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MOLECULAR DIAGNOSIS OF AFRICAN HORSE SICKNESS

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Dissertation summary

African horse sickness (AHS) is a viral disease of equids caused by the African horse sickness virus (AHSV), a double-stranded RNA virus of the genus *Orbivirus*. The disease had a devastating effect on the sporting horse industry, and many communities who rely on equids for subsistence farming.

In this study, which started in 2016, blood samples were collected from 81 horses in the KwaZulu Natal province of South Africa. Six horses (7%) were identified as AHSV positive and had their viral load quantified using a standard RT-qPCR assay. A semi-quantitative method, using digital gel image analysis, was evaluated as an alternative to qPCR in resource-limited settings. A significant correlation was found in the quantification data obtained using qPCR and digital gel analysis ($P < 0.01$), demonstrating the potential for its use in under-equipped diagnostic laboratories.

Vaccination is currently the main tool for control of AHS in endemic areas. Live attenuated vaccines (LAV) have the potential to disrupt effective disease monitoring by being a source of the detectable virus. The sensitivity of nucleic acid diagnostic assays allows for the detection of low levels of AHSV prior to the onset of clinical signs. A study was conducted on the effect that this vaccine has on viraemia levels and the disruptive effect this may have is discussed. Routinely vaccinated miniature mares (n=9) had their AHSV viraemia levels monitored following the use of a commercial polyvalent LAV. No AHSV was detected in the horses following vaccination. The absence of AHSV in the mares supports the concept that multiple vaccinations reduce the risk of vaccine-induced viraemia.

Flinders Technology Associates® filter paper cards (FTA® cards) are designed to store nucleic acid from fresh sample material in a stable matrix and may be a practical solution to disease surveillance in resource-limited settings, where conventional sampling methods are not always suitable. Storage and elution conditions of AHSV RNA from these cards have yet to be investigated. This study compared the efficacy of two elution reagents for AHSV RNA extraction from blood applied to FTA® cards. Cards with AHSV positive blood applied were stored at room temperature for one week. Amplified products were extracted using TE-buffer with an elution time of 24 hours. This study shows that FTA® cards are feasible for AHSV nucleic acid assays

but further investigation into standardised procedures for the storage and recovery of AHSV RNA from FTA® cards is required.

The AHSV genome is made up of ten double-stranded RNA segments with nine antigenically different serotypes of AHSV identified so far. However, there is limited information on the serotype prevalence of AHSV in endemic areas. Therefore, multivalent vaccines remain the mainstay for disease control in these regions. Improved epizootiological information can improve vaccination programs and control mechanisms. In this experiment, a novel approach was investigated for the assessment of AHSV nucleic acid variations using high-resolution melt analysis (HRMA). Blood infected with a wild strain of AHSV was used in a PCR that targeted regions of Segment 10 (S10) and Segment 2 (S2). Following this, high-resolution melting curves were generated from these PCR products. S10 primers amplified the expected 181 bp product but the other primer sets failed to amplify the S2 gene segment, warranting further evaluation and optimisation of the primer sets used to detect currently circulating AHSV strains.

Candidate's declaration

I, Evan Saul McColl, declare that:

I. The research reported in this dissertation, except where otherwise indicated, is my original research.

II. This dissertation has not been submitted in full or in part for any degree or examination to any other university.

III. This dissertation does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

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   a. Their words have been re-written but the general information attributed to them has been referenced;
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Dr Marion Young (Co-supervisor)

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DISSERTATION INTRODUCTION

African horse sickness (AHS) is an arthropod-borne disease causing high levels of mortality primarily affecting members of the Equidae family. This disease occurs in one of four forms distinguished by its clinical symptoms. Due to the range of clinical symptoms and similarities to other equine diseases, a laboratory diagnosis of AHS is necessary (OIE, 2017). AHS is a controlled and notifiable disease in South Africa (Act 35 of 1984) and is listed as notifiable by the World Organisation for Animal Health (OIE). There is no cure for AHS, and control relies on vaccination, vector management and effective control strategies.

AHS is caused by the African horse sickness virus (AHSV), an Orbivirus in the *Reoviridae* family. There are currently nine antigenically different serotypes of AHSV with limited cross-protection between serotypes, all of which are considered endemic in sub-Saharan Africa, (Howell, 1962; von Teichman *et al*., 2010).

Major outbreaks have occurred in North Africa, Asia and Europe (Mellor & Hamblin, 2004). These outbreaks have caused devastating losses to the equine industry both because of the disease, and because of the negative impact it has on the growth of the equine industry due to the mandatory implementation of strict quarantine procedures. In addition to the significant impact AHS has on the South African racehorse and sporting horse industry, AHS affects a large population of mostly unvaccinated equids which are relied on for transport and draught power in rural areas.

Control of AHS is limited to vaccination, and vector and movement control because there is no cure for the disease (Zientara *et al*., 2015). Advancements in the accuracy, speed and affordability of diagnostic techniques for AHS will improve disease surveillance and the timely implementation of control strategies in endemic areas (Weyer *et al*., 2015).

The research presented in this dissertation aimed to address several aspects related to the molecular diagnostics of AHS: To review and summarise the available literature pertaining to AHS molecular diagnostics; to investigate the potential disruptive effect the vaccine has on disease surveillance; to assess alternative methods for sampling

and quantification of AHSV in resource-limited areas; and, lastly, to generate high resolution melt data on current AHSV strains as part of a potential serotyping assay.

Research objectives

The specific goals of this study were to:

- Collect and identify AHS positive blood samples in the KwaZulu-Natal province to obtain wild-type AHSV strains for subsequent analysis.
- Evaluate the use of digital gel analysis as a quantitative alternative to qPCR in resource-limited settings.
- Investigate the level of viraemia induced in mares by a commercial live attenuated vaccine using a standard qPCR assay to assess the impact this has on disease surveillance.
- Evaluate a protocol for extracting viable AHSV RNA for PCR amplification using dried blood spot filter paper technology as an alternative to conventional sampling methods which require stringent storage conditions.
- Develop a protocol for the use of high-resolution melts for a potential serotyping assay, using currently circulating strains of AHSV.

The referencing system used in the chapters of this dissertation follows the specific style used in the South African Journal of Animal Science.

The dissertation is in the form of discrete research chapters, each following the format of a stand-alone research paper. This is the dominant thesis format used by the University of KwaZulu-Natal as it facilitates the publishing of research from work more easily than a single document. As such, there is an unavoidable repetition of references and introductory information between chapters.

CHAPTER 1:

Literature review

1.1. Introduction

African horse sickness (AHS) is a severe viral disease affecting members of the Equidae family (OIE, 2013). AHS is a notifiable disease as classified by the World Organisation for Animal Health (OIE) due to the severe effect that it has on the equine industry, with high mortality rates (OIE, 2013). AHS has a significant impact on the South African racehorse and performance horse industries, as well as affecting many susceptible rural equids. Fully susceptible horses have up to 90% mortality rate, while zebra and donkeys rarely show clinical signs (Erasmus, 1972).

AHS is caused by the African horse sickness virus (AHSV), a double-stranded RNA virus of the *Orbivirus* genus from the *Reoviridae* family (Verwoerd *et al*., 1979). Nine antigenically distinct serotypes of AHSV (AHSV-1 to AHSV-9) have been isolated, the last of which was described by Howell in 1962. AHSV is classified as an arbovirus due to its ability to infect and multiply within haematophagous arthropods (Mellor, 2000). AHS is a vector-borne disease spread by members of the *Culicoides* species (biting midges), with *Culicoides imicola* (Diptera: Ceratopogonidae) being the most significant vector species in South Africa. The disease is most prevalent in warm, wet weather when the *Culicoides* vectors are most abundant. However, *Culicoides spp.* are found throughout winter in many endemic areas (Venter *et al*., 2014). *Culicoides* spp. capable of spreading AHSV have been found worldwide, forcing strict quarantine protocols to be implemented by the European Union governing equid movement (European Commission, 2008).

Climate change is proposed as a driving force for the emergence of Bluetongue virus (BTV) in Europe because of the strong link between the epizootiology and the growing number of suitable habitats for the vector (Guis *et al*., 2012). This virus is closely related to AHSV, sharing many similarities, including being vectored by the *Culicoides* spp. There is an increasing concern that the introduction of AHSV into Europe may result in its permanent establishment due to the already present vector species and endemic nature of BTV (Allison *et al*., 2009; Thompson *et al*., 2012). Vaccines are currently used annually in endemic areas and a stockpile of vaccines exists to manage an outbreak in Europe. The risk of the live vaccine reverting to

virulence makes this vaccine an unattractive option for Europe, where the entire equine population is susceptible (Savini *et al*., 2017). This concern is justified by a published case of vaccine virus reversion to virulence in South Africa in 2014 (Weyer *et al*., 2016). Therefore, the possibility of vaccine-induced viraemia and the disruptive effect that it may have on molecular disease surveillance has been explored in this research.

1.2. **Epizootiology**

AHSV is widely spread in sub-Saharan Africa, extending as far north as the Sahara Desert, which provides a barrier between southern and northern Africa (Mellor & Hamblin, 2004). Each of the nine serotypes of AHSV are found throughout, and are considered to be endemic, in Southern Africa. Since 2007, serotypes 2 and 7 have been found in West and East Africa, where only serotype 9 had been found previously (Zientara *et al*., 2015).

Beyond South Africa, Spain has had five outbreaks of AHS starting in 1966 that were caused by serotype 9. There was no sign of the virus in Europe for 20 years prior to the AHSV-4 discovery in Spain. Along with three additional outbreaks, these outbreaks were reported to have been caused by subclinical zebra imported from Namibia (Lubroth, 1988). Strict vaccination programs, using monovalent vaccines and animal control measures, eliminated the disease from the region after persisting for four years. In total, over 1300 horses died because of the Spanish AHS outbreaks (Mellor, 1993). Such outbreaks have resulted in the implementation of strict equine movement restrictions from endemic areas.

The international exportation of horses from South Africa to non-endemic countries is required from the AHS free zone in the Western Cape (Fig. 1.1.). This area has been declared AHS-free and exports can occur from this region, on condition that it is preceded by strict surveillance. The surveillance zone borders the AHS-free zone and is constantly monitored for controlled equine diseases. Outbreaks in the surveillance zone disrupt the international exportation of horses from South Africa, negatively impacting the equine industry. Recently, there have been AHS positive cases in the surveillance zone, with outbreaks of AHSV-1 in both Mamre in 2011 and Paarl in 2016 (Weyer *et al*., 2017).

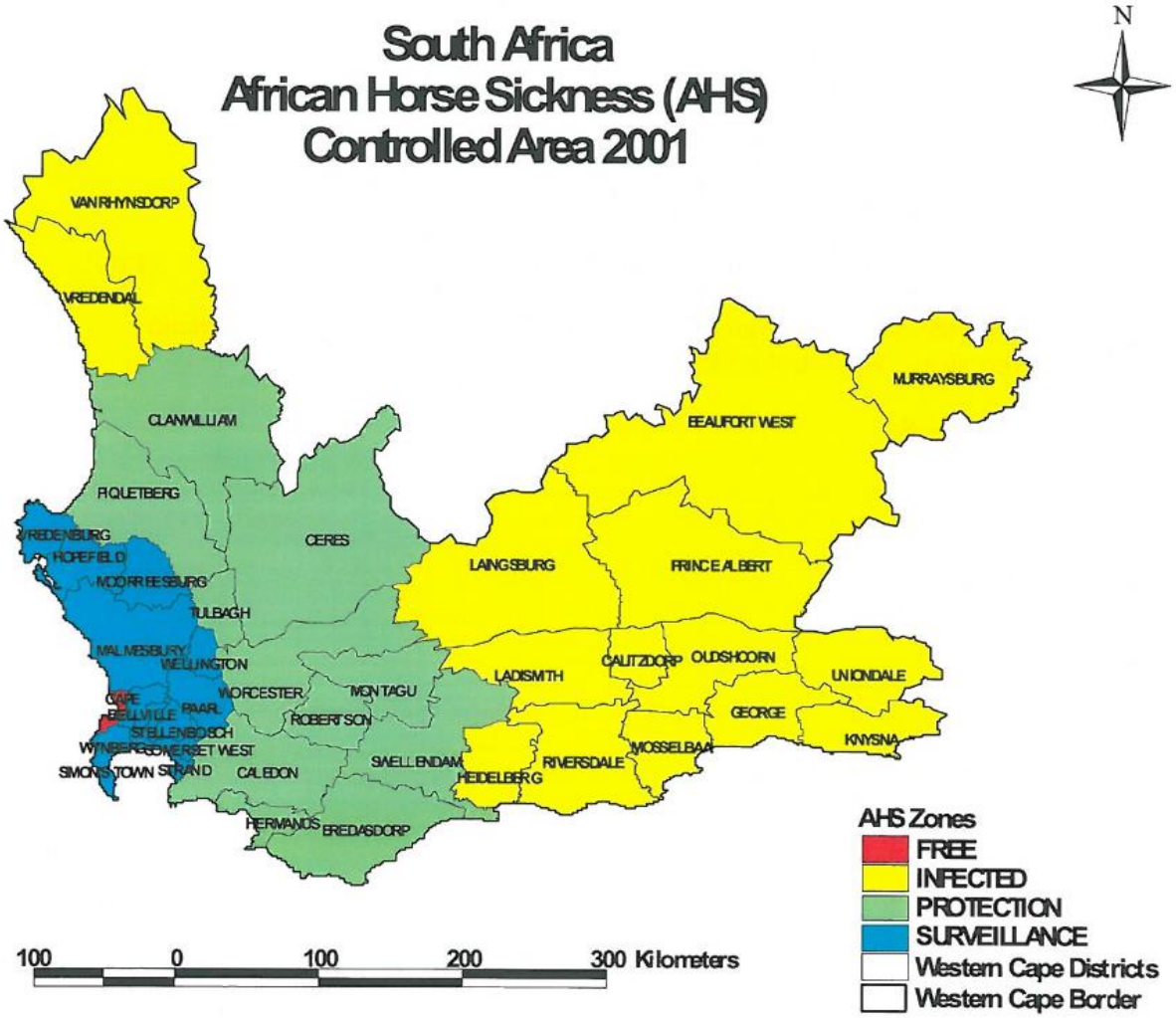

Figure 1.1 - Map showing the infected, protection, surveillance and free zones for African Horse sickness in South Africa (Western Cape Government, 2014).

Several orbivirus diseases in the northern hemisphere are transmitted by AHSV vectors (Howell *et al*., 2004). Together with the global spread of BTV, this has raised concerns of potential AHSV outbreaks outside of Africa and generated interest in assessing the potential impact of AHS outbreaks internationally (MacLachlan & Guthrie, 2010; Thompson *et al.*, 2012; Chapman *et al.*, 2017). Strict animal movement control is critical to prevent entry of AHSV into non-endemic regions.

1.3. **Host Range**

Horses, mules, zebra and donkeys are the most common hosts for AHSV (Zientara *et al.*, 2015). The mortality rate in horses is as high as 90% but fully recovered horses do not continue to be carriers of the virus (Coetzer & Guthrie, 2004). In zebra and donkeys, the disease usually remains subclinical and has a lower mortality rate

(Coetzer & Erasmus, 1994). Viral reservoirs allow for the permanent establishment of the virus and are important to the epizootiology of these diseases.

Zebra have been suggested as a reservoir for AHSV due to their low mortality and, therefore, caution is advised when relocating zebra to non-AHS endemic areas because zebra can create suitable conditions for AHSV (Barnard, 1998). The emergence of AHSV in West Africa, where zebra are not present, suggests the role of other species as reservoir hosts in these areas. Donkeys are unlikely to be long-term reservoirs for AHS as they have been shown to develop only a short period of viraemia following AHSV challenge (Hamblin *et al*., 1998). Dogs have also been shown to be susceptible to AHS by ingestion of infected meat and experimental infection (Theiler, 1910; Van Rensberg *et al*., 1981). It is generally accepted that dogs do not play a role in the spread of AHS, but a positive case of AHS in a dog through natural infection via a non-oral route was confirmed by Van Sittert *et al.* (2013).

Humans are not susceptible to natural infection of field strains of AHSV, although some neurotropic vaccine strains have caused encephalitis in humans and are consequently no longer in production (Swanepoel *et al*., 1992).

1.4. **Vector**

AHSV was initially suspected to have been transmitted through haematophagous arthropods but the exact species was not identified until 1944 when field-collected *Culicoides* were shown to carry the virus (Du Toit, 1944). *Anopheles* mosquitoes have been studied (Theiler, 1903; Mellor, 1994), but only the *Culicoides* spp. have been found to have a significant effect on the epizootiology of the disease (Mellor, 1994). The adult female *C. imicola* is the primary vector of AHSV in sub-Saharan Africa (Fig 1.2) (Mellor, 1994).

There are currently over 1400 known species of *Culicoides,* with 30 species of *Culicoides* thought to play some role in the transmission of BTV (Meiswinkel *et al*., 2004). Due to the similarities of BTV to AHS, there are concerns that BTV vectors found globally could spread AHS, and that currently established populations of *Culicoides* in AHS-free areas could cause a severe outbreak (Robin *et al.*, 2016).

Culicoides bolitinos (Diptera: Ceratopognidae) is morphologically similar to C. *imicola* and can also vector AHSV (Meiswinkel & Paweska, 2003). The larvae and pupae of

C. bolitinos inhabit the dung of cattle which brings additional risks to horses on cattle farms. Importantly, *C. bolitinos* will readily enter a stable to feed, reducing the efficacy of stabling as preventing contact between host and vector (Meiswinkel *et al*., 2000).

Following the Spanish AHSV outbreak, isolates of *C. obsoletus* and *C. pulicaris* tested positive for AHSV and may have been important vectors during this outbreak (Mellor, 1994). These midge species are endemic in northern and western Europe, and their ability to transfer AHSV create the requisite conditions for outbreaks of the disease.

The midges' ability to move a few kilometres from their breeding sites to feed emphasises the importance of movement control. In extreme cases, strong winds can carry midges much greater distances and have probably carried AHSV across the Strait of Gibraltar from Africa to Europe (Pedgley & Tucker, 1977).

The characteristics of the *Culicoides* vector are linked with the epizootiology of AHSV. The distribution of *C. imicola* has been demonstrated to be closely linked to the soil type, especially high-clay, moisture-retaining soils (Meiswinkel, 1998). Climatic conditions such as high levels of rainfall increase the number of breeding sites for the midge and outbreaks of AHS have often occurred when there has been heavy rainfall following a drought because increased breeding sites for the vector, combined with a high concentration of animal hosts at drinking holes, facilitated the rapid transmission of the virus (Jenkins & Young, 2010).

Figure 1.2 - Female *Culicoides* after feeding (Jenkins, 2008).

1.5. **Description of the virus**

The AHSV is a member of the *Orbivirus* genus of the *Reoviridae* family. Orbiviruses are non-enveloped viruses with ten double-stranded RNA segments coding for 11 viral proteins (VP) (Calisher & Mertens, 1998). The coding system used in this work, described by Mertens *et al.* (2006), is presented in Table 1.1.

Table 1.1 - Coding assignment and size of genome segments from Mertens *et al.* (2006)

Genome segment	Coding assignment (Mertens *et al.,* 2006)	Size (bp) (Mertens *et al*., 2006)
1	VP1	3965
2	VP2	3205
3	VP3	2792
4	VP4	1978
5	NS1	1748
6	VP5	1566
7	VP6	1169
8	VP7	1167
9	NS2	1166
10	NS3/NS3a	756

The virus has seven structural proteins (VP1-7) organised in a two-layer capsid (Fig. 1.3).

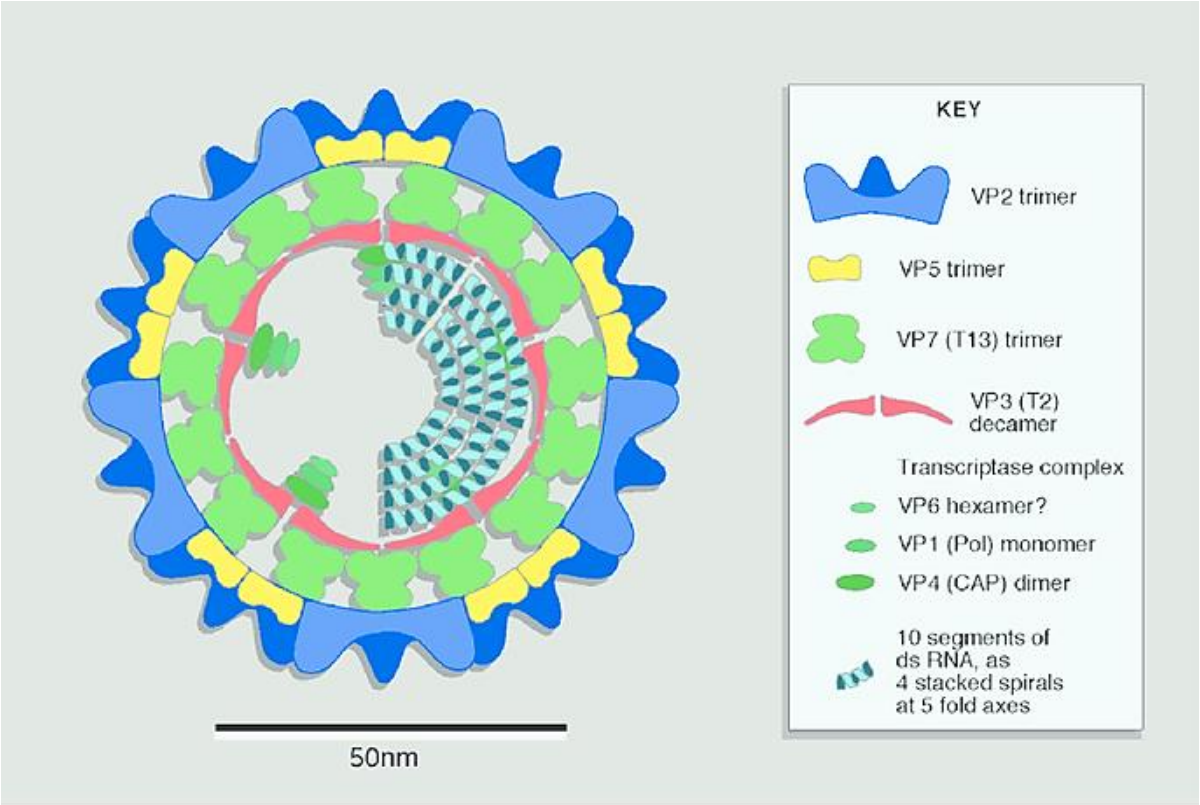

Figure 1.3 - Diagram of orbivirus structure showing structural proteins (Wilson *et al*., 2009)

The core particle of the virus is composed of major proteins, VP3 and VP7, and minor proteins, VP1, VP4 and VP6. The outer capsid is comprised of VP2 and VP5 (Bremer *et al*., 1990, Roy *et al*., 1994). The outer capsid protein, VP2, differs most between serotypes and is responsible for the antigenic diversity of the virus and as a result, is often the target for type-specific nucleic acid-based assays (Roy *et al.*, 1994). Segment 10 (S10) encodes for the non-structural proteins, NS3/NS3a, which are involved with the release of virus from infected cells (Martin *et al*., 1998). Phylogenetic studies into AHSV S10 have found homology in S10 from different serotypes, allowing serotypes to be grouped genotypically into one of three groups using this segment (Martin *et al*., 1998; Groenink, 2009).

So far, nine antigenically different serotypes of AHSV have been identified. The last of these serotypes were isolated in 1960, suggesting a degree of viral stability (Howell, 1962). This is important for the effective control of viral diseases because vaccines are

required to be updated in line with currently circulating serotypes (Mumford, 2007). The frequent emergence of new serotypes complicates this task. Genetic drift of AHSV was investigated by Groenink (2014) using phylogenetic analyses on available virus sequences at the time. Little to no genetic drift in AHSV sequences from 1960s-2000s was demonstrated. This is different to BTV, which has 28 serotypes, four of which have been identified in the last decade (Hofmann *et al*., 2008; Zientara *et al.*, 2014; Sun *et al*., 2016; Savini *et al*., 2017).

Reassortment is a system of genetic recombination which occurs in segmented RNA viruses. The segmented genome structure allows for segments of different viral strains to be exchanged during co-infections (McDonald *et al*., 2016). To date, there have been no reported cases of a multiple serotype co-infection for AHSV but a reverse genetics study showed that co-infection with two AHSV serotypes resulted in reassortment (Matsuo *et al.,* 2010). Live attenuated vaccines, as in the case with the AHS vaccine, contain multiple serotypes and may create suitable conditions for vaccine virus reassortment.

1.6. **Control**

1.6.1. **Treatment**

There is currently no prescribed treatment for AHS. Control of the spread of the disease is considered the best prophylactic means of minimising the impact of AHS (Zientara *et al.*, 2015).

1.6.2. **Vector control**

Vector control is one of the many approaches taken to reduce AHS transmission. The wide-ranging characteristics and sheer numbers of the *Culicoides* vectors add to the multifaceted problem of AHS.

Midge numbers are positively correlated with increasing ground moisture levels and sunlight (Jenkins, 2008). Larvicidal application at these breeding sites can decrease midge number in the area by eliminating the immature life stage of the vector (Page *et al*., 2014). Improving drainage of marshlands and standing water are environmental changes that can reduce the number of vectors in an area (Carpenter *et al*., 2008).

Other strategies to reduce transmission include contact restriction between host and vector through stabling of horses. While stabling is effective against *C. imicola,* it does not deter all *Culicoides* spp., and *C. bolitinos* will readily enter a stable to feed (Meiswinkel *et al*., 2000).

The efficacy of these strategies depends on good husbandry and persistent application of these measures, particularly during outbreaks and in favourable vector conditions.

1.6.3. **Vaccination**

Prior to 1930, vaccination was done by introducing virulent AHSV simultaneously with sera containing antibodies for AHSV (Theiler, 1915). The antibodies contained within the sera may have been intended for a different serotype or insufficient for the viral load, resulting in high animal mortality (Mellor & Hamblin, 2004). Virus attenuation for vaccine production was initially developed using suckling mouse brain passage but has since been changed to cell culture passage (Mellor & Hamblin, 2004).

Onderstepoort Biological Products (OPB) have produced a polyvalent, cell-cultured live attenuated vaccine (LAV) since 1994 (Fig. 1.4). This is the most commonly administered vaccine in most endemic areas. The vaccine is given in two doses 4 weeks apart. Dose 1 contains serotypes 1,3,4 and Dose 2 has serotypes 2,6,7,8. Two serotypes are not included in the multivalent LAV: AHSV-5 because of reports of deaths in vaccinated animals; and AHSV-9 because of cross-protection with AHSV-6 and its low occurrence in Southern-Africa (von Teichman *et al*., 2010). Horses may not move into AHS controlled areas less than 60 days after the second vaccination. This is because of the potential for the vaccine virus to be transmitted to serologically naïve animals within these areas (Weyer *et al.*, 2016).

The use of the LAV presents a challenge when using nucleic-acid based detection techniques for diagnosis, primarily because most RT-PCR assays are unable to distinguish between the LAV-virus and wild virus strains (Weyer *et al*., 2017). Therefore, vaccinated animals may test positive if the vaccine produces a detectable level of viraemia. Although, measurable viraemia following vaccination is uncommon in adult animals with prior vaccinations (Paweska *et al*., 2003, Weyer *et al*., 2017). The LAV is useful for reducing the severity of the disease but vaccinated animals are still susceptible to the disease in field conditions and multiple vaccinations may be necessary for adequate protection (Molini *et al*., 2015).

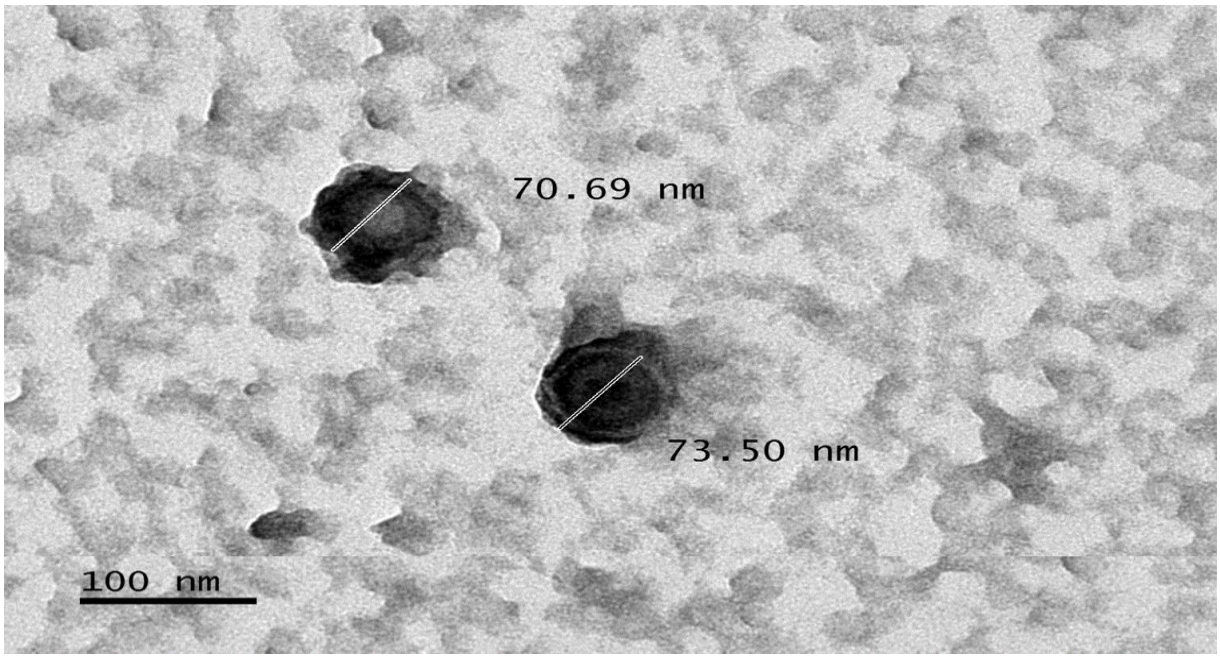

Figure 1.4 - Transmission electron microscope photographs of AHSV particle from the vaccine (Image taken by Evan McColl).

Attenuation through cell culture passage does not inhibit the vaccine-virus' ability to infect the *Culicoides* vector (Venter *et al*., 2009). This has been demonstrated by AHS outbreaks shown to be caused by virulent reversion of vaccine strains transmitted by this vector (Weyer *et al*., 2016). Multivalent vaccines may increase the potential for reassortment by introducing multiple serotypes simultaneously (Mertens *et al*., 2005).

Inactivated, or dead strain vaccines consist of virus particles that have been killed to prevent infection from the disease. Inactivated vaccines can effectively protect animals from AHS but multiple inoculations are needed as they do not confer a long-lasting immunity (House *et al*., 1994; Mellor & Hamblin, 2004; Lelli *et al*., 2013). Monovalent inactivated vaccines were used during the AHSV-4 epidemic in Spain, Portugal and Morocco that lasted from 1987-1991 (Zientara *et al*., 2015). Inactivated vaccines have the advantage of not causing viraemia, allowing vaccinated animals to be distinguished from naturally infected animals (House *et al*., 1994).

For decades, vaccines for AHS have relied on attenuation or inactivation of the virus. The production of a new generation of vaccines that are safer and more stable is being made possible by advancements in recombinant DNA and peptide synthesis technology (Nascimento & Leite, 2012). Recombinant vaccines are produced through the insertion of an antigen-encoding gene into a carrier virus, which stimulates an

immunological response. Several new generation AHS vaccines are being investigated as safe alternatives to conventional vaccines (Guthrie *et al*., 2009, Alberca *et al*., 2014, Maree *et al*., 2016, Lulla *et al*., 2017).

1.7. **Diagnostics**

The disease can have devastating effects on serologically naïve horse populations, with mortality rates in some cases as high as 90% (Zientara *et al.*, 2015). Clinical symptoms of AHS develop from problems with the circulatory and respiratory systems (Howell, 1962). The extent and severity of these symptoms categorise the disease into four separate forms (Table 1.2) (OIE, 2013).

Table 1.2 - African horse sickness disease forms (Wohlsein *et al*., 1998; OIE, 2013; Zientara *et al*., 2015).

Form	Incubation period (days)	Symptoms	Prognosis
Horse sickness fever	5-14	Fever (39-40.5 ° C).	Animal usually recovers
Cardiac	7-14	Fever (39-40 ° C). Swelling of eyelids, lips, cheeks, tongue, laryngeal region, fluid accumulation in the tissues. May shows signs of colic.	Death usually occurs 4-8 days following fever. Swelling lasts around 6 days in recovering animals. Mortality > 50 %
Pulmonary	3-5	Fever (39-41 ° C). Respiratory problems characterised by severe shortness of breath. Frothy fluid often released from nostrils. Swelling around eyelids.	Death from anoxia often occurs within 1 week. Highly fatal especially in horses with low or no immunity.
Cardio-pulmonary (mixed)	3-14	Fever (39-40 ° C). Mild pulmonary signs Fluid accumulation in the tissues.	Death usually occurs 3-6 days following fever. Mortality > 70 %

Animals that manage to recover from the disease usually develop a strong immunity to that specific serotype but will still be susceptible to other serotypes (CFSPH, 2015). This poses a challenge in Sub-Saharan Africa where multiple AHSV serotypes exist

simultaneously and animals can be exposed to and introduce new serotypes when being relocated within a region (Weyer *et al.*, 2016). Laboratory diagnosis of AHS is essential due to the similarity that the clinical signs have with other equine diseases (OIE, 2004). For example, equine encephalosis (EE) is caused by a closely related virus and share many clinical signs and epizootiological traits with AHS.

An important component of accurate diagnosis is safe transportation and storage of suitable samples without significant damage. The preferred sample for the laboratory diagnosis of AHS is whole blood collected in an anti-coagulant (OIE, 2004). However, the cold chain infrastructure required for the transport of these samples is not available in rural and remote areas of AHS endemic regions. Alternative options need to be considered for collecting and storing biological samples. One such example is the use of dried blood spots (DBS) on filter paper, which has been shown to be successful at storing viral RNA at ambient temperature for subsequent nucleic acid assays (Alvarez-Munoz *et al*., 2005, Uttayamakul *et al*., 2005).

Confirmation of AHS is diagnosed through identification of the infectious virus, viral nucleic acids, viral antigens or specific antibodies (OIE, 2004). There are several published techniques available for AHS diagnosis.

Viral neutralisation (VN) tests are the gold standard for AHSV isolation and serotyping (OIE, 2017). However, this serological test has the disadvantage of requiring the presence of AHSV antibodies, which are detectable between 10-14 days post infection (Zientara *et al*., 2015). This may make serological techniques unsuitable for outbreak management. Research into the diagnosis of AHSV has consequently shifted focus from VN techniques to nucleic-acid based assays, most notably PCR (Zientara *et al*., 2015).

1.7.1. **PCR**

The Polymerase Chain Reaction (PCR) is a molecular technique for amplifying and detecting nucleic acid sequences (Mullis, 1987). This PCR amplifies specific regions of a DNA strand utilising two oligonucleotide primers that border the DNA sequence being amplified. The PCR is carried out in small reaction tubes that are placed into a thermal cycler controlling the temperature of the reaction. Controlled heating and cooling are carried out in cycles causing enzymatic replication of the nucleic acid segment between the two primer binding sites. A variant of PCR called reverse

transcription polymerase chain reaction (RT-PCR) is used to detect RNA sequences such as the AHSV genome.

RT-PCR utilises the process of reverse transcription to transcribe RNA onto complementary DNA (cDNA) before being amplified in the following steps. Initially, RT-PCR assays for the detection of AHSV were labour-intensive, requiring restriction fragment length polymorphism (RFLP) analysis followed by RT-PCR assays (Zientara *et al*., 1993). The first AHSV RT-PCR assays published targeted the VP3 (Sakamoto *et al*., 1994), VP7 (Sailleau *et al*., 1997), NS1 (Mizukoshi *et al*., 1994), NS2 (Stone-Marschat *et al*., 1994) and NS3 genes (Zientara *et al*., 1998).

The first serotype-specific RT-PCR assay involved the use of nine pairs of primers targeting genome Segment 2 (Sailleau *et al*., 2000). This segment encodes for VP2, which is the protein responsible for the antigenic variability between serotypes, and as such, most PCR genotyping assays target this gene (Weyer *et al*., 2015). Concerns have been raised with using the divergent genome Segment 2 as targets for PCR due to its propensity for genetic change (OIE, 2017). Diagnostic assays targeting this region would require periodic evaluation to ensure that genetic change does not render these assays obsolete.

1.7.2. **qPCR**

Real-time or quantitative PCR (qPCR) is a variation of PCR in which the thermal cycler can detect changes of reporter fluorophore signals during amplification. Intercalating dyes and fluorescent probes are detected by sensors and the fluorescence is plotted against the cycle number, creating an amplification plot (Fig. 1.5.)

qPCR systems plot the accumulation of amplicons as the reaction takes place in real-time, allowing for the quantification of the amplified product. This also eliminates the need for some post-PCR processing steps such as electrophoresis gel analysis for confirmation. This reduces the risk of cross-contamination and reduces the assay time.

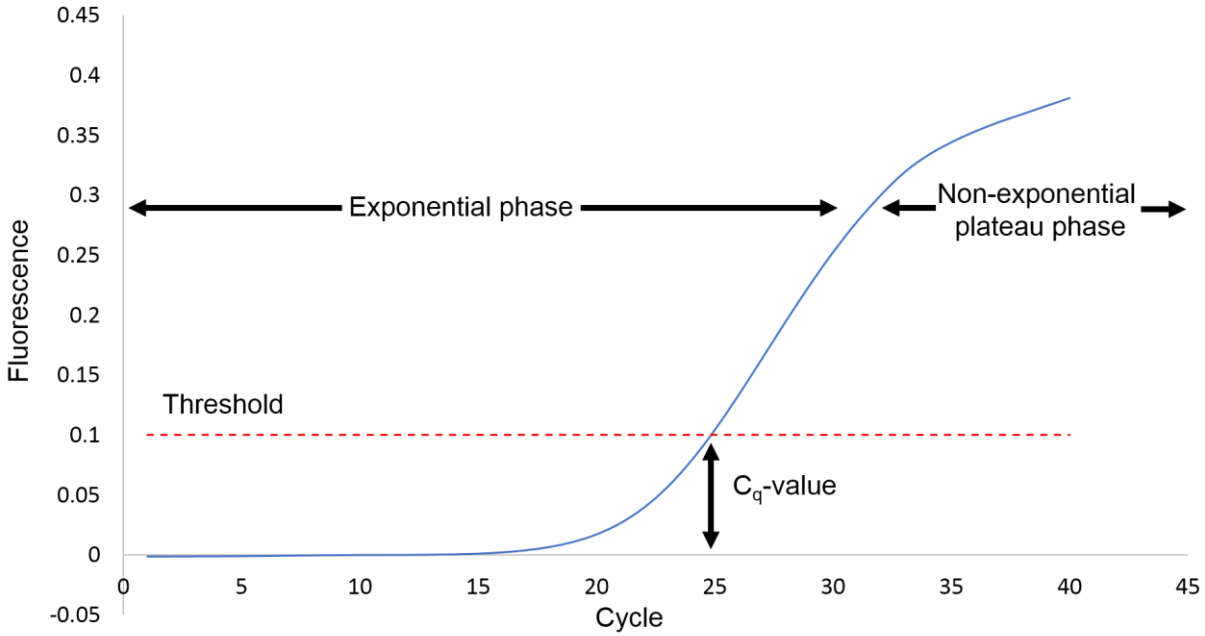

Figure 1.5 – Simplified plot demonstrating the increase of fluorescence as cycle number increases during PCR amplification.

To date, published RT-qPCR assays for AHSV target the VP2 (Weyer *et al*., 2015), VP7 (Aguero *et al*., 2008; Fernandez-Pinero *et al*., 2009; Quan *et al*., 2010), NS1 (Rodriguez-Sanchez *et al*., 2008) and NS2 (Quan *et al*., 2010) genes.

1.7.3. **High-Resolution Melt**

High-resolution melt (HRM) analysis is molecular technique based on the release of intercalating dye from amplicons through controlled heating. The unique sequence of the amplicon determines the rate at which the dye is released so that different amplicon sequences can be distinguished from one another by relative differences in these rates (Ririe *et al*., 1997).

Work by Groenink (2009 & 2014) focused on the development of an AHSV serotyping assay using HRM profiles from the PCR products of the nine serotypes. This approach used a primer pair to amplify a 181 bp region of Segment 10 and the generated HRM curves to group the sample into one of three bins. The serotype was then determined using Segment 2 clade-specific primers. This method was successful in correctly identifying the serotypes of viral reference strains and detected some reference strain anomalies, supported by the work of Weyer *et al.* (2015).

Accuracy and speed of detection of infectious diseases play a significant role in disease management. Early detection of the AHS serotype provides information needed for better and more efficient vaccination and control programs. Developments in speed and accuracy of AHS serotyping assays in combination with vaccination programs will aid in improving prophylaxis and control of this disease.

1.8. **Conclusion**

Control and diagnosis of AHS in endemic areas can be improved through improved sample handling techniques requiring less stringent transport conditions and safer vaccines. Effective prophylaxis of AHS is dependent upon good epidemiological models, early quarantining of viral ‘hot spots’, and measures that can be extended to vector control and good husbandry. Control of the disease will be improved by an increased understanding of the epizootiology and transmission of the disease. Therefore, improvements in the sensitivity, speed and accuracy of diagnostic assays will improve the effectiveness of control measures.

1.9. **References**

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CHAPTER 2:
A comparison of standard RT-PCR and RT-qPCR in the detection and quantification of AHSV

2.1. Abstract

African horse sickness (AHS) is a viral disease of equids caused by the African horse sickness virus (AHSV), a double-stranded RNA virus of the genus *Orbivirus,* which can devastate the sporting horse industry and the communities that rely on equids for subsistence farming. The diagnosis of AHS is often limited in endemic areas as diagnostics laboratories are under-equipped to perform the prescribed tests. Blood samples in EDTA were collected from horses in KwaZulu-Natal, South Africa. A semi-quantitative method for PCR product analysis was evaluated against a standard qPCR assay as a potential alternative in under-equipped diagnostic laboratories. There was a significant correlation between qPCR data and relative gel quantification of AHSV (R^2=0.768, P<0.01). With careful optimisation and calibrations, the assay described here could be useful where qPCR is not available.

2.2. Introduction

African Horse Sickness (AHS), caused by African Horse Sickness Virus (AHSV), is a vector-borne, infectious, non-contagious, often fatal viral disease affecting members of the Equidae family, with horses being the most susceptible (Zientara *et al.*, 2015). The disease is vector-borne with *Culicoides* biting midges (Diptera: Ceratopognidae) being the primary vector. Due to the massive numbers of *Culicoides* in South Africa, vector control is not possible for complete control of AHS (Esterhuizen, 2015). Vaccination and movement control of equids are essential for AHS-control in South Africa. The movement of susceptible animals from endemic regions to areas free from the disease needs to be done with caution as an AHS outbreak could severely affect populations of serologically naïve animals (Robin *et al*., 2016). Accurate detection of AHSV is crucial for movement control measures to be effective.

Reverse transcription-PCR (RT-PCR) was first used to detect AHSV in 1994 with a primer set targeting the NS2 gene (Stone-Marschat *et al*., 1994). Group-specific AHSV assays tend to target the more conserved genes: VP7 and NS2 (OIE, 2013). Real-time quantitative PCR (qPCR) allows for sensitive quantification of the target sequence without additional post-PCR work (Van Guilder *et al*., 2008). However, qPCR

capabilities are not standard in veterinary laboratories and may not be available for disease diagnostics in developing countries.

The aim of this study was to evaluate the use semi-quantitative agarose gel data compared to a commercially available RT-qPCR kit (PrimerDesign, Southampton, United Kingdom) for the detection and quantification of AHSV from field samples.

2.3. **Materials and methods**

2.3.1. **Sample population**

Whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vacutainers from 81 horses in KwaZulu-Natal, South Africa. Prior to analysis, blood was frozen and stored at - 20 °C as it was convenient for storage until analysis (Quan *et al*., 2010).

2.3.2. **RNA Extraction**

Total RNA was extracted from blood samples using the Quick-RNA™ Miniprep Plus Kit (Zymo Research, Irvine, USA), according to the manufacturers' recommendations. A volume of 500 µl of blood was transferred into a 2.5 ml microcentrifuge tube and an equal volume DNA/RNA Shield™ (2 X concentrate) was added to the sample. The mixture was vortexed before an 8 µl volume of Proteinase K (20 mg ml^{-1}) was added and incubated at room temperature for 30 minutes. To this solution, 1 ml of isopropanol was added and vortexed. The mixture was transferred to a Zymo-Spin™ III-CG column and collection tube and centrifuged at 15,000 x *g* for 30 s. To this column, 400 µl RNA Prep Buffer was added, centrifuged at 15,000 x *g* for 30 s, and the eluent was discarded. The column was then washed twice with 700 µl RNA wash buffer and centrifuged at 15,000 x *g* for 2 minutes. The column was transferred to an RNase-free tube. The final RNA was eluted by adding 50 µl nuclease free water to the column followed by centrifugation at 15,000 x *g* for 30 s. RNA that was not used immediately was stored at -80 °C. Absorbance readings between the wavelengths of 220 and 350 nm of the extracted RNA were taken using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, USA) to assess the quantity and purity of the extracted RNA.

2.3.3. **Complementary DNA (cDNA) synthesis**

Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA). In a PCR tube, 2 µl of template RNA was added to 1 µl of random hexamer primer (100 µM) and 9 µl of nuclease-free water. This was incubated at 95 °C for 1 minute and immediately transferred onto ice. The mixture was combined with 4 µl of 5 X reaction buffer, 1 µl RiboLock RNase Inhibitor (20 U μl^{-1}), 2 µl of 10 mM dNTP mix and 1 µl RevertAid M-MuLV Reverse Transcriptase (200 U μl^{-1}). The mixture was vortexed, spun down and incubated at 25 °C for 5 minutes followed by 42 °C for 60 minutes. The reaction was terminated by heating to 70 °C for 5 minutes. The cDNA was stored at -80 °C until further processing.

2.3.4. **Nucleic acid detection**

The PrimerDesign Genesig kit for AHSV VP7 detection was used in this study (PrimerDesign, Southampton, United Kingdom). The PCR was performed on a Rotor-Gene™ 6000 (Qiagen, Hilden, Germany).

Duplicate reactions were set up to a final volume of 20 µl containing 5 µl of template cDNA, 10 µl PowerUp™ Green Master Mix (Thermo Fisher Scientific, Waltham, USA), 4 µl nuclease-free water and 1 µl AHSV specific primer/probe mix (PrimerDesign, Southampton, United Kingdom). The PCR reaction conditions were as follows: an initial Uracil-DNA Glycosylase (UDG) activation step of 50 °C for 2 minutes, 95 °C for 2 minutes for initial denaturing, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Fluorescence data was collected at the end of each cycle.

A positive control (PC) containing VP7 template DNA with a concentration of $2x10^5$ copies μl^{-1} was included in each PCR run. The concentration of the PC in each run was kept constant. Therefore, to have comparable results between runs the threshold value was set to a value where the PC had a C_q of 23.0 across all the PCR reactions.

2.3.5. **Agarose gel electrophoresis**

A volume of 5 µl of PCR product was run on a 1.8% (w/v) agarose gel in 1 X TBE buffer containing 1 X SYBR ® Safe DNA gel stain (Invitrogen, Carlsbad, USA) to determine the molecular weight of the PCR products. Electrophoresis was run at 6.66 V cm^{-1} for 1 hour and visualised on a UV transilluminator. The DNA bands were sized using a GeneRuler™ 100 bp ladder marker (Thermo Fisher Scientific, Waltham, USA). The PCR band densities were digitally measured using ImageJ (Abramoff, 2004).

2.3.6. **Standard curve generation**

To calculate viral count in test samples, a standard curve was generated using a serial dilution of VP7 template DNA ranging from 2 x 10^5 – 2 x 10^1 copies μl^{-1} as provided in the RT-qPCR kit. Each dilution was analysed in duplicate on a Rotor-Gene™ 6000 using the Rotor-Gene™ 6000 series software.

2.3.7. **Analytical sensitivity**

Several methods for estimating qPCR assay sensitivity exist with no common consensus on the best method (Bustin *et al*., 2009). The limit of blank (LoB), limit of detection (LoD) and limit of quantification (LoQ) are important terms used to describe the level of analyte that can be reliably detected by an analytical assay (Clinical and Laboratory Standards Institute, 2004). The LoB is defined as the highest apparent concentration found for samples containing no target analyte. The LoD is the lowest concentration of analyte that can be reliably differentiated from the LoB. The LoQ is the lowest concentration of analyte that can be reliably quantified with an acceptable level of measurement variation. For this study, the LoQ was determined to be the lowest concentration calibrant which fitted the linear section of the standard curve. LoB and LoD were determined according to Armbuster & Pry (2008) as shown below.

$$\mathrm{LoB} = \bar{\mathrm{x}}\,(blank) + 1.645 \times \mathrm{SD}(blank)$$

$$\mathrm{LoD} = \mathrm{Lob} + 1.645 \times \mathrm{SD}(low\ concentration\ sample)$$

2.3.8. **Statistical analysis**

The results of digital gel analysis were compared with the standard qPCR (Calibrated with external standards) by simple linear regression analysis.

2.4. **Results**

2.4.1. **Electrophoresis gel analysis**

PCR products were run on electrophoresis gels to confirm product size (Fig. 2.1). Clear bands of the expected size of 118 bp were present in six samples (H35, H46, H48, H57, H58, H64). The PC included in each gel shows a clear band of the same size.

Contamination or large amounts of primer dimer result in some faint streaks visible around 100 bp and below in the gel image for samples H13-H24 shown in panel B (Fig. 2.1.) The same streaks are seen in the NTC for that gel.

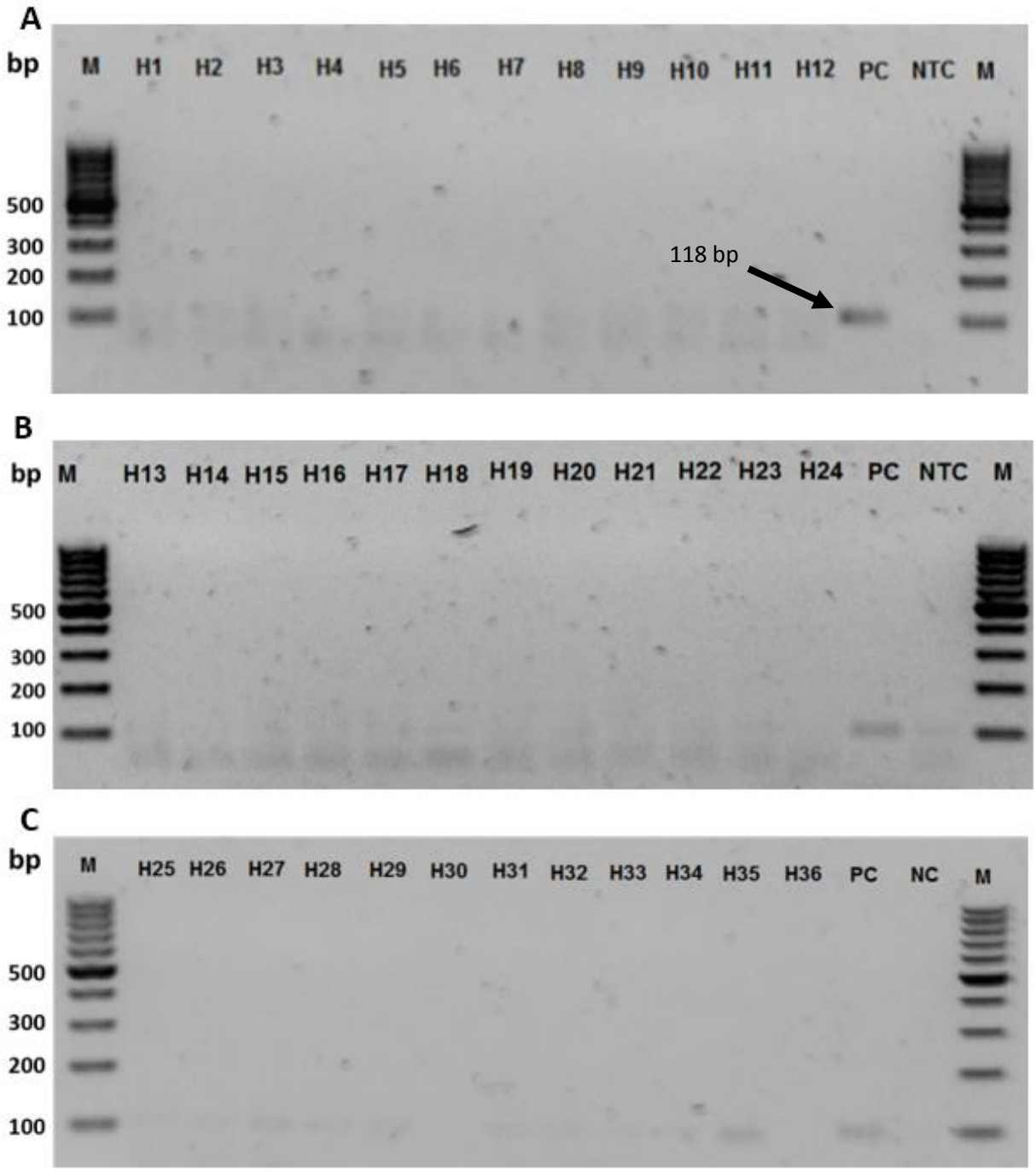

Figure 2.1 - Analysis of RT-PCR products using VP7 primers to amplify a 118 bp: (A) H1-H12; (B) H13-H24; (C) H25-H36; A 1.8% agarose gel was stained with Invitrogen™ SYBR™ Safe DNA Gel Stain. Lane M - Thermo Scientific™ GeneRuler™ 100bp DNA Ladder; Lanes H1-H71: Samples H1-H71; Lane PC: Positive control; Lane NTC: No template control.

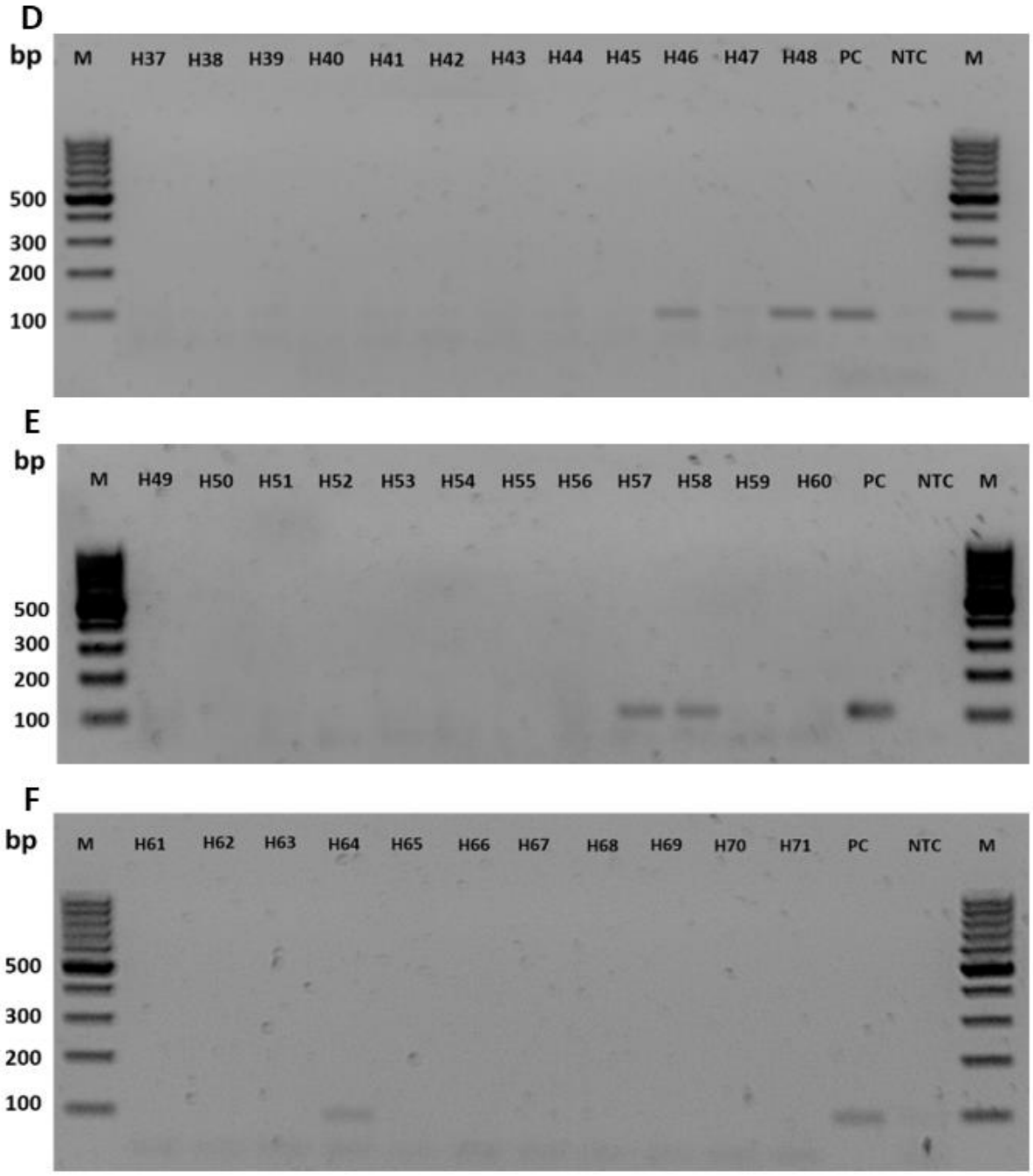

Figure 2.1 cont. - (D) H37-H48; (E) H49-H60; (F) H61-H71.

The gel images were analysed using ImageJ to quantify the band density of the PCR products. Table 2.1 shows the band density values after standardising them to the constant PC included in each gel.

Table 2.1 – Relative band density values as determined by digital electrophoresis gel analysis

Sample	Relative Band Density (%)	Sample	Relative Band Density (%)
H1	6.24	**H42**	4.04
H2	8.47	**H43**	2.60
H3	0.61	**H44**	0.30
H4	0.91	**H45**	0.42
H5	1.26	**H46**	46.90
H6	0.66	**H47**	8.17
H7	0.66	**H48**	78.14
H8	0.82	**H49**	0.37
H9	0.53	**H50**	1.06
H10	8.78	**H51**	1.08
H11	7.55	**H52**	0.78
H12	2.70	**H53**	0.04
H13	2.86	**H54**	0.10
H14	3.82	**H55**	0.07
H15	4.13	**H56**	0.44
H16	3.87	**H57**	39.93
H17	4.98	**H58**	32.94
H18	0.61	**H59**	1.79
H19	4.74	**H60**	1.15
H20	11.93	**H61**	0.35
H21	3.20	**H62**	0.26
H22	4.50	**H63**	0.83
H23	2.09	**H64**	49.77
H24	4.60	**H65**	0.98
H25	3.06	**H66**	1.83
H26	1.30	**H67**	2.44
H27	0.74	**H68**	1.52
H28	3.55	**H69**	1.43
H29	4.27	**H70**	0.85
H30	5.40	**H71**	1.41
H31	10.28	**H72**	0.61
H32	58.06	**H73**	1.18
H33	0.55	**H74**	0.88
H34	3.11	**H75**	0.70
H35	3.05	**H76**	0.87
H36	5.91	**H77**	0.68
H37	1.67	**H78**	0.74
H38	3.28	**H79**	0.97
H39	6.24	**H80**	0.79
H40	8.47	**H81**	0.97
H41	0.61	**PC**	100.00

2.4.2. **Standard curve**

Fig. 2.2 shows the graph of the fluorescence vs cycle number for the 10-fold dilution series. The fluorescence curves of the qPCR data in Fig. 2.2 show a delay in amplification take-off as the concentration of the template DNA decreases, with the longest delay being seen in the no template control (NTC). The variations in the fluorescence curves between replications increase as the number of copies in the template decrease.

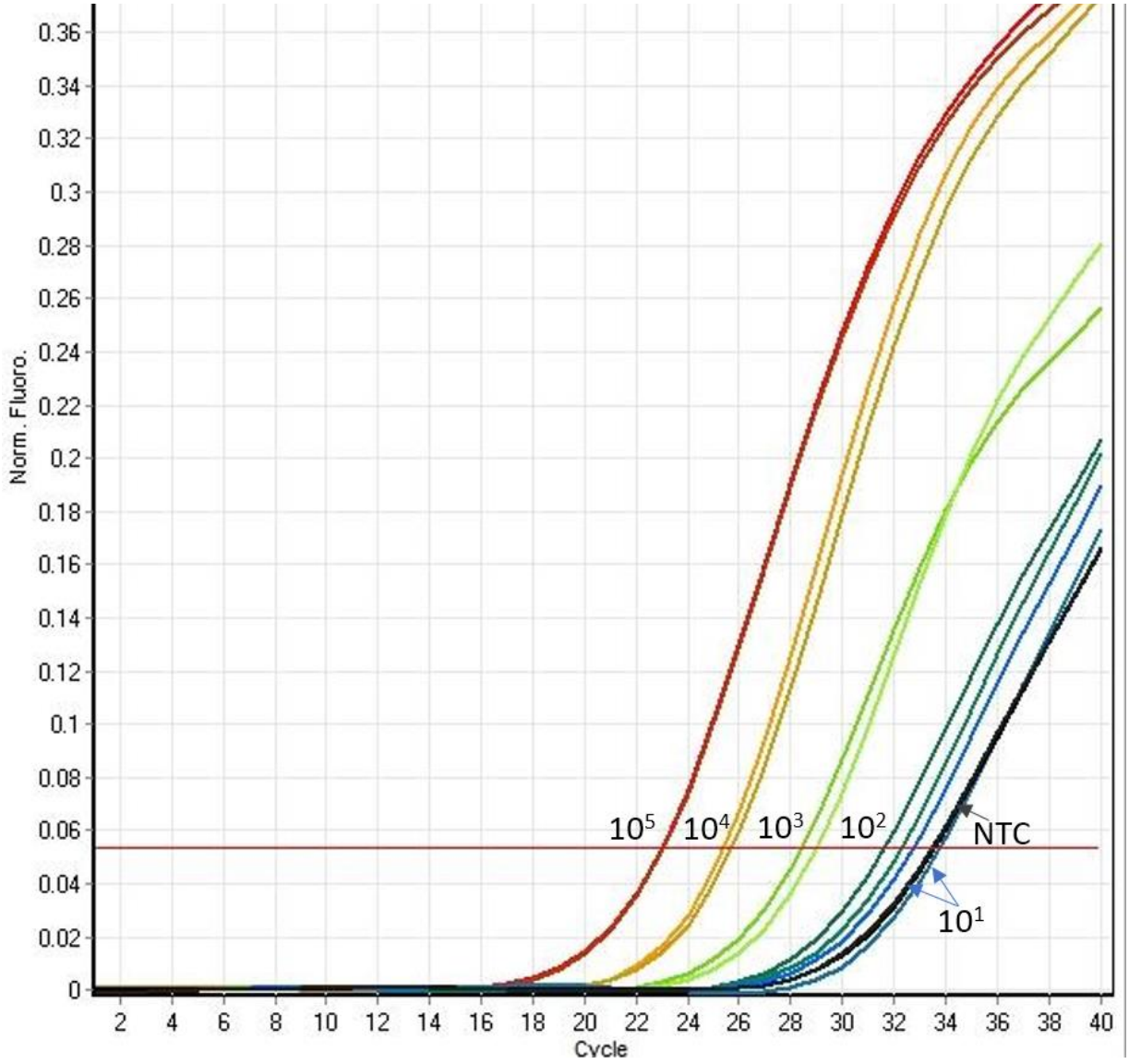

Figure 2.2 - Real-time PCR results for the amplification of a 10-fold dilution series for the generation of a standard curve. The plot represents cycle number vs fluorescence. Duplicate curves are represented by individual colours, representing a concentration range from 2×10^5 – 2×10^1 copies μl^{-1}. No template controls (NTC) containing nuclease-free water in place of template were included.

The LoB and LoD were measured by using test replicates of samples known to contain no analyte and a low concentration of analyte, respectively. Since C_q-values are the observed measurement with the RT-qPCR assay, the C_q-values were used in the calculation of analytical sensitivity and could translate to copy number if required. The LoB was calculated to be 33.5. The LoD was then calculated at 32.5. Samples were regarded as AHSV positive if the C_q-value of the sample was below the LoD.

A standard curve was constructed to measure the sensitivity of the qPCR assay and allow for quantification of samples (Fig. 2.3). The two lowest concentrations of the serial dilution (2 x 10^1 and 2 x 10^0 copies μl^{-1}) are excluded from the standard curve generation as their C_q-values are not below the calculated LoD. Linear regression analysis performed on the data from linear range (2 x 10^5 – 2 x 10^2 copies μl^{-1}) had an $R^2 > 0.99$. Based on the slope of the curve, the PCR efficiency was calculated to be 114%. The LoQ was determined to be the lowest concentration of the dilution series which fit the linear section of the standard curve. The LoB, LoD and LoQ are indicated on the curve.

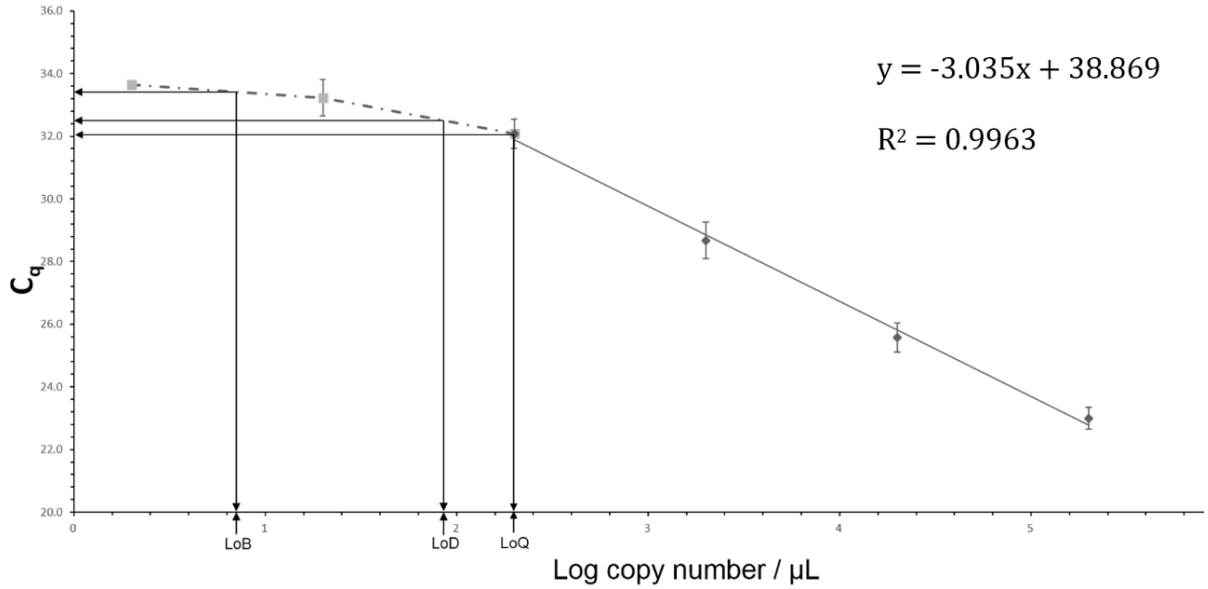

Figure 2.3 - Standard curve generated using 10-fold dilution series of external control of known concentration (2 x 10^5 copies μl^{-1}). The plot represents C_q against the log concentration of the template. The two lowest calibrants (2 x 10^1 – 2 x 10^0 copies μl^{-1}) which are not included in the standard curve generation are shown connected with a dotted line. The LoB (LoB; C_q = 33.5), LoD (LoD; C_q = 32.5) and LoQ (LoQ; C_q = 32.0) are annotated.

The C_q-values reported are defined as the cycle number at which the normalised fluorescence intercepts the threshold. Fig. 2.4 shows the fluorescence data from the

Rotor-Gene™ 6000 software for the RT-qPCR of extracted RNA, using H48 and H49 as examples of a positive and negative result respectively. Included in the figure are the amplification plots for the PC and NTC. The NTC and H49 have similar amplification curves with C_q-values above the LoD.

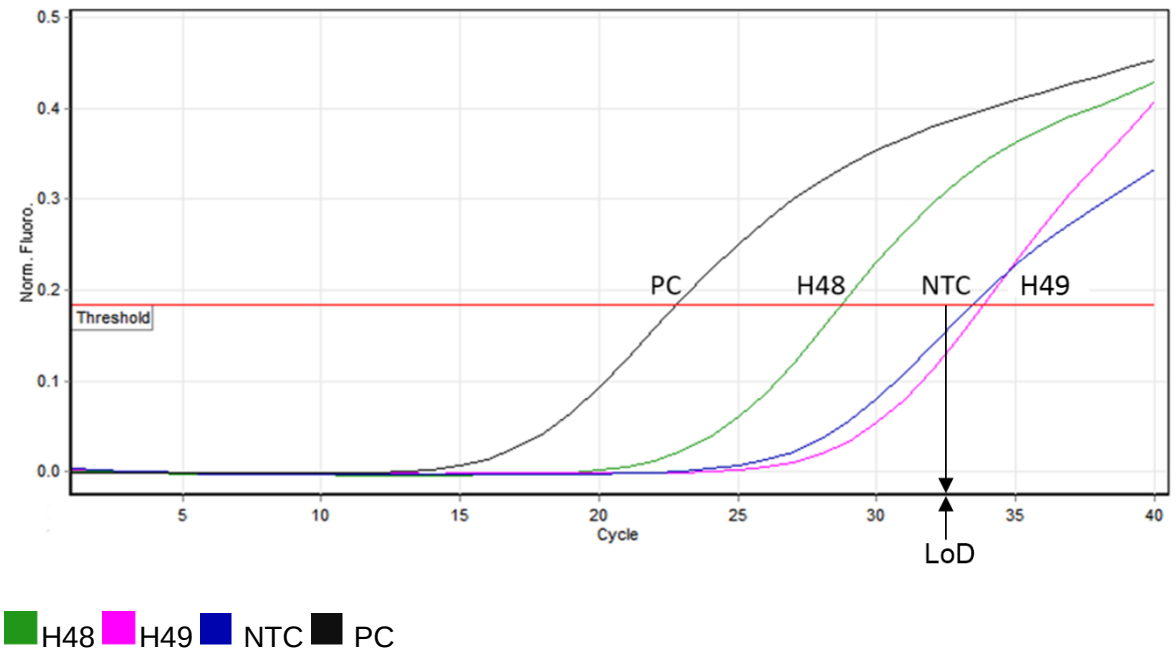

Figure 2.4 - Rotor-Gene™ 6000 data for the amplification of the 118 bp region of AHSV Segment 7 extracted from H48 and H49 with Positive (PC) and No template controls (NTC). The LoD is indicated on the graph.

The C_q-values for H1 - H81 are summarised in Table 2.2. The lowest C_q was 26.7 for H35. Six out of the 81 tested samples had C_q-values lower than the LoD of 32.5. The same samples had clear bands of expected product size on electrophoresis gels (Fig. 2.1). The mean C_q-value (± SD) for the NTC across the 3 separate runs was 36 (± 1.0).

Table 2.2 - Quantification cycle (C_q) values of qPCR for sampled horses H1 - H81. N indicates that the fluorescence threshold was not reached.

Sample	C_q	Sample	C_q
H1	37.8	H42	35.9
H2	37.7	H43	36.0
H3	37.1	H44	N
H4	39.5	H45	34.8
H5	36.7	H46	**29.9**
H6	N	H47	35.1
H7	37.2	H48	**30.1**
H8	39.0	H49	34.3
H9	37.4	H50	N
H10	37.0	H51	33.7
H11	36.5	H52	36.0
H12	35.6	H53	34.4
H13	37.1	H54	35.0
H14	36.3	H55	34.1
H15	35.5	H56	36.2
H16	N	H57	**31.4**
H17	N	H58	**30.7**
H18	34.8	H59	34.8
H19	37.0	H60	35.3
H20	35.5	H61	33.5
H21	36.9	H62	34.6
H22	36.4	H63	32.6
H23	36.4	H64	**31.1**
H24	36.3	H65	33.0
H25	35.8	H66	33.0
H26	34.8	H67	34.1
H27	34.8	H68	36.8
H28	34.9	H69	36.6
H29	37.3	H70	33.9
H30	35.2	H71	35.3
H31	37.1	H72	34.0
H32	37.8	H73	34.9
H33	37.5	H74	33.0
H34	39.0	H75	34.6
H35	**26.7**	H76	33.5
H36	37.2	H77	34.7
H37	37.8	H78	34.3
H38	37.5	H79	37.2
H39	39.0	H80	36.0
H40	33.3	H81	34.1
H41	38.1	**PC**	**23.0**

2.4.3. **Comparison of RT-qPCR and gel quantification**

Simple linear regression analysis was performed on the data of band density and C_q-values for samples H1-H81. A clump of 75 data points included in panel A (Fig. 2.5), represent samples which were considered negative. These samples had low band density and high C_q-values. Linear regression analysis indicated there was a significant correlation between the qPCR data and the digital gel analysis data ($P < 0.01$) with an $R^2 = 0.556$. Panel B of Fig. 2.5 is the simple linear regression with the negative results excluded and shows a higher coefficient of determination ($R^2 = 0.768$).

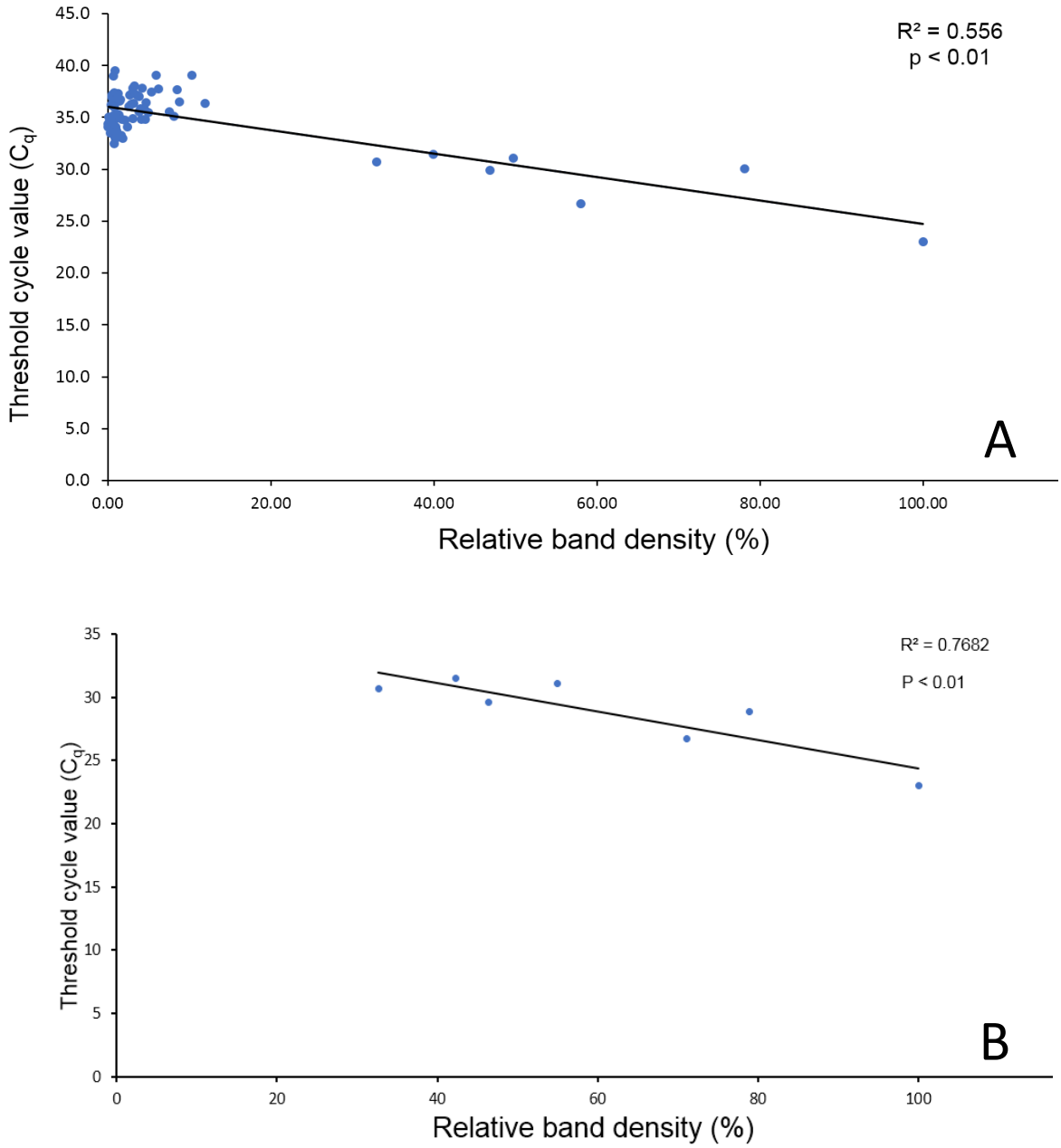

Figure 2.5 – Simple linear regression analysis of threshold cycle value (C_q) vs. relative band density. A – All samples included in the regression analysis; B – Negative samples removed from the regression analysis

2.5. **Discussion**

The objective of this study was to evaluate the use of gel image analysis as a method for AHSV quantification of field-collected samples. A commercially available qPCR assay was used as a reference technique for comparison.

The gel images shown in Fig. 2.1 showed six cases of distinguishable bands of 118 bp. Some evidence of contamination or primer dimer is shown in panel B (Fig. 2.1), these samples are most likely negative as the band appears as more of a smear than a solid band, which would be expected after 40 amplification cycles of a specific region. A similar smear is seen in the NTC of the same gel.

Digital analysis of these bands was performed using ImageJ, as described by Antiabong *et al.*, (2016). The band density values produced using this method are measured relative to all the band densities in the included image. Therefore, measured values from samples on different gels cannot be directly compared and each laboratory would need to calibrate the procedure for their equipment. To account for variations between gels, the PC was used as a reference to calculate the relative band density as it was included in each gel at the same concentration. Additionally, all gels were run under identical conditions using the same equipment, settings and reagents. The relative band densities from different gels could then be normalised and compared. The ability to quantify bands is useful because it allows information that is stored in a gel image to be saved in a more condensed format. After 40 cycles, PCR amplification is likely to have reached a plateau making concentration differences in the original test samples less apparent. Optimisations such as terminating this reaction during the exponential amplification stage may provide more meaningful quantification data and need further investigation should this method be used in the future.

For the RT-qPCR assay, a LoD had to be determined with the laboratory-specific conditions (Fig. 2.3). In some assays, a C_q cut-off value is determined to reduce the risk of interpreting a high C_q as a positive. If used, a C_q cut-off value should be specifically evaluated for different assays and PCR equipment to minimize the number of false positives and negatives. For this assay, a C_q cut-off corresponding to the observed LoD was selected (C_q-value > 32.5). Values higher than this indicated that the assay was unable to detect AHSV nucleic acid. Previous studies which have used this genome segment for quantification have generally used higher C_q cut-off values (Guthrie *et al*., 2013; Weyer *et al*., 2017). It is, however, difficult to compare C_q-values between laboratories using different equipment and reagents because these values are instrumental readings and not biologically meaningful (Bustin *et al*., 2009). The standard curve calibrated with external standards presented in Fig. 2.3 can be used to convert the laboratory-specific C_q-values to viral load. For this study, the SYBR green

intercalating dye bound to primer dimers, increasing background noise, which reduced the analytical sensitivity. With qPCR, the probability of false positive results increases as the analyte concentration approaches the LoD. For low viral titre, additional electrophoresis gel analysis may be required for confirmation.

In total, six AHSV-positive samples were detected in the sampled population. The six samples with C_q-values lower than the LoD (Table 2.2) were the same six to have clear bands present on a gel at the same position as the positive control (Fig. 2.1). Digital analysis of electrophoresis gel images and the RT-qPCR assay showed a statistically significant relationship ($P < 0.01$) in the quantity of amplified product (Fig. 2.5). There was a high degree of correlation ($R^2 = 0.768$) between the results of the two methods. While qPCR is a more sensitive quantification tool than gel analysis (Schmittgen *et al*., 2000), the method assessed above is a more affordable way to add valuable information to the conventional interpretation of PCR results by of gaining information on the relative quantity of amplified product. This may be particularly important in developing countries with veterinary laboratories lacking qPCR equipment.

2.6. **Conclusion**

Quantification using digital gel analysis will not replace the more sensitive qPCR assays but may prove to be a useful substitute in resource-limited settings with under-funded diagnostic laboratories. The ability to quantify viral load improves empirical decision making with regards to disease control and management.

2.7. **References**

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CHAPTER 3:

Investigation of the effect of a commercial live attenuated vaccine on viral load in mares

3.1. Abstract

Vaccination is the main mechanism of control of African horse sickness (AHS) in endemic areas. Improvements to the sensitivity of nucleic acid diagnostic assays allow for the detection of low levels of African horse sickness virus (AHSV) prior to the onset of clinical signs. A limitation of these assays is that they may detect RNA from live attenuated vaccines (LAV). This experiment examined the effect of the vaccine on viral load using an RT-qPCR diagnostic assay. Routinely vaccinated miniature mares (n=9) had their AHSV viraemia levels monitored following vaccination with a live-attenuated vaccine. No AHSV was detected in the horses following vaccination. The absence of AHSV in the mares supports vaccination as a viraemia reduction strategy.

3.2. Introduction

Vaccination remains the most practical method for prevention of viral diseases. The World Organisation for Animal Health (OIE) lists African horse sickness (AHS) as important to international trade. Countries and zones free from the disease are officially recognised by the OIE affecting the movement of animals between these areas (OIE Code Commission, 2012).

A live attenuated vaccine (LAV) manufactured by Onderstepoort Biological Products (OBP) is available in AHS endemic areas. This vaccine consists of two separate doses administered at least three weeks apart. The first vaccine contains Serotypes 1, 3, 4 and the second contains Serotypes 2, 6, 7, 8. Cross-protection reportedly exists between AHSV-5 and AHSV-8, as well as, AHSV-6 and AHSV-9 (von Teichman *et al*., 2010) therefore AHSV-5 and AHSV-9 are not included in either vaccine.

For a vaccine strain to be accepted in South Africa it cannot cause viraemia levels of above 10^3 PFU ml^{-1} (Paweska *et al*., 2003). However, a problem arises because the level of viraemia needed to infect the *Culicoides* vector is unknown. Thus, it is not definitively known if the level of viraemia induced by vaccination is low enough to prevent transmission from host to vector. It has been recently reported that AHS-vaccine virus has been transmitted through the normal AHS vectors (Weyer *et al.*,

2016). In addition, the OBP AHSV-LAV has caused outbreaks of virulent AHSV through genetic re-assortment and reversion to virulence (Weyer *et al*., 2016), indicating that serologically naïve equids are at risk of contracting AHS by the movement of vaccinated horses into AHS-free zones. The use of the vaccine should, therefore, be limited to periods of low vector activity and if possible not used in AHSV free zones.

Molecular diagnostics based on the amplification of nucleic acids, i.e, PCR and RT-PCR, can rapidly detect the presence of the virus. This has a range of applications within infectious disease diagnostics and has been used to detect and serotype the AHSV (Sailleau *et al*., 2000). However, RT-PCR is unable to distinguish between the attenuated vaccine virus and natural infection which may result in false reporting of the disease in vaccinated animals (Weyer *et al*., 2017).

In this study, a group of mares received vaccination with a commercial AHSV-LAV, and their viral load was monitored through RT-qPCR to determine if the AHSV-LAV could elicit detectable viraemia.

3.3. **Materials and methods**

3.3.1. **Study population**

Routinely vaccinated miniature mares (n = 9) aged 6 - 12 years of age were used in this study. Horses were housed at University of KwaZulu-Natal (UKZN), Ukulinga Research farm in the KwaZulu-Natal, South Africa. Ethical approval for the blood retrieval from these animals was granted by the Animal Research Ethics Committee of UKZN (Appendix 1).

3.3.2. **Vaccination**

The horses were vaccinated with the Onderstepoort Biological Products (OBP) vaccine in two doses (Day 0 and Day 28), as per the manufacturer's recommendations.

3.3.3. **Blood Collection**

Blood samples were collected in EDTA-containing vacutainers (Becton Dickinson, New Jersey, USA). Blood samples were taken on vaccination days prior to blood collection and then as detailed in Fig. 3.1. The blood samples were placed on ice before being transported to the laboratory for processing.

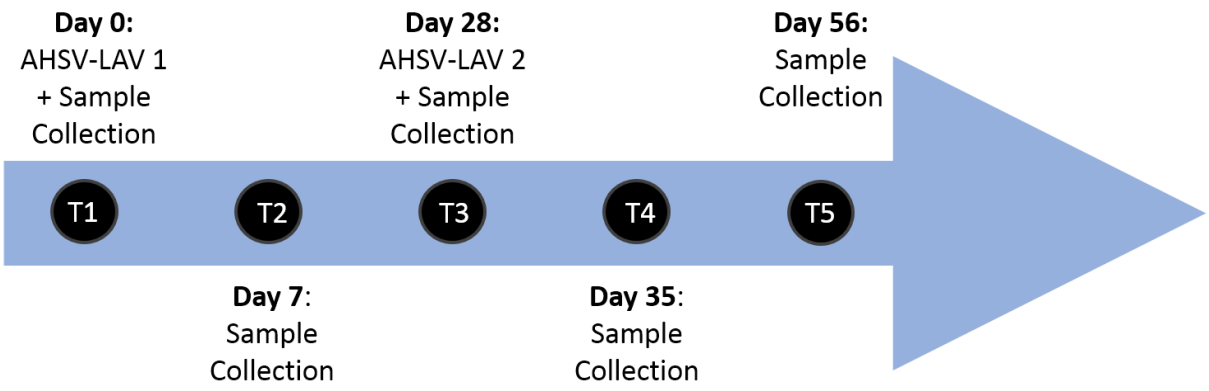

Figure 3.1 - Timeline of sampling and vaccination program

3.3.4. **RNA Extraction**

Total RNA was extracted from blood samples using the Quick-RNA™ Miniprep Plus Kit (Zymo Research, Irvine, USA), according to the manufacturer's recommendations: A volume of 500 µl of blood from each sample was transferred into a 2.5 ml microcentrifuge tube and an equal volume DNA/RNA Shield™ (2 X concentrate) was added to the sample and vortexed. An 8 µl volume of Proteinase K (20 mg ml^{-1}) was added to each mixture and incubated at room temperature for 30 minutes. To this solution, a 1 ml aliquot of isopropanol was added and vortexed. The mixture was transferred to a Zymo-Spin™ III-CG column (Zymo Research, Irvine, USA) and collection tube and centrifuged at 15,000 *x g* for 30 s. To this column, 400 µl RNA Prep Buffer was added, centrifuged at 15,000 *x g* for 30 s, and the eluent was discarded. The column was then washed twice with 700 µl RNA wash buffer and centrifuged at 15,000 *x g* for 2 minutes. The column was transferred to an RNase-free tube. The final RNA was eluted by adding 50 µl nuclease free water to the column followed by centrifugation at 15,000 *x g* for 30 s. RNA that was not used immediately was stored at -80 °C. Absorbance readings between the wavelengths of 220 and 350 nm of the extracted RNA were taken using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, USA) to assess the quantity and purity of the extracted RNA.

3.3.5. **Complementary DNA (cDNA) synthesis**

Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA). In a PCR tube, 2 µl of template RNA was added to 1 µl of random hexamer primer (100 µM) and 9 µl of nuclease-free water. This was incubated at 95 °C for 1 minute and transferred immediately onto ice. The

mixture was combined with 4 µl of 5 X reaction buffer, 1 µl RiboLock RNase Inhibitor (20 U μl^{-1}), 2 µl of 10 mM dNTP mix and 1 µl RevertAid M-MuLV Reverse Transcriptase (200 U μl^{-1}). The mixture was vortexed and incubated at 25 °C for 5 minutes followed by 42 °C for 60 minutes. The reaction was terminated by heating to 70 °C for 5 minutes. The complementary DNA (cDNA) was stored at -80 °C for further processing.

3.3.6. **Nucleic acid detection**

The detection of the AHSV VP7 gene was performed using the Genesig AHSV kit (PrimerDesign, Southampton, United Kingdom). The PCR for each sample was performed on a Rotor-Gene™ 6000 (Qiagen, Hilden, Germany). Duplicate reactions were set up to a final volume of 20 µl containing 5 µl of template cDNA, 10 µl PowerUp™ Green Master Mix (Thermo Fisher Scientific, Waltham, USA), 4 µl nuclease-free water and 1 µl AHSV specific primer/probe mix (PrimerDesign, Southampton, United Kingdom). The PCR reaction conditions were as follows: an initial Uracil-DNA Glycosylase (UDG) activation step of 50 °C for 2 minutes, 95 °C for 2 minutes for initial denaturing, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Fluorescence data was collected at the end of each cycle.

A positive control (PC) containing VP7 template DNA with a concentration of 2 x 10^5 copies μl^{-1} was included in each PCR run. The concentration of the PC in each run was kept constant. Therefore, to have comparable results between runs the threshold value was set to a value where the PC had a C_q of 23.0 across all the PCR reactions.

3.3.7. **Agarose gel electrophoresis**

A volume of 5 µl of PCR product was run on a 1.8% (w/v) agarose gel in 1 X Tris-borate-EDTA (TBE) buffer containing 1 X SYBR ® Safe DNA gel stain (Invitrogen, Carlsbad, USA) to determine the molecular weight of the PCR products. Electrophoresis was run at 6.66 V cm^{-1} for 1 hour and visualised on a UV transilluminator. The DNA bands were sized using a GeneRuler™ 100 bp ladder marker (Thermo Fisher Scientific, Waltham, USA).

3.3.8. **Standard curve**

To calculate viral quantity in test samples, a standard curve was generated from a known concentration of plasmid DNA provided as an external positive control (PC) with the PCR kit. The standard curve was generated using a 10-fold dilution series of the

template DNA ranging from 2 x 10^5 – 2 x 10^1 copies μl^{-1} The LoQ was determined as the lowest concentration of the serial dilution which fits on the linear range of the standard curve. LoB and LoD were determined according to Armbuster & Pry (2008).

3.3.9. **Data analysis**

Analysis of variance (ANOVA) was used to analyse the C_q differences over the study period. A P-value < 0.05 was considered significant.

3.4. **Results**

3.4.1. **RNA extraction**

Spectrophotometric analysis of extracted RNA produced A_{260}: A_{280} ratios in the range of 1.7 - 2.0. RNA quantity ranged from 12.0 – 65.5 ng μl^{-1}.

3.4.2. **Analytic sensitivity**

The LoB and LoD were calculated using replicates of samples known to contain no analyte and low concentration of the analyte. No template controls (NTC) were included in each run alongside tested samples to test account for inter-run variation. Since C_q-values are the observed measurement with the RT-qPCR assay, the C_q-values were used in the calculation of analytical sensitivity and could translate to copy number if required. The LoB was calculated to be 33.4. The LoD was then calculated at 32.4.

3.4.3. **Nucleic acid detection**

Fig. 3.2 shows the gel image for the sample taken from Peppa on Day 0. Amplification products were detected for the PC (expected size of 118 bp), but no equivalent product was detected for any of the samples or NTC. No amplification products were detected on gels from the additional eight mares in the trial (data not shown). This demonstrates that there was no detectable AHSV RNA prior to vaccination.

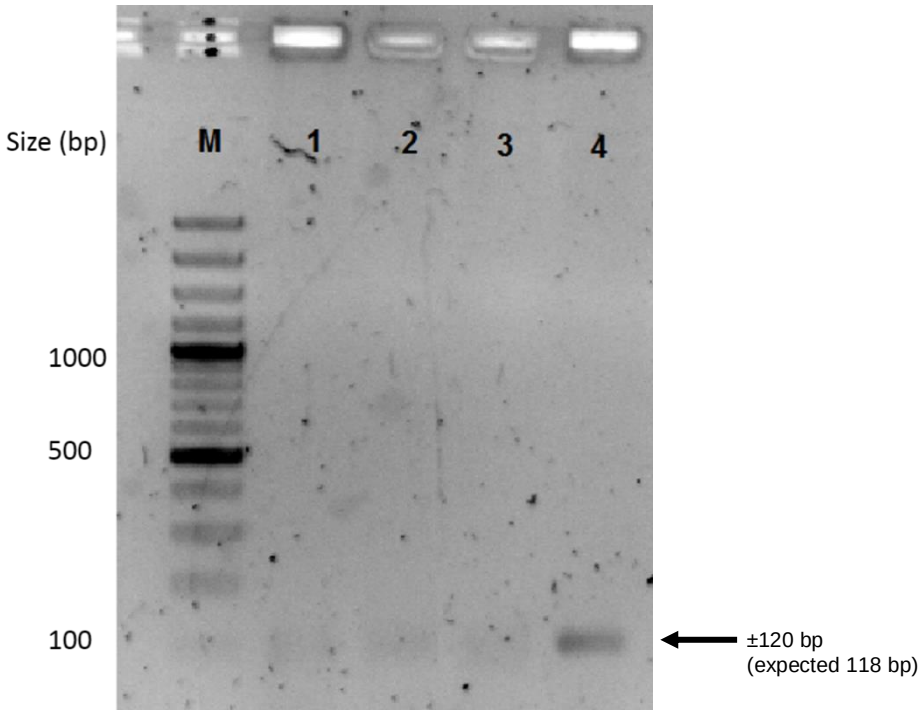

Figure 3.2 - Analysis of RT-PCR products of Horse 5 (Peppa) amplifying a 118 bp fragment of the genome segment encoding VP7. A 1.8% agarose gel was stained with Invitrogen™ SYBR™ Safe DNA Gel Stain. Lane M: Thermo Scientific™ GeneRuler™ 100bp Plus DNA; Lane 1 & 2: Horse 5 (Peppa); Lane 3: NTC; Lane 4: PC.

Four runs in the Rotor-Gene 6000 were completed in total to analyse a total of 90 samples. The ANOVA (Table 3.1) shows that there was no significant change in C_q-values for the vaccinated adult mares between sampling periods ($P > 0.05$), indicating that there was no evident increase in the viral load following vaccination.

Table 3.1 – Results of the ANOVA of standardized RT-qPCR C_q-values following vaccination of adult mares.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11.69717632	5	2.339435	1.043963	0.397924	2.33021
Within Groups	177.0325813	79	2.240919			
Total	188.7297576	84				

The line plot in Fig. 3.3 shows the mean and 95% confidence interval of C_q-values for the qPCR results of all horses across the 56-day period, indicating that no significant rise in AHSV occurred as all the means are within each other's confidence interval. Samples with C_q-values lower than the LoD were classified as positive.

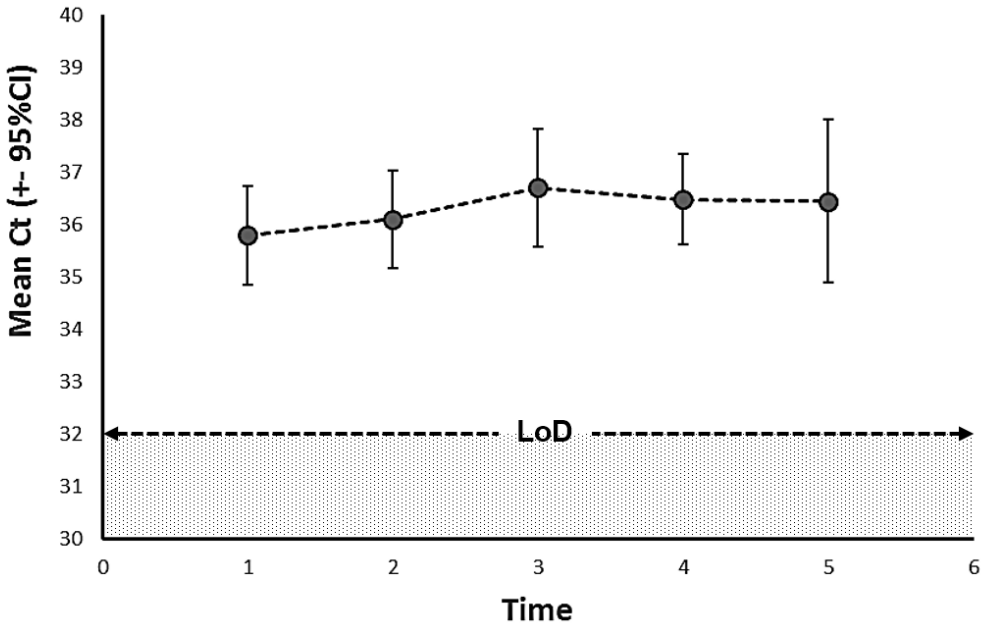

Figure 3.3 - Line plot describing C_q-value distributions of the RT-qPCR targeting the VP7 gene of AHSV in adult mares following routine vaccination.

The results showed that vaccination did not result in detectable levels of AHSV in the sampled animals.

3.5. **Discussion**

The aim of this study was to investigate whether the AHSV-LAV could elicit viraemia of a high enough level to be detected and quantified by RT-qPCR, and the potential this has for disrupting AHSV surveillance. All mares tested negative for AHS prior to vaccination (Fig. 3.2) and continued to test negative throughout the trial (Table 3.1 & Fig. 3.3). This result is supported by recent studies of viral load response in routinely vaccinated mares using the same vaccine (Weyer *et al*., 2017). However, in the work by Weyer *et al*., (2017), AHSV RNA was detected in weanling foals and yearlings for up to 16 weeks following vaccination. This is important because it shows that the LAV causes a considerable level of viremia in young animals. In addition to complicating surveillance, the vaccine virus may then be transmitted by the *Culicoides* vector to unvaccinated or susceptible animals. It has been demonstrated previously that *in vitro*

attenuation of AHSV may not eliminate the virus's ability to infect the *Culicoides* vector (Venter & Paweska, 2007; Venter *et al*., 2009). In addition, the likelihood of vaccine virus reverting to virulence is increased by its ability to survive and multiply within the vector (Venter *et al.*, 2009).

The virulent reversion of AHSV-LAV was confirmed to be the cause of an AHS outbreak in South Africa in 2014, where recently vaccinated animals were moved into a serologically naïve area of the Western Cape (Weyer *et al*., 2016). Transmission of the virus was thought to have occurred through the normal *Culicoides* vectors. This exhaustive genetic analysis, which consisted of the sequencing and comparison of over 50 whole genomes, highlighted the importance of the continued development of alternative vaccines. Until these vaccines become commercially available the AHSV-LAV will remain the mainstay for AHS control in endemic areas.

The results reported in the present study indicate a low impact on the disease surveillance of previously vaccinated older animals. The age and vaccination history should be recorded when sampling and can be used to aid in interpreting RT-qPCR data.

3.6. **Conclusion**

The annually vaccinated mares in this study showed no signs of viral RNA in the weeks following immunization, confirming that vaccine-induced viraemia is low in previously vaccinated older horses. Information on the age and vaccination history of the animal is therefore important for the effective surveillance of AHSV and should be considered when analysing RT-qPCR data.

3.7. **References**

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CHAPTER 4:

The use of filter paper card technology in molecular African Horse Sickness Virus diagnostics

Abstract

Flinders Technology Associates® filter paper cards (FTA® cards) are designed to store nucleic acid from fresh sample material in a stable matrix. This may offer a practical solution to disease surveillance in resource-limited settings where conventional sampling methods are not always suitable. Storage and elution conditions of African horse sickness virus (AHSV) RNA from these cards was investigated. A pair of elution reagents for AHSV RNA extraction from blood applied to FTA® cards were compared. Cards with AHSV positive blood applied was stored at room temperature for one week. Amplified products were successfully detected by RT-PCR after elution with Tris-EDTA buffer. This work demonstrated that FTA® cards can be used to store and transport AHSV for RT-PCR analysis.

4.1. Introduction

RNA integrity is an important factor in obtaining and evaluating genomic information. Current sampling methods rely on an effective cold-chain which may be unavailable to under-equipped veterinary laboratories. Spoiled samples, degraded during transportation from remote areas to laboratories for analysis, may be difficult to distinguish from normal samples and will be processed as such. False negatives due to sample spoilage will cause underreporting of the disease. In the case of the often-fatal AHS, a misdiagnosis could lead to the spread of the disease and subsequent equine deaths.

The popularity of dried blood spots (DBS) in resource-limited settings for the detection of viral diseases is increasing because of the advantages they have over conventional sampling methods (Muthukrishnan *et al*., 2008; Sakai *et al*., 2015). Flinders Technology Associates (FTA®) cards are designed to store nucleic acid from fresh sample material in a stable matrix. The chemicals contained within the matrix denature unwanted proteins and lyse cells to release nucleic acids and may prove to be a good

alternative sampling technique in nucleic acid based assays for RNA viral detection (Sigma-Aldrich, 2017). An advantage with these cards is the inactivation of the virus, which allows for safe transport of non-infective samples while keeping the nucleic acid available for downstream analysis. This does, however, have limitations in that live virus particles cannot be isolated and cultured if required.

FTA® cards have been used successfully in trials for the detection of viral RNA of Foot-and-mouth disease virus (Muthukrishnan *et al*., 2008), rabies (Picard-Meyer *et al*., 2007; Sakai *et al*., 2015) and Avian influenza virus (Abdelwhab *et al.,* 2011, Jóźwiak *et al*., 2016). DBS samples have also been used for the detection of a related orbivirus, Bluetongue virus (BTV) (Martinelle *et al*., 2015). The use of FTA® cards for storage of blood for AHSV detection could reduce sample spoilage during transportation and storage, thus increasing the accuracy of testing and disease reporting.

In this study, the use of FTA® cards was investigated as a method of storing blood for the detection of AHSV through RT-qPCR. The study compared methods for extracting AHSV RNA from FTA® cards.

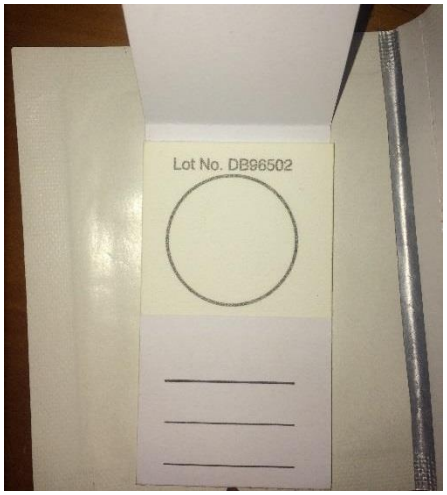

Figure 4.1 - Unused FTA® Micro Card

4.2. **Materials and methods**

4.2.1. **Preparation of virus stored on FTA® cards**

FTA® classic and FTA® micro cards (Whatman/GE Healthcare Europe, Freiburg, Germany), were used for blood storage. To the cards, 200 µl of AHSV positive blood, previously identified through group-specific RT-qPCR, was added to the card sampling

area. The cards were stored in sealed foil envelopes at room temperature for one week.

4.2.2. **RNA Extraction**

Total RNA was extracted from blood samples using the Quick-RNA™ Miniprep Plus Kit (Zymo Research, Irvine, USA). Two different eluents were compared: TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and RNA Lysis buffer (RL-buffer) (Zymo Research, Irvine, USA). RNA extractions were done in triplicate for the pair of eluents. Dried blood spots were cut out of each card using a scalpel and placed in nuclease-free 1.5 ml microcentrifuge tubes. To this, 300 µl of each eluent was added, mixed well and incubated at 4 °C for 24 hours. After 24 hours, the eluate was removed, placed into a Spin-Away Filter (Zymo Research, Irvine, USA) and centrifuged to remove gDNA. To the flow-through, 200 µl ethanol was added and mixed well. The mixture was transferred to a Zymo-Spin III-CG column (Zymo Research, Irvine, USA) in a collection tube and centrifuged at 15,000 x *g* for 30 s. To this column, 400 µl RNA Prep Buffer was added, centrifuged, and the flow-through was discarded. The column was then washed twice with 700 µl RNA wash buffer and centrifuged for 2 minutes. The column was transferred to an RNase-free tube and the final RNA was eluted by adding 30 µl nuclease free water to the column followed by centrifugation at 15,000 *x g* for 30 s.

4.2.3. **Complementary DNA (cDNA) synthesis**

Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA). In a PCR tube, 2 µl of template RNA was added to 1 µl of random hexamer primer (100 µM) and 9 µl of nuclease-free water. This was incubated at 95 °C for 1 minute and transferred immediately onto ice. The mixture was combined with 4 µl of 5 X reaction buffer, 1 µl RiboLock RNase Inhibitor (20 U μl^{-1}), 2 µl of 10 mM dNTP mix and 1 µl RevertAid M-MuLV Reverse Transcriptase (200 U μl^{-1}). The mixture was vortexed, spun down and incubated at 25 °C for 5 minutes followed by 42 °C for 60 minutes. The reaction was terminated by heating to 70 °C for 5 minutes. The cDNA was stored at -80 °C for further processing.

4.2.4. **Nucleic acid detection**

The PrimerDesign Genesig kit for AHSV VP7 detection was used in this study (PrimerDesign, Southampton, United Kingdom). The PCR for each sample was performed on a Rotor-Gene™ 6000 (Qiagen, Hilden, Germany). Duplicate reactions

were set up to a final volume of 20 µl containing 5 µl of template cDNA, 10 µl PowerUp™ Green Master Mix (Thermo Fisher Scientific, Waltham, USA), 4 µl nuclease-free water and 1 µl AHSV specific primer/probe mix (PrimerDesign, Southampton, United Kingdom). The PCR reaction conditions were as follows: an initial Uracil-DNA Glycosylase (UDG) activation step of 50 °C for 2 minutes, 95 °C for 2 minutes for initial denaturing, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Fluorescence data was collected at the end of each cycle. HRM was subsequently performed from 60 °C - 95 °C at 0.1 °C 2 s^{-1}.

A positive control (PC) containing VP7 template DNA with a concentration of 2 x 10^5 copies μl^{-1} was included in each PCR run. The concentration of the PC in each run was kept constant. Therefore, to have comparable results with the standard curve, the threshold value was set to a value where the PC had a C_q of 23.0 the PCR reactions.

4.2.5. **Agarose gel electrophoresis**

A volume of 5 µl of PCR product was run on a 1.8% (w/v) agarose gel in 1 X TBE buffer containing 1 X SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, USA) to determine the molecular weight of the PCR products. Electrophoresis was run at 6.66 V cm^{-1} for 1 hour and visualised on a UV transilluminator. The DNA bands were sized using a GeneRuler™ 100 bp ladder marker (Thermo Fisher Scientific, Waltham, USA).

4.3. **Results**

No AHSV was detected with RT-qPCR when using elution times of 30 minutes. TE-buffer and RLB (Zymo Research, Irvine, USA) then had their AHSV RNA elution capabilities compared when extending elution time to 24 hours at 4 °C.

Fig. 4.2 shows the amplification plots of products from two different eluents. All the TE-buffer eluted samples amplified before the NTC while all the RL-buffer eluted samples amplified after the NTC, indicating that viral RNA could not be detected in the RLB eluted sample.

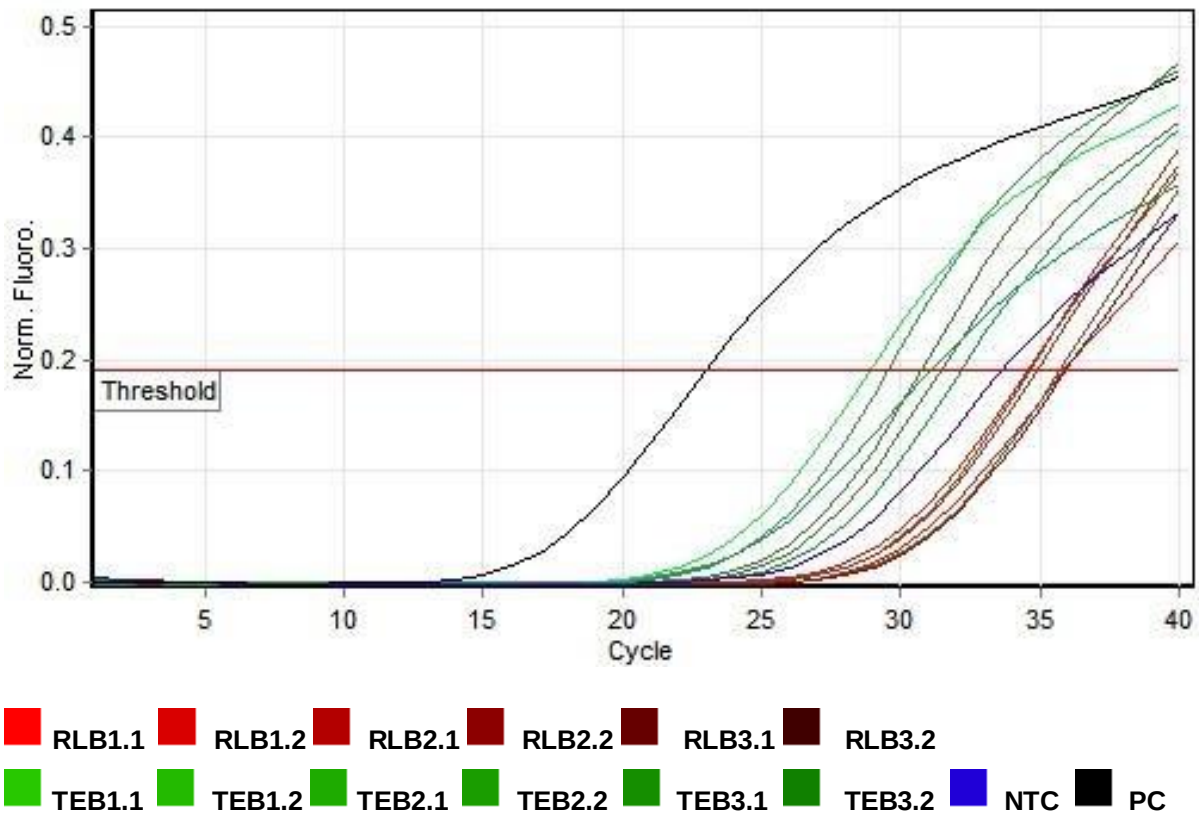

Figure 4.2 - Rotor-Gene™ 6000 data for the amplification of the 118 bp region of AHSV Segment 7 extracted from dried blood spots. RLB - RNA Lysis Buffer; TEB – TE Buffer; NTC – No template control; PC – Positive control

C_q-values obtained for the eluents used are summarised in Table 4.1. Samples with C_q-values < 32.5 were considered as AHSV positive.

Table 4.1 - C_q for different eluents used for RNA extracted from FTA® cards

Eluent	C_q (± standard deviation)
TE-buffer 1	29.69 (±0.08)
TE-buffer 2	31.02 (±0.49)
TE-buffer 3	32.16 (±0.13)
RL-buffer 1	36.35 (±1.26)
RL-buffer 2	35.35 (±0.68)
RL-buffer 3	35.47 (±0.83)
No template control	34.15
Positive control	23.00

Amplified products had their product sizes confirmed by running a 1.8 % agarose gel in 1 X TBE (Fig. 4.3). Viral RNA eluted using TE-buffer yielded the expected bands of 118 bp in all three tested samples after RT-qPCR (Lanes 1 - 3). No product was evident for the RL-buffer eluted samples (Lanes 4 - 6).

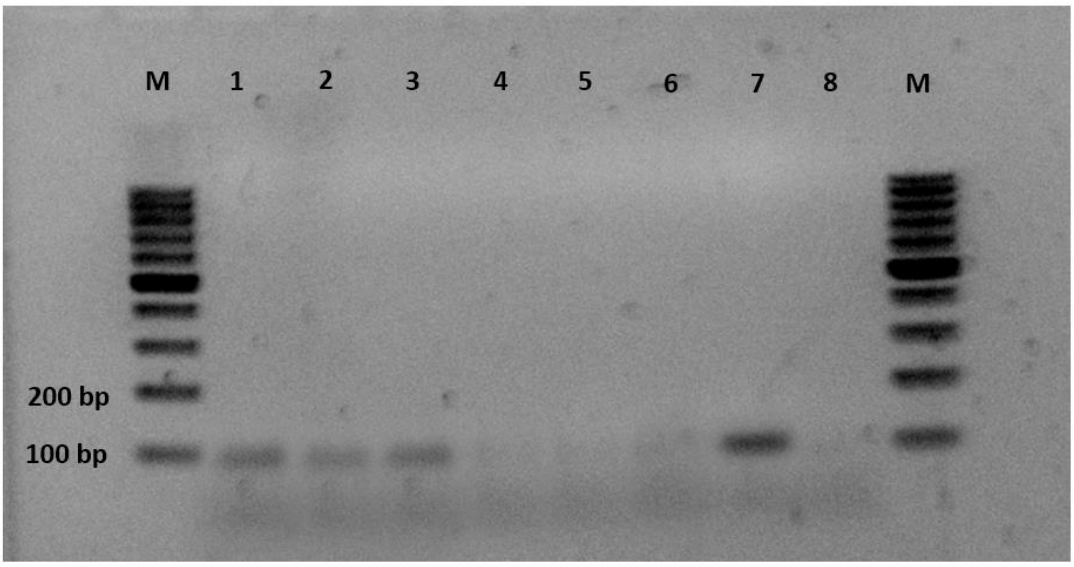

Figure 4.3 - Analysis of RT-PCR products of AHSV RNA using VP7 primers to amplify a 118 bp product. A 1.8% agarose gel was stained with Invitrogen™ SYBR™ Safe DNA Gel Stain. Lane M - Thermo Scientific™ GeneRuler™ 100bp DNA; Lanes 1-3: TE-buffer; Lanes 4-6: RL-buffer; Lane 7 - PC; Lane 8: NTC.

4.4. **Discussion**

Two different eluents were evaluated for their ability to elute AHSV RNA from DBS on FTA® cards. RL-buffer is supplied with the Quick RNA Mini-Prep (Zymo Research) and although it is designed for cells, tissue and blood, it was unable to elute AHSV RNA from DBS on FTA® cards when following the manufacturer's recommendations, and with the elution time increased to 24 hours. TE-Buffer is a non-proprietary, laboratory product and was found to be successful in eluting viral RNA from DBS on FTA® cards (Fig. 4.3). TE-Buffer has been found to be suitable for extracting viral RNA from DBS from Rabies virus and Human enterovirus (Li *et al*., 2012; Sakai *et al*., 2015).

Picard-Meyes *et al.* (2007*)* reported successful elution of rabies virus using Dulbecco's Modified Eagle Medium (DMEM) when the virus suspended in DMEM was applied to FTA® cards. The use of DMEM for elution of viral RNA from dried blood spots has, to the author's knowledge, yet to be investigated. Another untested eluent which may prove useful is RNA Rapid Extraction Solution, the recommended eluent for RNA

extraction from FTA® cards. While TE-Buffer was successful, a more in-depth evaluation of eluents is necessary to determine the most suitable for the extraction of AHSV RNA.

The FTA® cards used were stored at room temperature for 1 week before processing. Previous studies looking at storage conditions of FTA® cards on viral RNA degradation found significant RNA damage after 1 - 2 weeks of card storage at room temperature but found no damage after 3 months when the cards were stored at -20 °C or -80 °C (Sakai *et al*., 2015). The ability to transport biological samples from remote areas at room temperature without sample degradation increases regional access to diagnostic tests and reduces costs associated with cold chain shipment.

A potential downside to DBS sampling is the low input volume which may be a concern for detecting low levels of virus.

There is a lack of epizootiological data for many endemic regions affected by AHS. Improvement to the ease of collection and transport, together with greater safety and reliability of transporting biological samples using FTA® cards will improve epidemiological knowledge.

4.5. **Conclusion**

This study demonstrates that FTA® cards have the potential to be a powerful research tool for AHS. However, for viral load assays using dried blood spots to be comparable, standardised procedures for the collection, storage and extraction of viral RNA from these cards need to be established. It is unlikely that dried blood spots will replace the need for conventional sampling but may be useful to collect in addition to regular samples due to the relative sampling ease and demonstrated RNA stability.

4.6. **References**

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CHAPTER 5:

High resolution melt analysis of AHSV genome Segments 10 and 2

5.1. Abstract

African horse sickness (AHS) is caused by the African horse sickness virus (AHSV), a double-stranded RNA virus of the genus *Orbivirus* in the family *Reoviridae.* The viral genome is made up of ten double-stranded RNA segments. Nine antigenically different serotypes of AHSV have been identified so far. However, there is limited information on the serotype prevalence of AHSV in endemic areas. Therefore, multivalent vaccines remain the mainstay for disease control in these regions. In this experiment, a novel approach to assessing variations in AHSV nucleic acid using high-resolution melt analysis (HRMA) was investigated. Blood infected with a wild strain of AHSV was used in a PCR targeting regions of Segment 10 (S10) and Segment 2 (S2). Following this, high-resolution melting curves were generated from these PCR products. S10 primers amplified the expected 181 bp product but the other primer sets failed to amplify the S2 gene segment warranting, further evaluation and optimisation of these primer sets used to detect currently circulating AHSV strains.

5.2. Introduction

The African horse sickness virus (AHSV) genome comprises ten double-stranded RNA segments each coding for at least one viral protein (Grubman & Lewis, 1992). Genome Segments 2 (S2) and 10 (S10) are responsible for the genetic diversity between serotypes and are usually the regions targeted by nucleic acid-based serotyping assays (Venter *et al*., 2002). The last of the antigenically different serotypes were isolated in 1960, indicating that AHSV is not a rapidly reassorting virus (Howell, 1962).

Serotype determination is important in both epidemiological studies and vaccination programs. Sailleau *et al.* (2000) designed the first reverse transcription PCR (RT-PCR) assay for the serotyping of AHS. This assay targeted different regions of Segment 2 according to the serotype using nine primer pairs in separate reactions. Confirmation of product was done on agarose gel electrophoresis. A recent assay developed in South Africa used three triplex AHSV real-time RT-PCR assays to identify individual serotypes (Weyer *et al*., 2015). This assay, although accurate, is costly due to the multiple fluorescent probes required.

High-resolution melt (HRM) is a fast, cost-effective and powerful post-PCR technique used to identify genetic variation. HRM uses precise heating of PCR product to release an intercalating dye that is measured through sensitive light detectors. The resulting melt curves that are generated can be used to detect differences in amplicon sequences. A serotyping assay for AHSV using HRM was investigated by Groenink. (2014). This assay uses a combination of S10 and S2 primers to determine serotype. The HRM profile generated from melting of the 10-190 bp region of S10 was used to group samples into one of three bins. The serotype was subsequently identified from these bins using melt curves obtained from Segment 2 clade-specific primers.

The aim of this study was to test the primers sets described by Groenink (2014) on AHSV positive blood containing a currently circulating strain of AHSV to obtain a set of melt curves.

5.3. **Materials and methods**

5.3.1. **Sample material**

For this assay to be used to serotype AHSV, reference HRM curves for the nine AHSV serotypes would need to be generated for the specific reagents and equipment used. Due to the lack of a complete reference strain set, HRM curves for field strains can be generated, but can not be used for serotyping.

AHSV positive blood from two different horses, which had been identified previously by group-specific RT-qPCR was used for this study (H35 and H48).

5.3.2. **RNA Extraction**

Total RNA was extracted from the blood samples using the Quick-RNA™ Miniprep Plus Kit (Zymo Research, Irvine, USA), according to the manufacturer's recommendations: A volume of 500 µl of blood from each sample was transferred to a 2.5 ml microcentrifuge tube and an equal volume DNA/RNA Shield™ (2 X concentrate) was added to the sample and vortexed. An 8 µl volume of Proteinase K (20 mg ml^{-1}) was added to each mixture and incubated at room temperature for 30 minutes. To this, 1 ml of isopropanol was added and mixed by vortex. The mixture was transferred to a Zymo-Spin™ III-CG column (Zymo Research, Irvine, USA) in a collection tube and centrifuged at 15,000 x *g* for 30 s. To this column, 400 µl RNA Prep Buffer was added centrifuged at 15,000 x *g* for 30 s and the eluent was discarded. The column was then

washed twice with 700 µl RNA wash buffer containing ethanol and centrifuged at 15,000 x *g* for 2 minutes. The column was then transferred to an RNase-free tube. Final RNA was resuspended by adding 50 µl nuclease free water to the column followed by centrifugation at 15,000 x *g* for 30 s. The eluted RNA was either used immediately or split up and stored at -80 °C. Absorbance readings between the wavelengths of 220 and 350 nm of the extracted RNA were taken using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, USA) to assess the quantity and purity of the extracted RNA.

5.3.3. **Complementary DNA (cDNA) synthesis**

In a PCR tube, 2 µl of template RNA was added to 1 µl of random hexamer primer (100 µM) and 9 µl of nuclease-free water. This was incubated at 95 °C for 1 minute and transferred immediately onto ice. The mixture was combined with 4 µl of 5X reaction buffer, 1 µl RiboLock RNase Inhibitor (20 U μl^{-1}) (Thermo Fisher Scientific, Waltham, USA), 2 µl of 10 mM dNTP mix and 1 µl RevertAid M-MuLV Reverse Transcriptase (200 U μl^{-1}). The mixture was vortexed, spun down and incubated at 25 °C for 5 minutes followed by 42 °C for 60 minutes. The reaction was terminated by heating to 70 °C for 5 minutes. The cDNA was stored at -80 °C for further processing.

5.3.4. **Primers**

Standard VP7 primers (PrimerDesign, Southampton, United Kingdom) were used to confirm the presence of AHSV RNA in the test samples. Table 5.1 describes the primer sets used in this study, as previously described by Groenink (2014).

The S10 primers were designed to amplify a 181 bp product across all nine serotypes with differences in the amplicon sequences between primer binding sites depending on the serotype. These amplicon differences result in unique melt curves according to the serotype, allowing each sample to be placed into one of three clades (Table 5.2). A second set of primers, the clade-specific primers (A, B or C) can then be used to distinguish the serotype from the clade using discriminant analysis to compare the sample HRM data to reference data (Groenink, 2014). Due to the lack of reference strains, these melt curves would be generated but not used for serotyping.

Table 5.1 - List of primers used for the analysis of AHSV by HRM as defined in Groenink (2014)

	Name	Sequence (5'-3')	T_m (°C)	Size (bp)
Segment 10 (S10)	10_F	ATCCCTTGTCATGARTCTWGCT	57.7	181
	10_R	CTTGACATKGCTTGRTTAAGTATCC		
Segment 2 (S2): Clade-specific	A_F	ATTCACYATGGCTTCKGA	51.8	319
	A_R	CYACYCTTAYTTGRTTRTCATTTC		
	B_F	TTGGGTTGAWTGGGTYGT	54.9	249
	B_R	TTTGGRAACATYTGKGAWACDG		
	C_F	AGYGGNTGGMTYCCDTA	52.4	189
	C_R	CARTTYGARCCRATCCANG		

Table 5.2 - Serotypes per clade as defined in Groenink (2009)

Clade	Serotypes
A	3, 7
B	1, 2, 8
C	4, 5, 6, 9

5.3.5. **Nucleic acid amplification**

The protocol was the following for all primer sets: For a 20 µl reaction, 10 µl KAPA SYBR® FAST qPCR Master Mix (2 X), 0.8 µl forward and reverse primer (final concentration 400 nM), 6.4 µl nuclease-free water and 2 µl of cDNA template was combined in an extra-clear PCR tube. The tube was vortexed and spun down. The following PCR conditions were used: 95 °C for polymerase activation, followed by 40 cycles of 95 °C for 5 s for denaturation and 60 °C for 30 s for annealing and extension. HRM was subsequently performed from 60 °C - 95 °C at 0.1 °C 2 s^{-1}. PCR reactions were carried out in a Rotor-Gene™ 6000 (Qiagen, Hilden, Germany).

5.4. **Results**

PCR products for positive AHSV blood samples (Fig. 5.1) showed bands of suitable quality and size expected for both the VP7 and S10 regions.

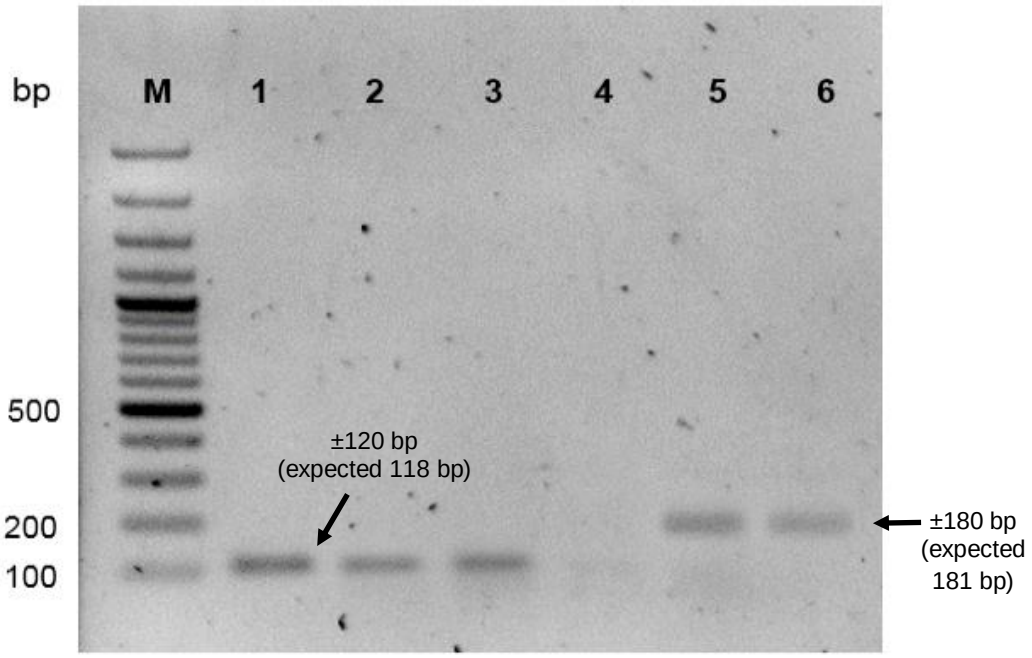
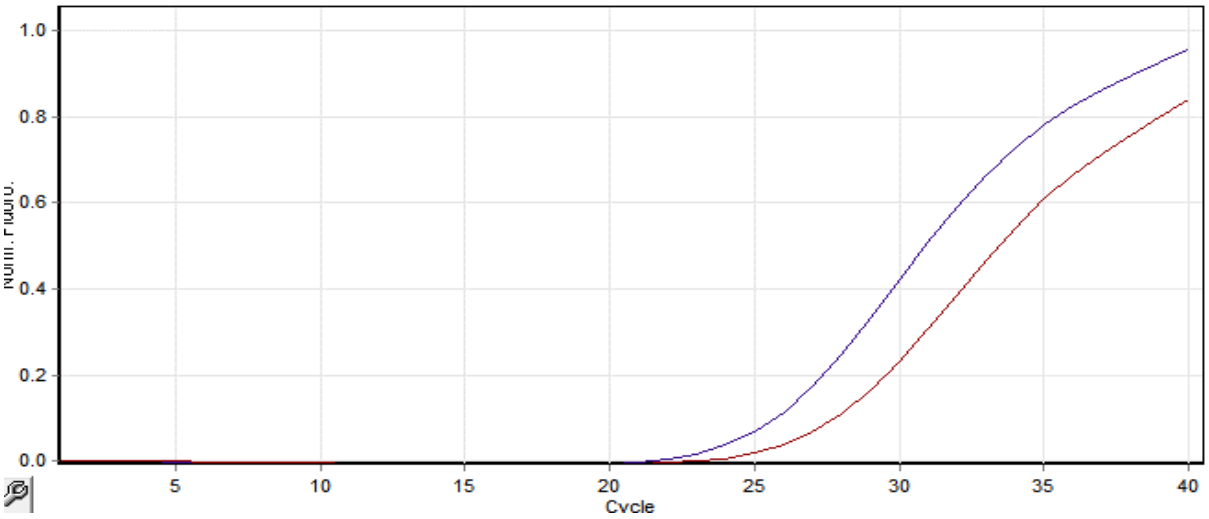


Figure 5.1 - Analysis of RT-PCR products of AHSV RNA using primers to amplify a 118 bp product of VP7 and a 181 bp product of S10. A 1.8% agarose gel was stained with Invitrogen™ SYBR™ Safe DNA Gel Stain. Lane M - Thermo Scientific™ GeneRuler™ 100bp DNA; Lane 1: H35 VP7; Lane 2: H48 VP7; Lane 3: PC VP7; Lane 4 – NTC VP7; Lane 5: H35 S10; Lane 6: H48 S10.

Fig. 5.2 is the graph of the normalised fluorescence from the qPCR of the cDNA from AHSV positive blood samples using the S10 primers. Amplification of product is shown from Cycle 22. The product was confirmed as being 181 bp by running a 1.8% agarose gel (Fig. 5.1).



■ - H35 ■ - H48

Figure 5.2 - Rotor-Gene™ 6000 data for the amplification of the 181 bp region of AHSV S10 extracted from AHSV positive samples using primers 10F and 10R.

Figure 5.3 is the HRM curves generated from the 181 bp fragment of S10. The two samples have similar curves both with double melt peaks indicating that the amplicon sequences from both samples are similar.

There was no amplification of either sample using primer sets A, B or C (data not shown).

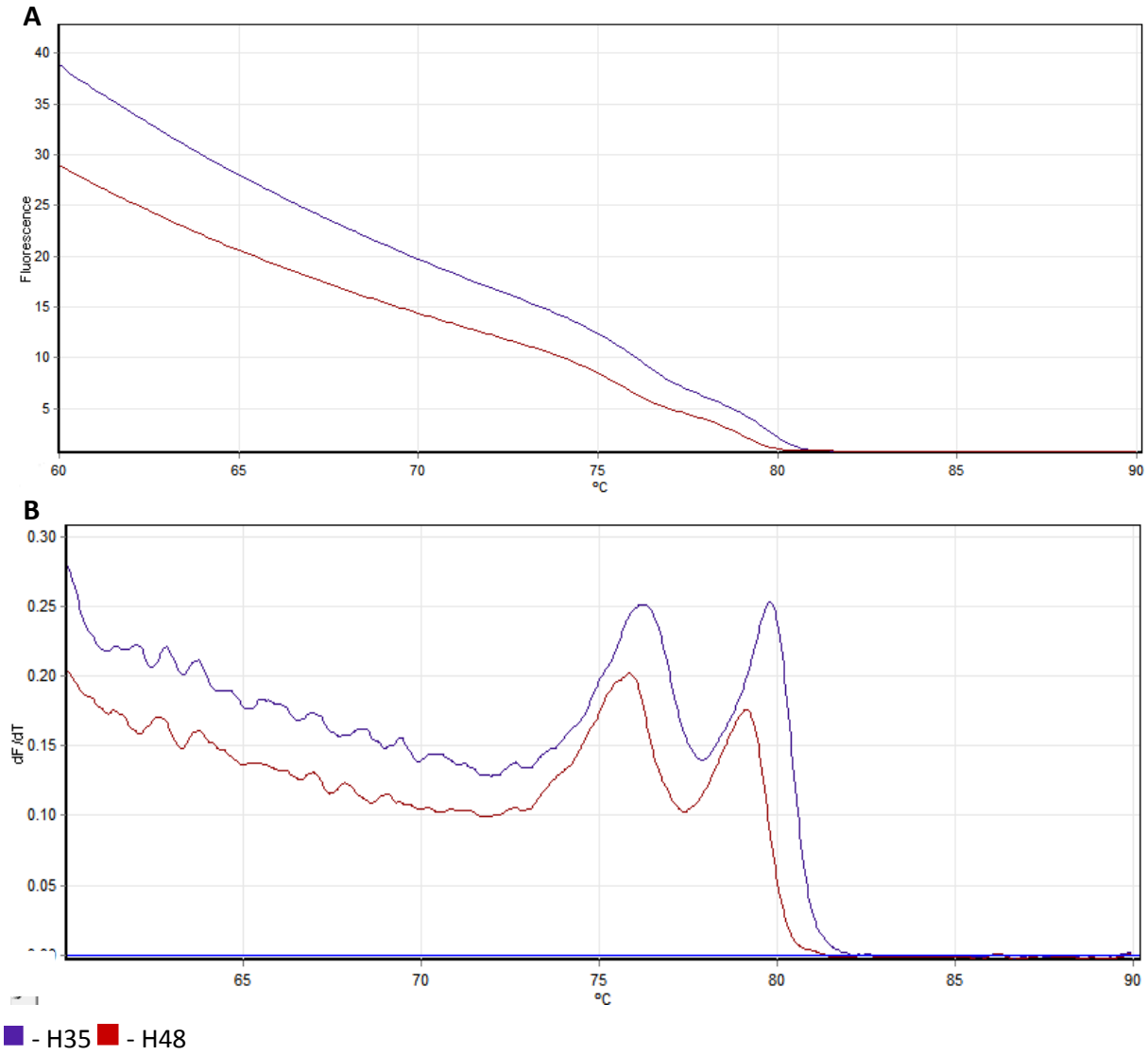


Figure 5.3 - Real-time RT-PCR and HRM results for the 181 bp region of S10. A: HRM curve. B: dF dT^{-1} of HRM curve.

5.5. **Discussion**

An advantage in using random hexamer primers with the described protocol during cDNA synthesis, as recommended by Quan *et al*. (2010), is that the cDNA can be stored and used with multiple primer sets or other nucleic-acid studies if required. This may be important when analysing index cases in AHS-free zones as multiple PCR primer sets can be used on the same cDNA stock. In addition, as cDNA is more stable than viral RNA, it might be a more robust alternative to the long-term storage of RNA sequences (Huang *et al*., 2017).

Primers outlined in this chapter (Table 5.1) were used in RT-PCR on selected AHSV positive blood samples. Internationally recognised VP7 primers were used as a

standard test to confirm the presence of AHSV RNA in these samples (Fig. 5.1). The S10 primers are designed to amplify a 181 bp region across all nine serotypes with differing sequences between the primer binding sites depending on the serotype. This allows HRM to detect differences in sequences of the amplified products that a gel would not be able to distinguish. S10 primers amplified a 181 bp region from both positive samples (Fig. 5.1). The successful amplification of target regions from both S10 and VP7 confirm the presence of AHSV. The HRM analysis of the 181 bp product revealed a double-peaked melt curve for each of the tested samples (Fig. 5.3b). This was most likely a result of multiple melt domains in the product because non-specific product amplification was not evident in the gel images (Fig. 5.1). The original study describing the primers only found double-peaked melt curves from serotypes in clade B (1, 2 or 8) (Groenink, 2014). Therefore, Clade B primers were expected to amplify a 249 bp product. No amplification was evident when using any of the clade-specific primer sets on the AHSV cDNA (Data not shown).

It is possible that sequence variation in genome Segment 2 prevented the amplification of the expected product. The successfully amplified regions (VP7 and S10) are reportedly more conserved and are unlikely to undergo significant genetic drift (Zientara *et al*., 2015). *Bluetongue virus* (BTV), the prototype species of the genus, has been extensively studied since its outbreak into Europe and 28 serotypes have been identified so far (Sun *et al*., 2016; Savini *et al*., 2017). Like AHSV, VP2 determines the serotype and is of relevance due to the increasing number of studies documenting the genetic reassortment of BTV (Shaw *et al*., 2013; Nomikou *et al.*, 2015).

There is also the possibility of a false positive caused by vaccine virus if the sampled horse was young and recently vaccinated. As the predominating vaccine used in South Africa is a multivalent vaccine, multiple serotypes would be present, all contributing the common genes (VP7 and S10), which are the targets for the group-specific assays. The serotype-specific (S2) genes would have been at a much lower level and may have been below the limit of detection. This result has been reported in previous AHSV serotyping studies where AHSV could be confirmed by VP7 confirmation but not serotyped by RT-qPCR targeting genome Segment 2 (Weyer *et al*., 2017).

Several other avenues were investigated as to the reason for the failed PCR including retesting using different reagent sets and reordering the primers from multiple

suppliers. The original study which described and tested these primer sets worked with relatively pure AHSV isolated from cell culture supernatant as opposed to blood stored in EDTA. Additional optimisations for the use with blood-derived samples will have to be investigated more before further conclusions can be made.

5.6. **Conclusion**

Before more conclusions can be made regarding these primers, further investigations into optimisations of the assay on fresh blood-derived samples need to be undertaken. If possible, these samples should be sourced from reference laboratories and be of a known serotype to avoid the problems that arise from sourcing wild-type viruses.

The possibility of genetic variation in Segment 2 of currently circulating field strains cannot be overlooked. Significant genetic variation in this genome segment is important because changes in it may result in several nucleic-acid based serotyping assays becoming obsolete or at least require re-evaluation.

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Dissertation Overview

African horse sickness (AHS) is a deadly disease affecting equids caused by African horse sickness virus (AHSV). The best practices for controlling AHS include vaccination and movement control. However, in epizootic outbreaks, these strategies do not provide adequate control. Molecular diagnostics of AHSV have proven to be successful in investigating previous outbreaks, but further development is needed for these tests to be useful in resource-limited endemic regions. There is also a growing concern regarding the use of live-attenuated vaccines in AHS control.

The research presented in this dissertation aimed to address several components related to the molecular diagnostics of AHS: 1) To review and summarise the available literature relating to AHS molecular diagnostics; 2) To investigate viraemia levels following routine vaccination of mares; 3) To investigate the suitability of dried blood spot filter paper technology for the sampling of blood for AHSV detection; 4) Generate high resolution melt data on current AHSV strains as part of a potential serotyping assay.

Chapter 2: A comparison of standard RT-PCR and RT-qPCR in the detection and quantification of AHSV

Major findings:

- There was a significant correlation between qPCR data and relative gel quantification of AHSV (R^2=0.768, P<0.01).

Implications:

The correlation between the C_q-values and the relative band density suggests the technique may be a valuable tool in quantifying viral load of AHSV where qPCR equipment is not available. Further optimisation and lab-specific calibration are required if this protocol was to be used.

Chapter 3: Investigation of the effect of a commercial live attenuated vaccine on viral load in mares

Major findings:

- Mares in this study, which have been annually vaccinated, showed no signs of viral RNA in the weeks following immunization.

Implications:

No detectable viraemia in the adult mares suggests that there is low risk for the live-attenuated vaccine to disrupt molecular diagnostics of AHS. Information on the age and vaccination history are therefore important considerations when analysing RT-qPCR data.

Chapter 4: Use of filter paper (FTA®) technology for sampling and recovery of African horse sickness virus RNA for molecular diagnostics

Major findings:

- Increasing the RNA elution time from 30 mins to 24 hours and using TE-buffer for RNA extraction allowed for the successful extraction of AHSV RNA.
- AHSV RNA was detected from blood stored on FTA® cards for 1 week at room temperature.

Implications:

Dried blood spot filter paper technology may be useful for AHSV diagnostics, especially in remote endemic areas. The aim of this study was to develop a protocol for the storage and recovery of AHSV RNA from FTA cards. A combination of having a 24-hour RNA elution time and using TE-buffer allowed for the successful extraction of AHSV RNA.

Chapter 5: HRM analysis of melt curves

Major findings:

- AHSV was detected using a novel primer set, targeting genome Segment 10 (S10). This primer set has up until now not been used on wild AHSV RNA extracted from blood.
- High-resolution melt curves were generated from the PCR products of the 181 bp S10 fragment.

- There was no RT-qPCR amplification when using three type-specific primer sets designed to amplify regions of Segment 2.

Implications:

The failure of the RT-qPCR assay indicates that further research into this assay is required. Future work should address the following:

- As this is the first study using this primer set on blood-derived samples, PCR conditions may be improved for these sample types.
- There could be a substantial genetic variation in Segment 2 between the current wild-type AHSV strains and the reference strain sequences on which the primers were based. Genetic variation over time can cause antigenic differences in viruses which may also make current diagnostic assays obsolete. Revaluation of these primer sets may be necessary to be in line with current reference strain sequences.
- The vaccination status and age of the animals from where the AHS positive blood originated was unknown. These may have been young animals receiving their first multivalent live-attenuated vaccine. If this was the case, conserved regions (VP7) which are present in all serotypes may have been detected by the standard RT-qPCR, but the serotype-specific regions (VP2), targeted by the primers used in this study, may have been below the limit of detection. If serotyped positive blood samples could be sourced from reference laboratories it will eliminate the problems that arise with sourcing these positive samples from wild cases.

Appendix

Appendix 1 – Ethical clearance certificate for the vaccination trial at UKZN

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

16 May 2016

Dr Marion Belinda Young
School of Agricultural, Earth & Environmental Sciences
Pietermaritzburg Campus

Dear Dr Young,

Protocol reference number: AREC/023/016
Project title: Field trial application of Groenik (2014) for molecular serotyping of African Horse sickness virus

Full Approval – Research Application

With regards to revised your application received on 11 May 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 16 May 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Dr Sanil Singh
Deputy Chair: Animal Research Ethics Committee

/ms

Cc Dean & HoS: Professor Albert Modi
Cc Academic Leader Research: Professor Onisimo Mutanga
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc Ukulinga Farm – Ms Alet Botha

Animal Research Ethics Committee (AREC)
Ms Marlette Snyman (Administrator)
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Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix 2 – Letter of no objection from state vet regarding AHS research in KwaZulu-Natal

agriculture
& rural development

Department:
agriculture
& rural development
PROVINCE OF KWAZULU-NATAL

To Whom It May Concern

Letter of no objection concerning African horse Sickness research at the University of Kwazulu Natal, reference number AREC/023/016

The Umgungundlovu district of the province of KwaZulu-Natal is located in the African Horse Sickness (AHS) Infected Zone, that is to say, neither the controlled zone nor the free zone. There are usually a number of cases during the summer months and at times movement of horses to the Western Cape is not permitted in order to reduce the risk of bringing the disease into the protected area.

I have no objection to blood samples being taken from horses suspected of having African horse sickness for research purposes and tested at UKZN, provided the horses themselves are not transported and provided that it is understood by the owner that a negative test result will not constitute permission to move the horse.

For more information on this matter please contact me.

Sincerely

Dr Allen Still
State Vet Umgungundlovu District
0825753929
0333476245
Allen.Still@kzndard.gov.za

REPUBLIC OF SOUTH AFRICA
DEPARTMENT OF AGRICULTURE
OFFICIAL VETERINARIAN
16 JAN 2017
DR A.D. STILL
REG NO: 014/11179
PROVINCE OF KWAZULU-NATAL

Appendix 3 – Permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No 35 of 1984)

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/5

Dr M.B Young
University of KwaZulu Natal
Tel: 082 419 3380
Email: youngm@ukzn.ac.za; 211529753@stu.ukzn.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Dr Young

Your application sent with the email on 3 February 2017 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. No part of the study may be performed unless valid ethical approval has been obtained from the relevant South African authority in writing;
4. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
5. Written consent from the owners of animals to be used in the study must be obtained before sampling;

6. This permit is only valid for sampling in the areas of Kwa-Zulu Natal province that have supplied a state veterinary letter of no restriction to the researcher and DAFF. No sampling may take place in any other area or province without written permission from the Director of Animal Health. Please apply in writing to HerryG@daff.gov.za;
7. Samples must be packaged and transported in compliance with the National Road Traffic Act 1996 (Act no 93 of 96);
8. African horse sickness (AHS) positive control samples may only be sourced from Deltamune (Pty) Ltd and only local field strains of AHS may be used;
9. As AHS is a controlled disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84) and the research test is not performed in a DAFF approved laboratory, the results may not distributed as a possible diagnostic test result to anyone other than the responsible State Veterinarian;
10. All potentially infectious material utilised or generated during or by the study is to be destroyed at completion of the study by incineration;
11. Only a registered waste disposal company may be used for the removal of waste generated by or during the study;
12. Records must be kept for five years for auditing purposes;

Title of research/study: Application of Groenink 2014 for the molecular serotyping of AHS virus.
Researcher: Dr M.B Young
Institution: Cytology Lab 244, Agricultural Campus, University of KwaZulu Natal.
Our ref Number: 12/11/1/1/5
Your ref: N/A

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -03- 0 1

Table 3.2 - Group-specific RT-qPCR results for the mares over the 56-day study period. The C_q-value obtained for the NTC of that run is included. N indicates that the fluorescence threshold was not reached.

Horse Name	Sampling time (day)	C_q	C_q Replicate	NTC C_q	Positive Control C_q
1. Thombi	0	34.86	36.02	32.38	23.00
	7	36.72	37.26	32.38	23.00
	28	38.38	39.86	32.38	23.00
	35	N	N	32.38	23.00
	56	N	39.10	32.38	23.00
2. Flikka	0	35.97	36.37	36.34	23.01
	7	34.05	35.26	36.34	23.01
	28	37.96	37.79	37.06	23.05
	35	37.28	37.59	37.06	23.05
	56	36.79	N	37.06	23.05
3. Spikkle	0	36.75	36.56	36.34	23.01
	7	34.74	36.69	36.34	23.01
	28	37.28	39.10	37.06	23.05
	35	37.48	37.07	37.06	23.05
	56	36.69	35.67	37.06	23.05
4. Lily	0	34.62	35.22	36.34	23.01
	7	34.43	35.88	36.34	23.01
	28	37.27	36.39	37.06	23.05
	35	35.68	N	37.06	23.05
	56	N	34.98	37.06	23.05
5. Peppa	0	34.08	35.31	36.34	23.01
	7	34.90	38.83	36.34	23.01
	28	37.19	35.58	37.06	23.05
	35	36.96	36.50	37.06	23.05
	56	36.48	36.51	37.06	23.05
6. Paska	0	36.31	34.43	36.35	23.01
	7	38.46	38.05	32.38	23.00
	28	35.89	34.84	37.06	23.05
	35	34.92	35.01	37.06	23.05
	56	N	N	37.06	23.05
7. Storm	0	37.37	36.94	32.38	23.00
	7	37.61	36.44	32.38	23.00
	28	37.53	35.25	37.06	23.05
	35	37.26	37.97	37.06	23.05
	56	37.56	39.10	37.06	23.05
8. Donkey	0	38.02	37.50	32.38	23.00
	7	N	35.05	36.35	23.02
	28	34.11	36.76	36.35	23.02
	35	36.57	33.93	36.35	23.02
	56	35.29	34.00	36.35	23.02
9. Zena	0	34.95	33.03	32.38	23.00
	7	34.44	35.89	36.35	23.02
	28	34.09	35.32	36.35	23.02
	35	34.92	38.85	36.35	23.02
	56	36.32	34.45	36.35	23.02