. Huismans, H. & Erasmus, B.J. 1981. Identification of the serotype specific and group-specific antigens of bluetongue virus. *Onderstepoort Journal of Veterinary Research*. 48: 51-58.

40. Hwang, G.Y. Chiou, J.F. Yang, Y.Y. & Li, J.K. 1993. High-sequence conservation among the United States bluetongue viruses cognate M2 genes which encode the nonstructural NS1 tubule protein. *Virology*. 192: 321-327.

41. Hyatt, A.D. Zhao, Y. & Roy, P. 1993. Release of bluetongue virus-like particles from insect cells is mediated by BTV non-structural protein NS3/NS3A. *Virology*.193:592-603.

42. Iwata, H. Yamagawa, M. & Roy, P. 1992. Evolutionary relationships among gnat-transmitted orbiviruses that cause African horse sickness, bluetongue, and epizootic haemorrhagic disease as evidenced by their capsids protein sequences. *Virology*. 191: 251-261.

43. Jeffreys, A.J. Wilson, V. Neumann, R. & Keyte, J. 1988. Amplification of human minisatellites by the PCR: towards DNA fingerprinting of single cells. *Nucleic Acids Research*. 16: 10953-10971.

44. Jiménez- Clavero, M.A. Agüero, M. Miguel, E.S. Mayoral, T. López, M.C. Ruano, M.J. Romero, E. Monaco, F. Polci, A. Savini, G. & Gómez-tejedor, C. 2006. High throughput detection of bluetongue virus by a new real-time fluorogenic reverse transcription- polymerase chain reaction: Application on clinical samples from current Mediterranean outbreaks. *Journal of Veterinary Diagnostic Investigations*. 18: 7-17.

45. Kar, A.K. Bhattacharya, B. & Roy, P. 2007. Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. *BMC Molecular Biology*.8:4

46. Katz, J.B. Alstad, A.D. Gustafson, G.A. & Moser, K.M. 1993. Sensitive identification of bluetongue virus serogroup by a colorimetric dual oligonucleotide sorbent assay of amplified viral nucleic acid. *Journal of Clinical Microbiology*. 31: 3028-3030.

47. Kawasaki, E.S. Clark, S.S. Coyne, M.Y. Smith, D.S. Champlin, R. Witte, O.N. & McCormick, F.P. 1988. Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukaemia-specific mRNA sequences amplified *in vitro. Proceedings of the National Academy of Science. USA*. 85: 5698-5702.

48. Koekemoer, J.J.O. & van Dijk, A.A. 2004. African horsesickness virus serotyping and identification of multiple co-infecting serotypes with a single genome segment 2 RT-PCR amplification and reverse line blot hybridization. *Journal of Virological Methods*.122: 49-56.

49. Laviada, M.D. Babin,M. Dominguez, J. & Sanchez-Vizcaino, J.M. 1992. Detection of African horsesickness virus in infected spleens by a sandwich ELISA using two monoclonal antibodies specific for VP7. *Journal of Virological Methods*. 38: 229-242.

50. Lekanne Deprez, R.H. Fijnvandraat, A.C. Ruijter, J.M. & Moorman, A.F.M. 2002. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR Green I depends on cDNA synthesis conditions. *Analytical Biochemistry*. 307:63-69.

51. Lunt, R.A. White, J.R. & Blacksell, S.D. 1988. Evaluation of a monoclonal antibody blocking ELISA for the detection of group-specific antibodies to bluetongue virus in experimental and field sera. *Journal of General Virology*. 69: 2729-2740.

52. Maan, S. Maan, N.S. Ross-Smith, N. Batten, C.A. Shaw, A.E. Anthony,S.J. Samuel, A.R. Darpel, K.E. Veronesi, E. Oura, C.A.L. Singh, K.P. Nomikou, K. Potgieter, A.C. & others. 2008. Sequence analysis of bluetongue virus serotype 8 from the Nertherlands 2006 and comparison to other European strains. *Virology*. 377:308-318.

53. Mackay, I.M. Arden, K.E. & Nitsche, A. 2002. Real-time PCR in virology. *Nucleic Acids Research*.30: 1292-1305.

54. MacLachlan, N.J. Osburn, B.I. & Stott, J.L. 1989. Is bluetongue virus infection of cattle a truly persistent viral infection? *Proceedings of the U.S. animal health association*. 94: 89-98.

55. MacLachlan, N.J. 2004. Bluetongue: A review and global overview of the only OIE List A disease that is endemic in North America. *In: 55th Annual meeting of the American College of Veterinary Pathologists (ACVP) and 39th Annual meeting of the American Society of Clinical Pathology (ASVCP), 2004 Orlando, FL, USA, ACVP and ASVCP (Eds.).*

56. Maree, F.F. & Huismans, H. 1997. Characterization of tubular structures composed of non-structural protein NS1 of African horsesickness virus expressed in insect cells. *Journal of General Virology*. 78:1077-1082.

57. McIntosh, B.M. 1956. Complement fixation with horsesickness viruses. *Onderstepoort Journal of Veterinary Research*.27:165-169.

58. McIntosh, B.M. 1958. Immunological types of horsesickness virus and their significance in immunization. *Onderstepoort Journal of Veterinary Research*. 27: 465-538.

59. Meiswinkel, R. 1998. The 1996 outbreak of African horse sickness in South Africa-the entomological perspective. *Archives of Virology. Supplementum*. 14: 69-83.

60. Meiswinkel, R. Nevill, E.M. & Venter, G.J. 1994. In: Coetzer, J.A.W. Thomson, G.R. & Tustin, R.C. (Eds.), Infectious diseases of Livestock with special reference to Southern Africa. Vol1, Oxford University Press, Cape Town, pp. 68-89.

61. Mellor, P.S. & Hamblin, C. 2004. African horse sickness. *Veterinary Research*. 35: 445-466.

62. Mellor, P.S. & Boorman, J. 1995. The transmission and geographical spread of African horse sickness and bluetongue viruses. *Annals of Tropical Medicine and Parasitology*. 89: 1-15.

63. Mellor, P.S. 1990. The replication of bluetongue virus in Culicoides Vectors. *Current Topics in Microbiology and Immunology*. 162: 143-161.

64. Mertens, P.P.C. Maan, N.S. Prasad, G. Samuel, A.R. Shaw, A.E. Potgieter, A.C. Anthony, S.J. & Maan, S. 2007. The design of primers and use of RT-PCR assays for typing European BTV isolates: differentiation of field and vaccine strains. *Journal of General Virology*. 88:2811-2823.

65. Mertens, P.P.C. & Mellor, P.S. 2003. Bluetongue. *State Veterinary Journal*. 13: 18-25.

66. Mertens, P.P.C. Arella, M. Attoui, H. Belloncik, S. Bergoin, M. Boccardo, G. Booth, T.F. Chiu, W. Diprose, J.M. Duncan, R. Estes, M.K. Gorziglia, M. Gouet, P. Gould, A.R. and others. 2000. Reoviridae. In: Van Regenmortel, M.V.H. Fauquet, C.M. Bishop, D.H.L. Calisher, C.H. Carsten, E.B. Estes, M.K. Lemon, S.M. Maniloff, J. Mayo, M.A. McGeoch, D.J. Pringle,C.R. & Wickner, R.B. (Eds) Virus Taxonomy. Seventh report of the International Committee for the Taxonomy of Viruses. New York, Academic press, pp 408-421.

67. Molenkamp, R. van der Ham, A. Schinkel, J. & Beld, M. 2007. Simultaneous detection of five different DNA targets by real-time Taqman PCR using the Roche Lightcycler480: Application in viral molecular diagnostics. *Journal of Virological Methods*. 141: 205-211.

68. Monaco, F. Cammá, C. Serini, S. & Savini, G. 2006. Differentiation between field and vaccine strain of bluetongue virus serotype 16. *Veterinary Microbiology*.45-52.

69. Mullis, K.B. & Faloona, F.A. 1987. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods Enzymology*. 155: 335-350.

70. Nel, L.H. Piccard, L.A. & Huismans, H. 1991. A characterization of the nonstructural protein from which the virus –specified tubules in epizootic haemorrhagic disease virus –infected cells are composed. *Virus Research*. 18: 219-230.

71. OIE, 2004. Manual of diagnostic tests and vaccine for terrestrial animals, African horse sickness, Office International des epizooties. Chapter 2.1.11.

72. OIE, 2004. Manual of diagnostic tests and vaccine for terrestrial animals, Bluetongue, Office International des epizooties. Chapter 2.1.9.

73. Ong, W.T. Omar, A.R. Ideris, A. & Hassan, S.S. 2007. Development of a multiplex real-time PCR assay using SYBR Green 1 chemistry for simultaneous detection and subtyping of H9N2 influenza virus type A. *Journal of Virological Methods*. 144: 57-64.

74. Pornprasert, S. Phusua, A. Suanta, S. Saetung, R. & Sanguansermsri, T. 2008. Detection of alpha-thalassemia-1 Southeast Asian type using real-time gap-PCR with SYBR Green 1 and high resolution melting analysis. *European Journal of Haematology*. 80: 510-514.

75. Potgieter, A.C. Cloete,M. Pretorius,P.J & van Dijk, A.A. 2003. 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. *Journal Of General Virology*. 84: 1317-1326.

76. Purse, B.V. Mellor, P.S. Rogers, D.J. Samuel, A.R. Mertens, P.P.C. & Baylis, M. 2005. Climate change and the recent emergence of bluetongue in Europe. *Nature Reviews Microbiology*. 3: 171-181.

77. Reddington, J.J. Reddington, G.M. & MacLachlan, N.J. 1991. A competitive ELISA for detection of antibodies to the group antigen of bluetongue virus. *Journal of Veterinary Diagnostic Investigations*. 3:144-147.

78. Ririe, K.M. Rasmussen, R.P. & Wittwer, C.T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry.* 245: 154-160.

79. Rodriguez, M. Hooghuis, H. & Castaño, M. 1993. Current status of the diagnosis and control of African horse sickness. *Veterinary Research*. 24: 189-197.

80. Rodriguez-Sanchez, B. Fernandez-Pinero, J. Sailleau C. Zientara, S. Belak, S. Arias, M. & Sanchez-Vizcaino, J.M. 2008. Novel gel-based and real-time PCR assays for the improved detection of African horsesickness virus. *Journal of Virological Methods*. Doi:10.106/j.jviromet.2008.03.029.

81. Roy,P. Mertens, P.P. & Casal, I. 1994. African horsesickness virus structure. *Comparative Immunology Microbiology and Infectious Diseases*.17: 243-273.

82. Roy, P. 1992. Bluetongue virus proteins. *Journal of General Virology*. 73: 3051-3064.

83. Roy,P. & Gorman, B.M. Bluetongue virus.1990. *Current Topics in Microbiology and Immunology*. 62:1-200.

84. Roy, P. Marshall, J.J.A. & French, T.J. 1990. Structure of bluetongue virus genome and its encoded proteins. *Current Topics in Microbiology and Immunology*. 162: 43-87.

85. Roy, P. 1989. Bluetongue virus genetics and genome structures. *Virus Research*. 13: 179-206.

86. Saiki, R.K. Scharf, S.J. Faloona, F. Mullis, K.B. Horn, G.T. Erlich, H.A. & Arnheim, N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science*. 230: 1350-1354.

87. Sailleau, C. Hamblin, C. Paweska, J.T. & Zientara, S. 2000. Identification and differentiation of the nine African horse sickness virus serotypes by reverse transcription and PCR amplification of the serotype specific L2 gene. *Journal of General Virology*. 81: 831-837.

88. Santhosh, S.R. Parida, M.M. Dash, P.K. Pateriya, A. Pattnaik, B. Pradhan, H.K. Tripathi, N.K. Ambuj, S. Gupta, N. Saxena, P. & Lakshmana Rao, P.V. 2007. Development and evaluation of SYBR Green I- based one-step real-time RT-PCR assay for detection and quantitation of Japanese encephalitis virus. *Journal of Virological Methods*. Doi:10.1016/j.jviromet.2007.02.011.

89. Scanlen, M. Paweska, J.T. Verschoor, J.A. & van Dijk, A.A. 2002. The protective efficacy of a recombinant VP2-based African horsesickness subunit vaccine candidate is determined by adjuvant. *Vaccine*. 20:1079-1088.

90. Schröter, M. Zöllner, B. Schäfer, P. Laufs, R. & Feucht, H. 2001. Quantitative detection of Hepatitis C Virus RNA by Light Cycler PCR and comparison with two different PCR assays. *Journal of Clinical Microbiology*.39: 765-768.

91. Seipp, M.T. Durtschi, J.D. Liew, M.A. Williams, J. Damjanovich, K. Voelkerding, K.V. & Wittwer, C.T. 2007. Unlabeled oligonucleotides as internal temperature controls for genotyping by amplicon melting. *Journal of Molecular Diagnostics*. 9:284-289.

92. Shaw, A.E. Monaghan, P. Alpar, H.O. Anthony, S. Darpel, K.E. Batten, C.A. Guercio, A. Alimena, G. Vitale, M. Bankowska, K. Carpenter, S. Jones, H. Oura, C.A.L. King, D.P. Elliot, H. Mellor, P.S. & Mertens, P.P.C. 2007. Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. *Journal of Virological Methods*. Doi:10.1016/j.jviromet.2007.05.014.

93. Spreull, J. 1902. Report from veterinary surgeon Spreull on the result of his experiments with the malarial catarrhal fever of sheep. *Agricultural Journal of the Cape of Good Hope*. 20: 469-477.

94. Spreull, J. 1905. Malarial Catarrhal fever (Bluetongue) of sheep in South Africa. *Journal of Comparative Pathology and Therapeutics*. 18: 321-327.

95. Stone-Marschat, M. Carvill, A. Skowronek, A. & Laegreid, W.W. 1994. Detection of African Horse Sickness Virus by Reverse Transcription-PCR. *Journal of Clinical Microbiology*.32: 697-700.

96. Theiler, A. 1921. African horse sickness (Pestis Equorum). *South African Department of Agricultural Science. Bulletin* 19.1-29.

97. Thompson, J.D. Gibson, T.J. Plewniak,K.F. Jeanmouqin, F. & Higgins, D.G. 1997. *Nucleic Acids Research*. 25:4876-4882.

98. Toussaint, J.F. Sailleau, C. Breard, E. Zientara, S. & De Clercq, K. 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *Journal of Virological Methods*. 140: 115-123.

99. Van Dijk, A.A. & Huismans, H. 1982. The effect of temperature on the *in vitro* transcriptase reaction of bluetongue virus, epizootic haemorrhagic disease virus and African horsesickness virus. *Onderstepoort Journal of Veterinary Research*. 49:227-232.

100. Van Staden, V. Theron, J. Greyling, B.J. Huismans, H. & Nel, L.H. 1991. A comparison of the nucleotide sequences of cognate NS2 genes of three different orbiviruses. *Virology*. 185: 500-504.

101. Varga, A. & James, D. 2006. Real-time RT-PCR and SYBR Green I melting curve analysis for the identification of *Plum pox virus strains* C, EA, and W: Effect of amplicon size, melt rate, and dye translocation. *Journal of Virological Methods*. 132:146-153.

102. Verwoerd, D.W. & Erasmus, B.J. 2004. *Bluetongue*. In: Coetzer, C.A.W., Tustin, R.C. (Eds.). Infectious diseases of Livestock, Vol 11, Oxford University Press, Cape Town, South Africa. pp 1201-1220.

103. Verwoerd, D.W. Huismans, H. & Erasmus, B.J. 1979. Orbiviruses. In: Franekel- Conrat, H & Wagner, R.R. (Eds). Comprehensive Virology.14:285-345.

104. Verwoerd, D.W. Louw, H. & Oelermann, R.A. 1970. Characterization of bluetongue virus ribonucleic acid. *Journal of Virology*. 5:1-7.

105. Verwoerd, D.W. 1970. Failure to demonstrate in vitro as opposed to in vivo transcription of the bluetongue virus genome. *Onderstepoort Journal of Veterinary Research*. 37:225-227.

106. Verwoerd, D.W. Els, H.J. De Villiers, E. & Huismans, H. 1972. Structure of the bluetongue virus capsid. *Journal of Virology*. 10:783-794.

107. Wade-Evans, A.M. Mertens, P.P.C. & Bostock, C.J. 1990. Development of the polymerase chain reaction for the detection of bluetongue virus in tissue samples. *Journal of Virological Methods*. 30: 15-24.

108. Wade-Evans, A. Woolhouse, T. O'Hara, R. & Hamblin C. 1993. The use of African horse sickness virus VP7 antigen, synthesized in bacteria, and anti-VP7 monoclonal antibodies in a competitive ELISA. *Journal of Virological Methods*. 45: 179-188.

109. Wetzel, H. Nevill, E.M. & Erasmus, B.J. 1970. Studies on the transmission of African horsesickness. *Onderstepoort Journal of Veterinary Research*.37:165-168.

110. Williams, C.F. Inoue, T. Lucus, A. de A. Zanotto, P.M. & Roy, P. 1998. The complete sequence of four major structural proteins of African horsesickness virus serotype 6:

evolutionary relationships within and between the orbiviruses. *Virus Research*. 53: 53-73.

111. Wilson, W.C. Ma, H.-C. Venter, E.H. van Dijk, A.A. Seal, B.S. & Mecham, J.O. 2000. Phylogenetic relationships of bluetongue viruses based on gene S7. *Virus Research*. 67: 141-151.

112. Wittwer, C.T. Hermann, M.G. Moss, A.A. Rasmussen, R.P. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques*. 22:130-138.

113. Zientara, S. Sailleau, C. Moulay, S. Wade-Evans, A. & Cruciere, C. 1995. Application of the polymerase chain reaction to the detection of African horse sickness viruses. *Journal of Virological Methods*. 53: 47-54.

114. Zientara, S. Sailleau, C. Moulay, S. & Cruciere, C. 1994. Diagnosis of the African horsesickness virus serotype 4 by a one-tube, one manipulation RT-PCR reaction from infected organs. *Journal of Virological Methods*. 46: 179-188.

115. Zientara, S. Sailleau, C. Moulay, S. & Cruciere, C. 1993. Diagnosis and molecular epidemiology of the African horsesickness virus by the polymerase chain reaction and restriction patterns. *Veterinary Research*.24: 385-395.