

Identifying B-cell epitopes of African horse sickness virus serotype 4 recognised by antisera of immunised horses

By

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Declaration

I, Evans Mantiri Mathebula, hereby declare that the work contained in this thesis that I hereby submit for the degree of Magister Scientiae to the University of Pretoria, is my own work and has not been previously submitted by me to this or any other tertiary institution.

Evans Mantiri Mathebula

Date

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List of Abbreviations

°C	Degrees Celsius
AHS	African horse sickness
AHSV	African horse sickness virus
Amp	Ampicillin
ARC-OVI	Agriculture Research Council Onderstepoort Veterinary Institute
ATP	Adenosine triphosphate
Balb/C	Laboratory-bred albino mouse strain C
BHK	Baby Hamster Kidney
bn	Billion
bp	Base Pairs
BTV	Bluetongue virus
cDNA	Complementary Deoxyribonucleic Acid
CFU	Colony Forming Units
CLP	Core-Like Particles
CTL	Cytotoxic T-lymphocyte
DAFF	Department of Agriculture, Forestry and fisheries
DAS-ELISA	Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay
DIVA	Differentiate between Infected and Vaccinated Animals
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
dsRNA	Double stranded Ribonucleic Acid
<i>E. coli</i>	Escherichia coli
EEV	Equine Encephalosis Virus
EHDV	Epizootic Haemorrhagic Disease Virus
ELISA	Enzyme-Linked Immunosorbent Assay
G+C	Guanine plus Cytosine

HRP	Horseradish Peroxidase
IFNAR-/-	Interferon Alpha Receptor knock-out
IgG	Immunoglobulin G
Kan	Kanamycin
Kb	Kilobase
LB	Luria Bertani
Ltd	Limited
min	Minute/s
MOI	Multiplicity of Infection
MP	Milk Powder
mRNA	Messenger Ribonucleic Acid
MVA	Modified Vaccinia Ankara
N	Normal
NGS	Next generation sequencing
nm	Nanometre
NMR	Nuclear Magnetic Resonance
NS	Non-structural protein
<i>ns</i>	Segment encoding non-structural protein
OBP	Onderstepoort biological products
OD	Optical density
OIE	The World Organisation for Animal Health
OPD	o-Phenylenediamine dihydrochloride
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pfu	Plague forming units
qPCR	Real-time polymerase reaction

RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
s	Second/s
TMB	Tetramethyl benzidine
TMVP	Tobacco Mosaic Virus protein
TUP	Target unrelated peptide
TY	Tryptone yeast
TYE	Tryptone yeast extract
U	Units
v/v	Volume per Volume
VIBs	Viral inclusion bodies
VLP	Virus-Like Particles
VNT	Virus neutralisation tests
VP	Viral Protein Number

Summary

Identifying B-cell epitopes of African horse sickness virus serotype 4 recognised by antisera of immunised horses

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African horse sickness is an infectious, insect-borne but non-contagious disease of equids responsible for more than 95% mortality in naïve horses. The African horse sickness virus has ten segments of double stranded RNA encoding seven structural and three non-structural proteins. There is currently no cure for the disease and one of the effective ways to control or prevent new infection is by vaccination. Although successful, the currently used vaccine is not registered for use outside the sub-Saharan region because of fears of reversal to virulence. Inactivated and subunit vaccines have been tested but are not commercially available. Research towards recombinant vaccines that will offer protection without these limitations is thus ongoing. Identification and characterisation of antigenic regions on proteins has always been essential in the development of vaccines and immunodiagnostic reagents. AHSV proteins have been shown to confer protection to model animals in

one form or another. This study was undertaken to investigate the global humoral immune reaction during immunisation in horses. This was done using sera from immunised horses and a genome-targeted phage display library. A phage library expressing a repertoire of AHSV-4 peptides large enough to represent the whole genome was constructed. The library was affinity selected with purified naïve (day 0) and immunised (day 28, day 52) horse IgGs and a pool of phages expressing binding peptides were isolated. The DNA inserts of the pool of phages were subjected to high throughput sequencing and sequences identified by matching them to the AHSV-4 genome. Analysis of the extensive data output selected by the naïve and immunised IgGs enabled a thorough examination of the panning process. Sequences were normalised by subtracting the naïve from the immunised sequences. Comparing the sequences selected with day 28 IgG enabled identification of 16 potentially antigenic regions recognised by most horses. Some of these regions could easily have been missed with the traditional clone picking approach. Some of the regions were also selected by most horses with day 52 IgG, indicating persistent antibodies. Potentially antigenic regions were identified without the functional re-testing which is common part of traditional phage display. This was due to lack of access to the single clones in the library. One way to confirm binding of selected regions is by peptide ELISA, which was started in this study. This study improved on all the phage display work already done on orbiviruses and offered more information on the immunogenicity of AHSV-4 in horses. Future work should focus on confirming antigenicity of the selected regions. The potential antigenic regions could be fused with T-cell epitopes, identified in a parallel study, to form a construct that might induce production of humoral and cellular immune responses.

CHAPTER 1: LITERATURE REVIEW

1.1. Importance of horses

The importance of horses in economic development dates back to antiquity when they were used for transport, military operations, draught power in agriculture and mining (Coetzer and Guthrie, 2004). Horses were in some sense the backbone of the economy until the introduction of mechanised transport. Horses are now still used in farming, entertainment and in recreational performances which include among others activities associated with racing where large prices are won (Biffa and Woldemeskel, 2006). Therefore, anything that has a negative impact on the well-being of horses threatens the economic and social development of a country. Animal diseases, such as African horse sickness (AHS) continue to affect the health of horses. The disease continues to thrive in South Africa, where it is endemic because of the availability of vectors and reservoirs like donkeys and zebras. As a result, restrictions in trading with international horses and limiting South African horses to compete internationally has had an impact on horses' sale price and ultimately the national economy, where the equine industry contributes R2.71 bn to Gross Domestic Product (Standish *et al.*, 2011).

1.2. African horse sickness

1.2.1. Hosts

African horse sickness (AHS) is an infectious but non-contagious and deadly viral disease transmitted by blood sucking insects of the species *Culicoides* (Mellor, 1993). It affects horses, mules, donkeys, and zebras with a varying degree of severity (Barnard, 1993). It was once regarded as the most dangerous viral disease of horses

(Coetzer and Erasmus, 1994). Naive horses are the most susceptible of the equid family with mortality rate exceeding 95% (Coetzer and Guthrie, 2004). Susceptibility is significantly less in mules (50%), as low as 10% in donkeys and almost asymptomatic in zebras (Barnard, 1993; OIE, 2012). Zebras are regarded as natural reservoirs after almost 100% of them tested in Tanzania and the Kruger National park were found to have antibody titers despite being asymptomatic (Barnard, 1993). Apart from equids, antibodies to African horse sickness virus (AHSV) were detected in dogs, goats, elephants, camelids and buffalo with immunoassays (Awad *et al.*, 1981; Lubroth, 1992; van Sittert *et al.*, 2013). However, although antibodies have been isolated in these non-equid species, their role in disease transmission had not been proven (McIntosh, 1955, CFSPH, 2015). African horse sickness is listed as a notifiable disease by the World organisation of Animal health (OIE) because of its ability to cause widespread death to susceptible equid populations and its impact on animal trade for affected countries worldwide (OIE, 2012).

1.2.2. AHS in South Africa

Although AHS is endemic and widely distributed throughout Africa and has been present for centuries, South Africa was free of the disease until the introduction of horses after the arrival of the first settlers of the Dutch East India Company of Good Hope in 1652 (Henning, 1956). They brought horses and donkeys which were not indigenous to South Africa and therefore naïve. An outbreak of AHS occurred in 1719, claiming approximately 1700 horses (Theiler, 1921). It was not until in 1853 -1855 that interest in the disease grew, after the largest outbreak which almost crippled the horse industry, with death of horses equalling 70 000 in the Cape of Good Hope (Bayley, 1856).

Other less severe outbreaks have occurred in South Africa since and data from 1955 to 1995 showed that the northern part had one outbreaks in summer every five to ten years, and very rare in the western part (Bosman *et al.*, 1995). The Western Cape is the least affected area in the South Africa and an AHS controlled area was established in agreement with the European Union, which consisted of the AHS free zone surrounded by the surveillance zone and protection zone to allow horse trade with the world (Fischler, 1997). In this demarcation, animals in the surveillance and free zone are unvaccinated. Only animals in the protection zone are vaccinated to ensure protection of the inner naïve zones. An animal infected in the surveillance zone signals an outbreak. Only animals in the AHS free zone are exported. Outbreaks in the surveillance zone have so far been reported in 1999, 2004 and 2011 (Bell, 1999; Sinclair *et al.*, 2006; Grewar *et al.*, 2013). Another outbreak occurred in 2014 also in the protection zone (Grewar, 2014). These outbreaks were attributed to illegal movement of horses to that area which cast doubt on the implementations of the control measures in place mentioned by Guthrie (1999). The other possibility was however, that the outbreak may have been initiated by movement of infected midges, which can be transported by prevailing winds for hundreds of kilometres and are difficult to control (Sellers *et al.*, 1977). The attack on this region by AHS is particularly worrying as outbreaks in a surveillance zone, leads to an embargo on the export of horses from an affected country and consequently leads to economic loss (Venter *et al.*, 2006). Almost all AHS outbreaks in South African were linked to the timing of the warm-phase El Nino/Southern oscillation, which brings about rainfall and drought, a perfect breeding ground for the vectors (Baylis *et al.*, 1999).

1.2.3. AHS worldwide

The disease has proven to be able to cross borders and cause disease outside sub-Saharan region. Outbreaks have been reported in countries previously AHS-free, like India (Henning, 1956), Spain during 1987-1987, Portugal and Morocco in 1989-1991 (Rodriguez *et al.*, 1992). The Spanish outbreak was attributed to importation of an infected zebra from Namibia (Lubroth, 1988). The most severe outbreak that occurred outside Africa, spread from the middle east, Pakistan, India, Saudi Arabia to Syria accounting for almost 300 000 deaths in 1959-1961 (Anwar and Qureshi, 1972). AHS outbreaks also occurred in Ethiopia in 2003, Senegal and Nigeria in 2007 and Namibia in 2011 (Sabirovic *et al.*, 2008; Molini *et al.*, 2015).

1.3. African horse sickness virus

African horse sickness virus (AHSV) belongs to the genus *Orbivirus* in the family *Reoviridae* (Verwoerd *et al.*, 1979) and infects the respiratory and gastric enteric organs (Urbano and Urbano, 1994). The virus shares similar properties to orbiviruses like bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV) and also with equine encephalosis virus (EEV) (Verwoerd *et al.*, 1979; Spence *et al.*, 1984). Although AHSV has not been shown to cross-react with any other closely related orbiviruses (OIE, 2012), its prototype the BTV cross-reacts with EHDV in agar gel immunodiffusion (AGID) and complement fixation assays (OIE, 2012). AHSV consist of nine worldwide recognised antigenically different serotypes confirmed by using serum virus neutralisation test in mice (Theiler, 1921; McIntosh, 1956; Howell, 1962). AHSV serotypes were identified and differentiated by the specificity of interactions between outer capsid protein (VP2) and neutralising antibodies in virus neutralisation assays and homology (Figure 1.1) (Howell, 1962; Potgieter *et al.*, 2003), but shared a

common complement fixing antigen (Gorman, 1979). Cross-reaction has been observed between AHSV serotypes 1 and 2, 3 and 7, 5 and 8, and 6 and 9 in serum neutralisation tests (Howell, 1962; Coetzer and Erasmus, 1994; Potgieter *et al.*, 2003; OIE, 2012). This cross-reaction informed the grouping of the currently used live attenuated vaccine discussed later.

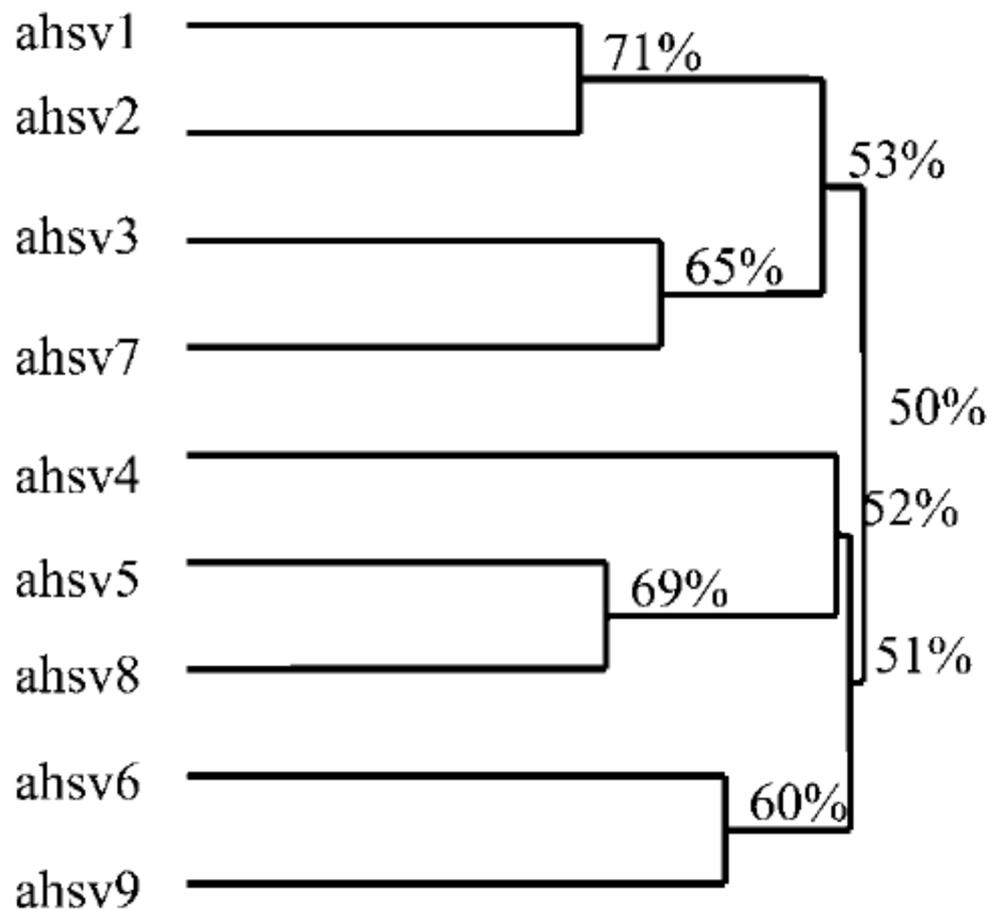


Figure 1.1: AHSV homology of serologically cross-reacting serotypes based on antibodies against VP2. Percentage homology between serotypes is indicated (Potgieter *et al.*, 2003).

The virus is transmitted between vertebrate hosts by certain adult female *Culicoides* midges of the family *Ceratopogonidae*, and replicate in both hosts (Roy, 1996). The principal vector is reported to be *Culicoides imicola* but *C. bolitinos* and *C. oxystoma*

have also been shown to transmit AHSV in the field (Meiswinkel, 1997; Fall *et al.*, 2015). AHSV has also been isolated in *C. pulicaris*, and *C. obsoletus*, but their potential to transmit the disease has been minimal (Mellor *et al.*, 1990). *C. pulicaris* and *C. obsoletus* have been reported to be competent in transmitting the closely related BTV (Boorman *et al.*, 1975., Mellor *et al.*, 1990). This may suggest that the ability of the different *Culicoides* vector species to transmit particular viruses may be geographically dependent (Mellor *et al.*, 1990). The insects become infected when they feed on sick animals during febrile and viraemic stages (Bosman *et al.*, 1995). Other potential vectors with the capability of carrying the virus include ticks, phlebotomine sand-flies and mosquitoes but their involvement in transmitting the virus has not yet been proven (Calisher and Mertens, 1998).

1.3.1. Viral particle structure

The purified structure of AHSV is similar to its prototype orbivirus of sheep, bluetongue virus (Murphy *et al.*, 1971). The first structure of an orbivirus was first correctly described for the complex BTV type 10 using electron microscopy and negative staining (Verwoerd *et al.*, 1972). The virion is a non-enveloped particle, 60-80nm in diameter and is made up of a two layered icosahedral capsid composed of 32 capsomeres (Bremer, 1976). It contains structural and non-structural proteins of different lengths and sizes surrounding the genome. Figure 1.2 illustrates the orbivirus structure (Wilson *et al.*, 2009).

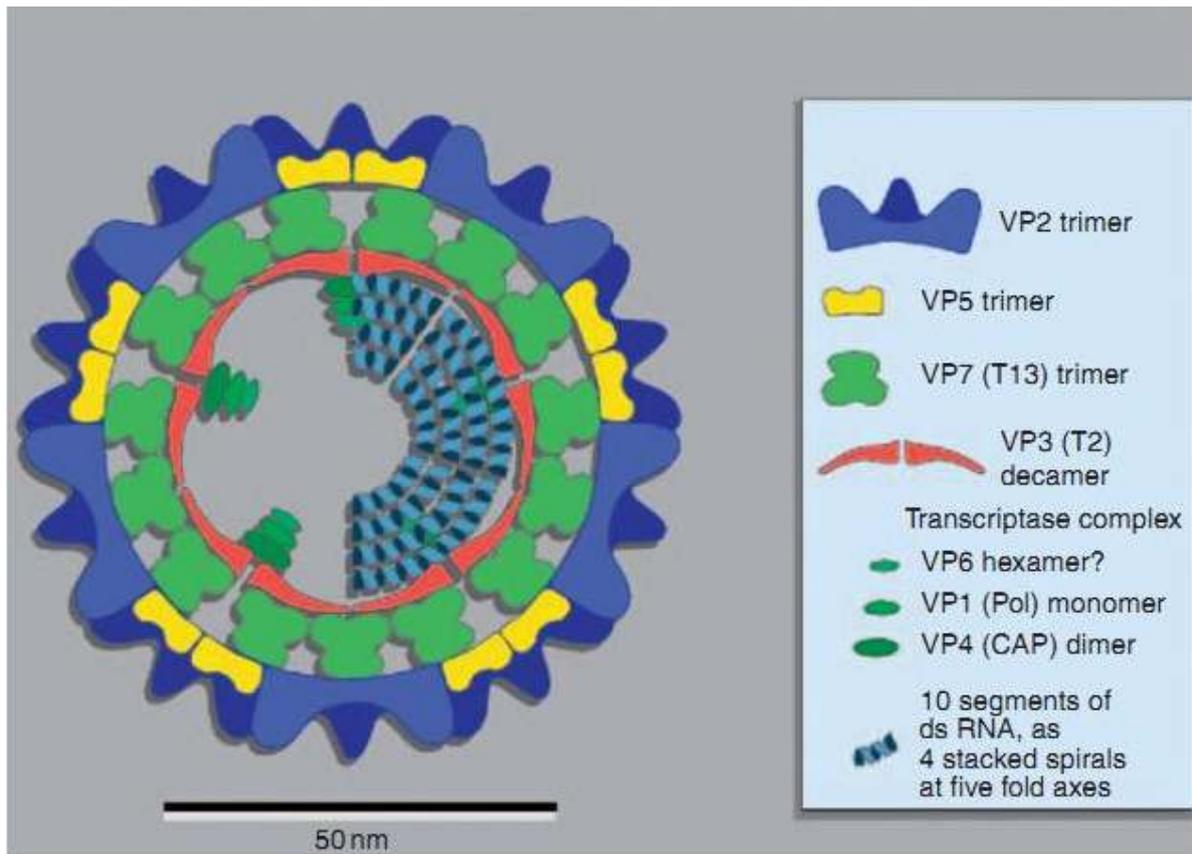


Figure 1-2: Diagram of the Orbivirus structure. Figure published in *Virus taxonomy: VIIIth report of the international Committee on Taxonomy of Viruses*, Mertens P.P.C., Attoui H., Duncan R., Dermody T.S. (Eds), Reoviridae, pp. 447-454, © Elsevier. Adapted from Wilson *et al.*, 2009.

The outer capsid consists of two major proteins, VP2 and VP5, which dissociate when the virus passes the cell membrane (Basak *et al.*, 1996). Iodine labelling experiments showed that VP2 is the major protein which is more exposed on the surface of the virion when compared to its association with VP5 (Lewis and Grubman, 1991). VP2 determines the different serotypes and together with VP5 are responsible for cell attachment, entry and membrane destabilisation and thus are targets for virus neutralisation activity (Martinez-Torrecuadrada *et al.*, 2001). They are critical in the initiation and severity of pathogenesis in the host (Huismans *et al.*, 2004). Protease treated virions generate infectious subviral particles which contain VP2 and are infective to mammalian cells like BHK cells (Burroughs *et al.*, 1994). The inner layer

(core particle) consists of major proteins VP3 and VP7 and encloses minor structural proteins VP1, VP4, VP6, non-structural proteins NS1 – NS3, and the ten segmented viral dsRNA genome. VP3 stabilised by VP7 anchors the three minor structural proteins forming the virus core (Grubman and Lewis, 1992; Mellor and Mertens, 2008). VP3 has also been shown to be conserved among the orbiviruses by sequence analyses (Roy *et al.*, 1994). VP7 is the major component of the inner layer of the core surface when compared to VP3 (Gould and Hyatt, 1994, Belhouchet *et al.*, 2011). All viral proteins have been sequenced for all nine AHSV serotypes (van Schalkwyk, unpublished). The VP7 sequence is highly conserved between all the nine serotypes thus has been used as a group-specific determinant and targeted for diagnostic purposes (Chuma *et al.*, 1992; Laviada *et al.*, 1992). The three minor structural proteins (VP1, VP4, and VP6) perform enzymatic activities that lead to the preparation and packaging of viral mRNA. VP1 is a RNA polymerase, VP4 a capping enzyme and VP6 is a helicase enzyme. These enzymes are essential for virus propagation, both in the vector and vertebrate hosts (Wilson *et al.*, 2009; Manole *et al.*, 2012). Non-structural proteins (NS1, NS2, NS3 and NS3a) are assisting proteins found in abundance during early infection (NS1, NS2), suggesting that they are essential in viral replication (Roy *et al.*, 1994). NS1 forms long tubules within the cytoplasm during infections that are characteristic of orbivirus infection (Mellor and Mertens, 2008). NS2 is said to be the major component of viral inclusion bodies (VIBs) representing the major site of viral RNA synthesis and particle assembly during replication of orbiviruses (Mellor and Mertens, 2008). NS3 and NS3a are found in high abundance on the cell membrane of host cells and are active in influencing the timing of virus particle release (Hyatt *et al.*, 1993; Van Staden *et al.*, 1995, O'Hara *et al.*, 1998). Information on function of recently identified non-structural protein NS4 is inconclusive

and ongoing (Belhouchet *et al.*, 2011). Core-like particles (CLPs) and virus-like particles (VLP) formation by BTV major capsid proteins in baculovirus expression systems in insect cells show that CLP formation by VP3 and VP7 is self-primed and although VP2 and VP5 can interact independently with preformed cores to form VLP, they fail to assemble to form CLPs in the absence of the scaffold (Maree *et al.*, 1998). Although there are limited reports on AHSV virion assembly, VP7 of AHSV is said to be highly insoluble (Roy *et al.*, 1991; Basak *et al.*, 1996). The majority of VP7 is sequestered into distinct flat crystalline-like structures irrespective of other proteins and is thus not available for virion assembly (Basak *et al.*, 1996; Bekker *et al.*, 2014). This may indicate one difference between the virion genes and assembly between BTV and AHSV which are thought to be very similar. The core of AHSV has been reported to be as infective as the complete virion when tested on certain cell types (Mertens *et al.*, 1996).

1.3.2. Genome

The genome of orbiviruses, described the first time by Verwoerd (1970) for BTV, comprises of 10 linear dsRNA segments encoding structural and non-structural proteins (Grubman and Lewis, 1992; Mertens *et al.*, 2005). The segments differ in length and size and are designated S1-S10 (Grubman and Lewis, 1992) based on their migration on polyacrylamide gel electrophoresis (PAGE) (Roy *et al.*, 1994). Migration profiles of all nine AHSV serotypes on 7% PAGE in Laemmli buffer systems show high variability among serotype in S2 and S10 which may suggest high variability in sequences (Bremer *et al.*, 1990).

Each dsRNA segment has a single open reading frame which encodes one protein product with the exception of S10, which contains two in-frame open reading frames which encodes two small non-structural proteins ~20 amino acids apart NS3/3a (Mertens *et al.*, 1984; van Staden and Huisman, 1991; Roy *et al.*, 1994). Recently another non-structural protein NS4, coded by S9 which also codes for VP6, has been described using immunohistochemistry and bioinformatics analysis (Belhouchet *et al.*, 2011; Zwart, *et al.*, 2015). The function of NS4 is unclear and still under investigation. The genome makes up 20% of the viral particle weight and its G+C content ranges from 42 – 44% (Gould and Hyatt, 1994). Using data derived with AHSV- 4, 6 and 9 genome analysis, sizes of the different AHSV fragments and their coding protein were determined (Table 1) (Bremer *et al.*, 1990; Roy *et al.*, 1994).

Table 1.1: Sizes of full length dsRNA genome segments and corresponding encoded protein length (Bremer *et al.*, 1990; Roy *et al.*, 1994).

Segment	Base pairs (bp)	Encoded proteins	Amino acids (aa)
S1	~3500-3965	VP1	1305
S2	~3205-3229	VP2	1053
S3	~2792	VP3	905
S4	~1987-1987	VP4	642
S5	~1748-1751	NS1	548
S6	~1566	VP5	505
S7	~1169-1179	VP7	354
S8	~1167	NS2	365
S9	~1100	VP6	369
S10	~756	NS3/3a	203/217

Transcription regulation rate of segments remains fairly constant for most viral proteins, with variation observed *in vitro* and *in vivo* for S5 which is expressed at twice the frequency of S2 and S10 expressed at the lowest rate, half the rate of the other proteins (Huisman, 1979). This could be the reason why NS1 (S5) is in high abundance during early stages of infection, indicating that it may be critical for initiation and propagation of disease. African horse sickness virus has a RNA polymerase dependant genome of which VP1 which has been proven to be an RNA polymerase (Vreede and Huisman, 1998; Bachanek-Bankowska *et al.*, 2014). Since it is a polymerase, its function is dependent on the temperature being between 27 and 42 °C and it is inactive at 12-15 °C (Wilson *et al.*, 2009). During infection, the genome of AHSV never leaves the viral core. Transcription of the genome continues in the core particle extruding multiple capped single stranded mRNA transcripts of each genomic segment into the host cytoplasm (Martin and Zweerink, 1972, Verwoerd *et al.*, 1972; Basak *et al.*, 1997). The mRNA is then used for synthesis of not only viral proteins, but also act as templates for synthesis of genomic dsRNA segments for progeny particles (Kaname *et al.*, 2013). Viral proteins, VP1, VP3, VP4 and VP6 are found associated with the genome and are cardinal in transcription of the genome. These proteins are largely concerned with core organisation, transcription, capping replication and packaging of viral RNAs (Wilson *et al.*, 2009).

Sequence variation and conservation has been reported among the different orbiviruses and within serotypes. A variable nucleotide sequence often leads to a variable protein product. A striking feature within the orbiviruses is the high conservation within the 5' and 3' terminal nucleotides of their mRNA strands. Using AHSV serotype 4 (AHSV-4) sequence genome analysis, 5' terminal sequences consists of 5'GUU^AUA....3' and the 3' consists of^AcC^AU^AC3' and the consensus

sequence is 5'GUUA/UAA/U.....ACA/UUAC3' (Roy *et al.*, 1994). These conserved 5' termini regions of the segments may serve as recognition signals to initiate transcription and RNA packaging (Rao *et al.*, 1983). Phylogenetic analysis of VP2 of the nine AHSV serotypes indicated a homology variation as high as 71% (Potgieter *et al.*, 2003). High variable regions were found to be in amino acid sequence regions associated with antigenicity and that play a role in virus neutralisation in AHSV-3 (Bentley *et al.*, 2000), AHSV-4 (Venter *et al.*, 2000) and AHSV-9 (Martinez-Torrecedrada *et al.*, 2001). Using sequencing results and hybridization analysis, Roy and colleagues (1994) reported that apart from S2 (VP2), other segments like S10 (NS3/3a) and S6 (VP5) are also variable among AHSV serotypes. Segment 1 and 3 have been found to be the most conserved and have been used as virus-species or serogroup specific antigens to develop a real-time assay for rapid, sensitive and reliable detection and typing of AHSV (Bachanek-Bankowska *et al.*, 2014). Similar work was previously reported by Sakamoto and colleagues (1993) where they developed a sensitive reverse transcriptase-polymerase reaction diagnostic reagent for AHSV VP3 and NS1. However, the most conserved segment among different serotypes is segment 7 and VP7 is used as diagnostic reagent. Segments which code for NS1 and NS2 (S5 & 8) are conserved to some degree as established using sequencing and hybridization analysis (Roy *et al.*, 1994).

Genome segment reassortment is one of the mechanisms, unique to segmented viral genomes, which is used for virus evolution. It occurs when two or more orbiviruses of the same serogroup infect a single cell or host (Mertens, 1994). The genome is transcribed into mRNA and transported in the cytoplasm for the synthesis of proteins and assembly of progeny viral particle. During this assembly of viral inclusion bodies, segment exchange may occur to give rise to a different viral particle genetically.

However, no reassortment has ever been observed with different species in the orbivirus group (Williams *et al.*, 1998). Genome reassortment could also result from genome interaction between live vaccine and wild-type viruses in either vector or mammalian host, to produce a different virus which could produce even enhanced virulence characteristics, leading to a more severe pathogenesis than before (Mellor and Hamblin, 2004). Rate of reassortment has been reported to be 6 times higher in insect when compared with vertebrate hosts (Mellor and Hamblin, 2004) which places insect vectors as high risk for perpetuating the generation of reassortant viruses.

1.3.3. Pathogenesis

Pathogenesis by AHSV infection depends primarily on the isolate virulence phenotype (Burrage and Laegreid, 1994). There is no susceptibility difference to AHSV in foals and old horses of different sexes (Maurer, and McCully, 1963; Laegreid *et al.*, 1993). Whether state of horse immunity influences pathogenesis prognosis or not by AHSV was questioned by Howell (1963) and Erasmus (1978) after observing high mortality rate in horses vaccinated with AHSV-4 attenuated heterotypic vaccine. These horses produced prolonged viraemia, high antibody titers but below detectable levels of neutralising antibodies and died when challenged with a virulent homotypic AHSV. AHSV strains have varying levels of virulence and the virulence is associated with cell tropism (McIntosh, 1958; Erasmus, 1973) indicated by the different clinicopathological forms of the disease. Erasmus (1963) and later Wohlsein and colleagues (1998) also reported that serial passage of AHSV isolated from a horse's lung tissue continued to produce the pulmonary form whereas passage using spleen homogenates from the same animal resulted in a milder form. Difference in virulence has also been observed among different serotypes in horses inoculated with AHSV-4 and 9, where serotype 4

produced more severe clinical signs than serotype 9 (Howell and Erasmus, 1963; Skowronek *et al.*, 1995; Sailleau *et al.*, 1997). Similar results were observed in immunodeficient interferon alpha receptor knock-out mice (IFNAR^{-/-}) (de la Poza *et al.*, 2013). Recently, de la Grandiere and colleagues (2014) reported virulence of AHSV-4 and 9 using immunodeficient IFNAR^{-/-}, immunocompetent Balb/C and 129 Sv/Ev mice strains inoculated with an equal dose subcutaneously and intranasally. They confirmed that AHSV-4 was more virulent and had higher clinical impact than AHSV-9 in the investigated strains. These studies suggest that serotype 4 is one of the highly virulent and highly pathogenic strains of African horse sickness virus. Although more than one serotype can infect during an outbreak, no isolation of more than one serotype from a natural infected animal had been recorded at the time using neutralisation tests (Howell, 1979). However, with the advent of sequencing and next generation sequencing technology, a mixture of sequences representing segments of AHSV-1 and AHSV-3 were identified when sequencing AHSV-1 genome isolated from spleen (Potgieter *et al.*, 2009). This mixture was attributed to segment reassortment during virus replication that may have happened during co-infection with different AHSV serotypes, possibly AHSV-1 and AHSV-3.

Pathogenesis is initiated by virus replication cycle when an infected female biting midge capable of transmitting the virus, principally *C. imicola*, feeds on an equid host. These midges feed on equid blood as they need it to provide a protein source for egg production (Wilson *et al.*, 2009). Upon AHSV entering its mammalian host, it attaches to erythrocytes, leading to primary viraemia (Theiler, 1921). AHSV does not replicate in mature red blood cells as it lacks the necessary cellular components (Clift and Penrith, 2010). The virus is disseminated to regional lymph nodes where replication takes place before spreading to pulmonary microvascular endothelial cells, disrupting

the endothelial cell-barrier function (Stoltz *et al.*, 1996). Using positive labelling in immunohistochemical assay and transmission electron microscopy of tissues from 196 experimental and natural infected cases of AHSV, it was shown that AHSV targets mainly microvascular endothelial cells and monocytes-macrophages for virus replication and that organs or tissues that are surrounded by such cells were rich in virus (Carrasco *et al.*, 1999; Gomez-Villamandos *et al.*, 1999; Clift and Penrith, 2010). More organs or tissues are implicated experimentally such as the heart, lungs, spleen, caecum, pharynx, choroid plexus, where they were found to be primary sites for secondary virus replication (Coetzer and Erasmus, 1994; Burrage and Laegreid, 1994; Clift and Penrith, 2010). Incubation period in secondary viraemia is between 9 and 21 days before clinical signs assume (Coetzer and Erasmus, 1994). The disease form exhibited by a horse depends on the virulence of the strain (Maurer and McCully, 1963; Newsholme, 1983), organ or tissue tropism and the degree of injury the virus causes on the endothelial cells, which results in increased vascular permeability of the cell membrane (Burrage and Laegreid, 1994).

1.3.4. Clinical forms

African horse sickness is characterised by pulmonary and cardiac system involvement and occurs in four different clinical forms. The different forms of the disease in descending virulence order include peracute i.e. pulmonary (dunkop) form, subacute i.e. cardiac (dikkop) form, the acute (mixed) form and the horse sickness fever (Theiler, 1921). Clinical forms in horses ensue because of a cascade of events that follow infection, starting by replication in and damaging of the endothelial cells in the heart, lungs and macrophages in circulation, fibroblasts, smooth muscle cells and pericytes,

and intravascular coagulation (Burrage and Laegreid, 1994; Carrasco *et al.*, 1999; Gomez-Villamandos *et al.*, 1999).

Mortality rate may reach 100% within 5 days of initial clinical signs in pulmonary form. It is characterised by the development of high fever (up to 45 °C), depression, severe respiratory distress, head hanging down, severe dyspnoea, coughing, sweating and a frothy discharge from the nostrils (Thompson *et al.*, 2012). There is minimal or no subcutaneous swelling of the head (dunkop – thin head) observed (Coetzer and Guthrie, 2004). AHSV-4 have been reported to be highly virulent as experimentally inoculated horses developed severe pulmonary form of AHS (Skowronek *et al.*, 1995; Sailleau *et al.*, 1997). Serotype 4 infection has been shown to almost always result in pulmonary form in naïve animals (de la Poza *et al.*, 2013; de la Grandiere *et al.*, 2014).

In the cardiac form mortality rate may exceed 70% in less than 8 days of fever onset. It is also characterised by fever (41 °C), swelling of the head (dikkop), loss of ability to swallow and sublingual petechiae (Coetzer and Guthrie, 2004), swelling of the neck, face, chest, and supraorbital fossae, ecchymotic haemorrhages on the tongue and colic (Coetzer and Erasmus, 1994). Necropsy reveals hydropericardium, petechial and ecchymotic haemorrhages of the endocardium, muscular edema in the head and neck and also myocardial necrosis (Maurer and McCully, 1963). This form has been associated with serotype 9 (Skowronek *et al.*, 1995; Sailleau *et al.*, 1997) and is common in horses in endemic areas (Roy, 2005).

The mixed form is the most common form of the disease and the mortality rate may exceed 50% within six days after onset of fever (Coetzer and Guthrie, 2004). It is a combination of the pulmonary and the cardiac forms of the disease, characterised by respiratory distress, moderate fever and swelling of the supraorbital fossae (Coetzer

and Erasmus, 1994). Serotype 9 has been shown to produce this form in experimental horses (Skowronek *et al.*, 1995; Sailleau *et al.*, 1997).

African horse sickness fever form of the disease is the least pathogenic and is usually seen in animals with a certain degree of immunity (Howell, 1963) where they are immune to one or more serotypes and becomes infected with one that has a cross-protection. This form also occurs in species which are clinically resistant to the disease such as donkeys and zebras (Coetzer and Guthrie, 2004). It is characterised by fever, swelling of the head which last less than 6-10 days. Loss of appetite, congestion of the conjunctiva and slight breathing difficulty are some of the transient signs observed in other horses (Howell, 1963). Animals suffering from this form usually recover to normal.

1.3.5. Diagnosis

Correct diagnosis is the first step in an attempt to control and prevent AHS. The epizootic nature of the disease, as well as the social and economic relevance of the disease make rapid and correct diagnosis an absolute essential. Several methods have been used to confirm the diagnosis of AHS since its discovery. How quickly a disease is detected and control measures instituted determines the extent of damage the disease will have on the affected population. AHSV has an incubation period of 3-7 days after infection (Laegreid, 1996) and a reliable method that will allow proper diagnosis as quick as possible is ideal (Sailleau *et al.*, 2000). Clinical signs and lesions, along with history and season of manifestation may be the first line of diagnosis, but they are not enough to give final diagnoses as other viral diseases may give similar clinical signs e.g. anthrax (Demissie, 2013). In some cases the disease develops fast

and the animal dies without high viremia and antibody titers (Laviada *et al.*, 1992; Martinez-Torrecedrada *et al.*, 1997). Hence other diagnostic methods need to be involved to aid in the diagnosis. The first laboratory method used in the diagnosis of AHS was based on inoculation of infected blood on stable cell lines and checking cytopathic effects which took more than ten and sometimes more days to give results (Hamblin *et al.*, 1990; Laviada *et al.*, 1992; Laegreid, 1994). This delayed implementation of control measures and administration of correct homologous vaccine in regions which are known to be AHS free. It is also not an ideal method of detection during an outbreak. Hence quicker methods of virus isolation and identification of organs with high titers to accelerate detection are desired. Erasmus (1973) found AHSV to be in high titers in the spleen, lung and lymph nodes early in infection and hence were the tissues of choice for viral isolation.

1.3.5.1. Serological methods

Serological methods depend on the reaction of the immune system to produce antibodies against the antigen. Methods used to detect these antibodies in blood serum or tissue homogenates include immunofluorescence and enzyme-linked immunosorbent assays (ELISA). However, the sensitivity of these methods was low on clinical tissue homogenates (Laegreid, 1994). Thus they were used together with complement fixation (McIntosh, 1956), agar gel immunodiffusion (House *et al.*, 1990), direct and indirect immunofluorescence (Davies and Lund, 1974) and virus neutralisation test (Howell, 1962) as confirmatory tests. VP7 protein is highly conserved among all nine AHSV serotypes and thus can be used as a group-specific determinant and became the most used protein for diagnostic purposes in ELISA based analyses (Hamblin *et al.*, 1991; Laviada *et al.*, 1992). VP7 specific antibodies

are conformational dependent and are detected 15 days post-infection with ELISA and immunoblotting assays, which undermines its use during outbreaks. This is in contrast to antibodies against NS2 and VP6 which were detected in early infection in horses, and thus could be potentially used in outbreaks (Martinez-Torrecuadrada *et al.*, 1997). However, more research into the use of antibodies against NS2 and VP6 for AHS diagnosis is still needed.

Double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs), which uses two phage displayed single-chain variable fragment (scFv) antibodies selected from a chicken library has been developed to detect AHSV. The method is both serotype and serogroup-specific (van Wyngaardt *et al.*, 2013). The advantage of DAS-ELISAs is that they can detect the AHSV directly (Wyngaardt *et al.*, 2013). However, further investigation into the use of this method during an outbreak still needs to be done using more serotypes as only two serotypes (serotype 3 and 8) were used in the study. More sensitive molecular biology methods to detect the genome of the virus have also been developed.

1.3.5.2. Nucleic acid techniques

Reverse transcription polymerase chain reaction (RT-PCR) is one method that has been used to detect AHSV in infected horses using segment 7 and to differentiate among the nine serotypes using segment 10 restriction fragment length polymorphism (Stone-Marschat *et al.*, 1994; Zientara *et al.*, 1994; Zientara *et al.*, 1995). They developed primers to amplify S8 (NS2) which proved to be group specific for AHSV after being compared with closely related species like BTV, EHDV or EEV (Laegreid,

1994). This method was highly sensitive and can detect very low copies of RNA molecules within 24 hours (Bremer *et al.*, 1998; Aradaib, 2009).

With the advancement of PCR and access to AHSV genome sequences, new diagnostic methods were developed and old methods improved. The detection and expression analysis of RNA in real time led to the development of a real-time PCR (qPCR) assay that targets both serogroup-specific conserved segments 1 and 3 and serotype-specific segment 2 (Bachanek-Bankowska *et al.*, 2014). The assay was able to detect AHSV and not the other closely related orbiviruses and discriminate amongst the nine serotypes which makes it virus-species-specific. This technique has proven to be reliable, rapid and sensitive for the detection and identification of AHSV RNA in infected samples of choice (Bachanek-Bankowska *et al.*, 2014). This is a desired method of diagnosis during an outbreak and will allow corrective action to be taken promptly before the disease spreads.

All the mentioned methods aid in accurate detection of the causative agent which assist in the selection and administration of an appropriate vaccine in countries where the disease is not endemic. They also assist in controlling trade and implementing control measures especially in countries where AHS is not endemic but also in countries where the disease is endemic where strict trade measure are in place.

1.3.6. African horse sickness control

Several methods have been employed to curb AHS since its discovery. There is no known cure for AHS therefore control and empirical preventive methods were used since antiquity (Henning, 1956). Some of the primitive methods included stabling the horses (still used today), use of nose bags, bleeding infected animals, burying

carcasses six feet under, cremation, drainage of swamps and ponds harbouring the “germ”, quarantine and slaughtering infected animals (Henning, 1956; House *et al.*, 1992). These measures aimed at limiting exposure to infection were discovered by horse owners and farmers and used before scientific information became available about the disease and its aetiology. Increase interest in AHS and the discovery that the causative agent was a virus led to the development of a vaccine.

1.3.7. Vaccine development

Vaccine (from Latin term “vacca”, meaning cow) was first coined by Edward Jenner in 1798 when he inoculated humans with a cowpox virus that conferred protection against smallpox virus (Willis, 1997). If a vaccine is used correctly, it may prevent clinical signs of disease after infection or help control, eliminate or even eradicate an infection at population level (Meeusen *et al.*, 2007). Vaccination and other control measures are currently used in many countries to protect horses and prevent epidemics from African horse sickness disease. In countries where the disease is not endemic, vaccination is done on a case by case using monovalent strains to avoid introducing new serotypes into the region. First the serotype is identified and a homologous vaccine strain is administered. Mass vaccination in these countries is often late when the disease has already spread, leading to huge losses. After the disease is controlled, countries with an AHS free status often embark on mass slaughtering of the vaccinated animals to recover the AHS-free status (Portas *et al.*, 1999).

The first AHSV vaccine, a live-virus vaccine developed by Theiler in the 1900s, used infective blood to inject naïve horses followed by hyper-immune serum. This was after

he observed that horses that recovered from natural infection were protected against a subsequent attack. This meant that these horses had developed some form of solid, lifelong protection. Information on immune systems was in its infancy and very little was known of how vaccines work. Although the injected material offered some level of immunity and was used for many decades, it was later discovered that immunisation with one strain did not offer full protection against all strains, as some horses still died after vaccination (Theiler, 1921). Similar results were observed later using BTV strains where immunisation with a particular strain resulted in lifelong immunity for the homologous strain but little to no immunity to other strains (Neitz, 1948). This led to the discovery of nine immunologically distinct AHSV serotypes. Currently, all nine AHSV serotypes are present in South Africa and for a vaccine to fully protect it must ideally confer protection to all nine serotypes.

1.3.7.1. Live attenuated vaccine

The demonstration that AHSV can be attenuated by serial intracerebral passage in mice greatly assisted in improving and simplifying the AHSV vaccine (Alexander *et al.*, 1936). RNA viruses are highly variable, have a high error rate of about 10^5 and passaging many times through a non-target host induces random mutations on its genome and may often lead to reduced virulence (Meeusen *et al.*, 2007; Pugachev *et al.*, 2004). Thus, it was observed that serial intra-cerebral passage of AHSV in mice converted virulent isolates to less virulent strains with retention of its capacity to be immunogenic (Alexander, 1938). This discovery led Alexander and colleagues (1936) to suggest that a highly effective attenuated vaccine could be produced (du Plessis *et al.*, 1991). Adult mouse brain passaged live virus vaccine was successfully used for quite some time. Nevertheless, post-vicinal complications like encephalitis in horses

and donkeys was observed as a side effect of the vaccine (Burrage and Laegreid, 1994). This necessitated improvement of the vaccine and attenuation by passage in cell culture was attempted (Mirchamsy and Taslimi, 1964).

This was developed by cultivation of mouse brain attenuated AHSV in cell cultures i.e. monkey kidney cells and BHK cells (Ozawa and Hazrati, 1964). It was in cell culture passage that they observed and demonstrated that plaque size is a marker for virulence where large plaques, low virulence strains were selected as vaccine candidates (Erasmus, 1978). Monovalent and polyvalent vaccines are currently in use derived from virus passage in cell culture. Research on AHSV vaccine strains indicated serological cross-reactivity and cross-protection among the serotypes. Serotype 1 was found to cross-react with serotype 2 and serotypes 3 with serotype 7. AHSV serotype 4 has been reported not to cross react with any other serotype and serotype 8 and serotype 9 confers cross protection to serotype 5 and serotype 6 (Howel, 1962; Erasmus, 1978; Coetzer and Erasmus, 1994; von Teichman *et al.*, 2010). This led to the pairing combinations of the currently widely used attenuated polyvalent vaccine produced at Onderstepoort biological products (OBP) given in two components: a trivalent (serotypes 1, 3, 4) and quadrivalent (serotypes 2, 6, 7, 8) (Du Plessis *et al.*, 1998; Coetzer and Erasmus, 1994).

Serotype 5 was initially grouped with serotypes 1, 3, 4 and 5 (House *et al.*, 1992) but was removed in 1993 from the combination after it was implicated in severe post vaccination reactions and deaths in the 1990 season (Coetzer and Erasmus, 1994). Serotype 9 is also excluded because serotype 6 provide very strong cross-protection (Coetzer and Erasmus, 1994). Cross-reactivity was explained using homology based on amino acid alignment of capsid protein VP2 of all serotypes which showed that there is high identity within antigenic regions of serotypes that serologically cross react

(Potgieter *et al.*, 2003). Success of this vaccines owes to the fact that because they are live, they are able to infect and replicate and induce both humoral and cellular immunity (Meeusen *et al.*, 2007). Although the vaccine enjoys great success, it has been a subject of some drawbacks.

A recent study compared the commercially available polyvalent and monovalent live-attenuated AHSV vaccine in foals. They found that most foals converted consistently to an anti-AHSV-1 response than to any other serotype in both monovalent and polyvalent vaccines, and a booster did not significantly increase neutralising antibodies to the other serotypes suggesting poor anamnestic response (Crafford *et al.*, 2014). Immunised horses have been shown to be affected by AHS and that more rounds of vaccination are required to reach the detectable serological immune response under field conditions (Weyer *et al.*, 2013; Molini *et al.*, 2015). This questions the protection offered by the currently used vaccine in endemic countries. It also cast doubts on the number of booster vaccinations required to achieve detectable antibody titers associated with protection. The roles other systems like cellular immunity may play cannot be ignored. Other limitations include the inability to prevent viremia of vaccinated animals and variable immunogenicity in animals (Burrage and Laegreid, 1994). Also, live vaccine cannot be given to pregnant mares, can only be used in endemic regions and have potential to revert to virulence through reassortment (Mellor and Hamblin, 2004). Thus the need to improve the vaccine for use especially in countries which only experience epidemics and to allow less restrictions in horse training. A vaccine that will be devoid of replicating mechanism but with retention of its immunogenicity would be desired, this lead to development of an inactivated or killed vaccine.

1.3.7.2. Inactivated or killed virus vaccines

Inactivated or killed virus vaccines are produced by heat or chemical inactivation. They offered an alternative to live attenuated vaccines because they do not replicate (Minke *et al.*, 2004), and thus are generally stable and do not pose risk of reversion to virulence (Meeusen *et al.*, 2007). Several studies have shown that formalin inactivated tissue cultured virus vaccine induce some level of antibody production but not enough to confer protection (Du Toit and Alexander, 1930; Walker, 1931; Kind, 1934). The observation that inactivated virus have lost their ability to infect and replicate may be responsible for the low protection. This may be because they can only activate humoral immunity and multiple vaccinations may be required to stimulate protection (Meeusen *et al.*, 2007). The first effective formalin inactivated cell culture virus vaccine (serotype 9) was developed by Ozawa and Bahrami (1966), albeit huge doses were used to obtain protection. Mirchamsy and Taslimi (1968) improved the vaccine by adding an adjuvant, aluminium hydroxide, to enhance the immunogenicity. Two injections administered 4 weeks apart were able to protect and neutralising antibodies persisted for over 6 months although the response decreased. Although successful, the use of whole virus as a vaccine has its disadvantages. Growth of large amounts of virus makes production very expensive as large doses are needed to induce protective antibody production (Laegreid, 1996). An attempt to use antigenic parts of the virus as a vaccine has been on-going.

1.3.7.3. Subunit vaccines

The requirement that vaccines should serologically allow differentiating infected from vaccinated animals (DIVA) has also made subunit unit vaccines an attractive area of research. This is to allow for diagnostic testing in horse trading from AHS free area to

endemic areas and vice versa. DIVA vaccines are developed from identifying and selectively deleting genes from a pathogen. The antibody repertoire induced by this vaccine strains with the deleted genes will be different from those induced by a wild type strain in an immunological assay. It is expected that when an animal is vaccinated with a DIVA vaccine and tested, antibodies against the “marker” should not be detected in the vaccinated animal but against wild type virus antibodies (Meeusen *et al.*, 2007). Thus the deleted genes serve as “markers”. Care should be taken in not using proteins that are expressed by genes used as “markers” in a subunit vaccine construct. Markers that are usually used are non-structural proteins (van Regenmortel, 2008) like NS3. The gene is expressed in *E. coli* and used as an antigen in ELISA (Laviada *et al.*, 1995). Recombinant subunit vaccines are safer and can be used in combination with other subunit vaccines without fear of reassortment. Huisman and colleagues (1987) demonstrated in BTV that purified outer capsid protein VP2 elicited neutralising antibodies and protected sheep against a virulent strain. This created a lot of interest in recombinant subunit vaccines and in 1992, baculovirus of insects were used as vectors in developing the first recombinant subunit vaccine (Roy and Erasmus, 1992). Efficiency of the subunit vaccine depends on the expression system (OIE, 2012). Some of the systems that have been used with successful expression of VP2 and VP5 of AHSV include canarypox vector system AHSV-4 (Guthrie *et al.*, 2009), BTV-17 (Boone *et al.*, 2007), baculovirus-expression systems (Roy and Sutton, 1998) poxvirus expression system (Guthrie *et al.*, 2009; Castillo-Olivares *et al.*, 2011) and modified vaccinia Ankara expression virus system (Alberca *et al.*, 2014). Various successes of using recombinant viral proteins to induce protective antibodies are reported in the literature. Problems with recombinant subunit vaccines were encountered in upscaling of production (Verwoerd, 2012).

This means identifying those antigenic regions on a protein responsible for inducing protective immunity will allow production of smaller components and help with upscaling. Insight into the horse's humoral immune response to AHSV could help with vaccine design and development. What is evident is that information of the immunogenicity of AHSV proteins is vital for the identification of these antigenic regions and development of a recombinant subunit vaccine construct.

1.4. AHSV immunogenicity and epitopes

The immunogenicity of a protein is determined by the ability of the antigenic determinants to provoke neutralising antibodies against that protein (van Regenmortel, 2008). An antigenic determinant or epitope is the portion on a protein that binds specifically to an antibody (Novotny *et al.*, 1983) and is found on restricted parts of the protein (van Regenmortel, 1986). To correctly identify an antigenic region on a protein antigen, its purity is important as small antigenic fragments may be obscured by big non-antigenic fragments and can be missed by methods used to delineate epitopes. This problem has been circumvented by the use of synthetic fragments (van Regenmortel *et al.*, 1988). The determinants of antigenicity in proteins can only be analysed operationally and the type of probe used in the analysis affects the results, hence a set of methods to delineate antigenicity have been suggested (van Regenmortel, 1986).

VP2 is said to contain the majority of the antigenic regions in most members of the Orbivirus genus (Huisman and Erasmus, 1981; Grubman *et al.*, 1983; Marshall and Roy, 1990). It is the major target of the neutralisation response by the host and induces neutralising antibodies (Ranz *et al.*, 1992; Burrage *et al.*, 1993). The gene encoding

VP2 protein, segment 2, has been cloned, sequenced and the protein expressed for all nine AHSV serotypes (Vreede and Huisman, 1994; Potgieter *et al.*, 2003) and is the most extensively studied viral protein of AHSV. The capacity of an expressed protein to be antigenic depends somehow on the expression system and purification system used (Martinez-Torrecuadrada *et al.*, 1996; Meeusen *et al.*, 2007). This means the ability of an expression system to express the protein in the correct conformation is critical. Recombinant baculovirus-expressed VP2 of AHSV and BTV has been shown to induce serotype-specific immunity in horses against virulent challenge (Martinez-Torrecuadrada *et al.*, 1994; Du Plessis *et al.*, 1998) and in sheep (Roy *et al.*, 1990) with AHSV-5 VP2 needing a saponin adjuvant to confer protection (Scanlen *et al.*, 2002). This immune response was even higher in sheep when baculovirus expressed BTV VP2 was combined with VP5 (Roy *et al.*, 1990; Martinez-Torrecuadrada *et al.*, 1996). Similar results are observed when recombinant canarypox virus vectored (ALVAC) co-expressing AHSV-4 VP2 and VP5 was used (Guthrie *et al.*, 2009). Protective immunity increased and lasted longer than 15 months when baculovirus-derived VP2, VP3, VP5 and VP7 viral-like particles (VLP) were combined as a candidate vaccine and induced full protection in immunised horses with no viremia (French *et al.*, 1990; Roy *et al.*, 1992; Martinez-Torrecuadrada *et al.*, 1996). However, when baculovirus expressed AHSV-4 VP2 and VP5 were purified by affinity chromatography, and administered singly or combined, no neutralising antibodies were induced in horses and thus failed to protect against virus challenge. Protection was observed when purified VP7 was added albeit with low production of neutralising antibodies (Martinez-Torrecuadrada *et al.*, 1996). This may mean that addition of VP7 to the cocktail induced or activated the cell-mediated response since its addition did not increase neutralising antibodies but conferred protection (Wade-Evans *et al.*,

1997). These results emphasise the need to properly select expression and purification systems for recombinant proteins. Baculovirus expressed VP2 of most AHSV tested induce significant titers of neutralising antibodies and confer protection. A combination of expressed VP2 of all nine AHSVs was evaluated. Individually recombinant baculovirus expressed VP2 of all nine AHSV serotypes was used to immunise guinea pigs. They produced much lower neutralising antibodies when compared to when immunized with a single VP2. This could be attributed to cross-reactivity and interference among genetically closely related AHSV serotypes (Kanai *et al.*, 2014) leading to low induction of neutralising antibodies.

AHSV VP5 is one of the serological markers in infection (Martinez-Torrecedrada *et al.*, 1997). Although purified or non-purified baculovirus expressed virus BTV VP5 in insect cells did not provoke production of neutralising antibodies (Mertens *et al.*, 1989; Roy *et al.*, 1990; Marshall and Roy, 1990), AHSV-9 VP5 expressed by the same system elicited significant AHSV-specific neutralising antibodies in rabbits and generated VP5-specific monoclonal antibodies capable of neutralising virus in a plaque reduction assay (Martinez-Torrecedrada *et al.*, 1999). Recombinant modified vaccinia Ankara expressed VP2 (MVAVP2) and VP7 (MVAVP7) injected in ponies (Chiam *et al.*, 2009) and in IFNAR^{-/-} mice (Castillo-Olivares *et al.*, 2011; Calvo-Pinilla *et al.*, 2014; Alberca *et al.*, 2014) induced protective antibodies detected by western blotting and virus neutralisation test. The experiment in mice indicated that this protection is increased by passive transfer of MVA-VP2 induced antiserum to mice 48 hours before or after challenge with virulent AHSV-4 (Calvo-Pinilla *et al.*, 2015). However, recombinant modified vaccinia Ankara expressed NS3 (MVANS3) failed to produce any antibodies detected by western blot (Chiam *et al.*, 2009). This means that not all viral proteins have the capacity to provoke the immune system as some proteins

are degraded after translation, and are not detected by the immune system. NS3 is an important protein involved in viral particle escape during replication and assembly, it would be expected that it is detected by the immune system as it is responsible for the timing of viral particle escape. Reports suggest that AHSV and BTV NS3 is poorly expressed in mammalian systems (Mertens *et al.*, 1984; Guirakhoo *et al.*, 1995; Chiam *et al.*, 2009), meaning its threshold is below immune system detection of mammals hence its poor capacity to induce neutralising antibodies. Results may be different when a different expression system and natural host are used, but it is suggested that the poor presence in expression in mammalian cells may be the property of mRNA of both closely related AHSV and its prototype BTV (Chiam *et al.*, 2009).

NS1 is another protein prominent during infection as it forms long tubules within the cytoplasm. It should therefore be antigenic as observed by the identification of multiple cytotoxic T-lymphocyte (CTL) epitopes in BTV NS1 and the addition of MVA-NS1 to MVA-VP2 and MVA-VP7 enhanced protection in BTV (Andrew *et al.*, 1995; Janardhana *et al.*, 1999; Calvo-Pinilla *et al.*, 2012). It is yet to be determined if AHSV NS1 has antigenic properties. Recombinant MVA (rMVA-VP2-NS1) of AHSV-4 administered to IFNAR^{-/-} not only produced significant levels of neutralising antibodies, but also stimulated specific T cell responses against virulent AHSV-4 and cross protected against AHSV-9 with no detectable viremia (de al Poza *et al.*, 2013). This suggests that addition of NS1 to a subunit vaccine construct increases immunogenicity by inducing both humoral and cellular immunity, which are highly desired properties for a vaccine. These properties, if they can be reproduced in horses, could be a breakthrough in vaccine design. Experiments to establish an effect of a recombinant construct of VP2, VP5, VP7 and NS1 in horses may thus add more

information on the immunogenicity, as reports suggested that the first two proteins induce humoral immunity and the last two seem to activate cellular immunity.

1.4.1. Antigenicity prediction

1.4.1.1. Hydrophilicity and Secondary structure

Methods that detect the hydrophilic regions of a protein are used to predict antigenicity (Hopp and Woods, 1981). Hydrophilic regions on a protein are found on the surface of the protein and interact easily with solvents and antibodies (van Regenmortel, 1986). Therefore a protein that has hydrophilic residues on its surface is likely to be antigenic (Stern, 1991). Hopp and Woods (1981), looking at myoglobin, showed that the most hydrophilic segments possessed antigenic properties. Using secondary structure prediction algorithms, the secondary structure loops and turns were found to generate high peaks in hydrophobic regions of the protein, which are then used as predictors of antigenicity (Garnier *et al.*, 1978; Chou and Fasman, 1978; Hopp, 1986).

1.4.1.2. Segment mobility

The importance of protein function dynamism has been well emphasised over the years (Gurd and Rothgeb, 1979; Karplus and McCammon, 1983; Ringe and Petsko, 1985). Information from nuclear magnetic resonance (NMR) studies and X-Ray crystallography (Debye-Waller factors) which measure the motion of protein side chains (Westhof *et al.*, 1984) showed the antigenicity of tobacco mosaic virus protein (TMVP) and myoglobin. Antibodies raised against peptide fragments of myohaemerythrin that showed highly mobile regions reacted strongly with the native

protein while antibodies to well-ordered regions did not, suggesting mobile regions are antigenic (Tainer *et al.*, 1984).

1.4.1.3. Surface accessibility

Protein surfaces are always in constant interaction with antibodies and therefore it makes sense that protein surface accessibility will influence antigenicity. Thornton *et al.*, 1986, using three proteins of known tertiary structure, reported that regions found on the protein surface or found to be protruding have antigenic properties correlating with segment mobility followed by hydrophilicity. Similar results were observed with 133 hexapeptides of myohaemerythrin (Geysen *et al.*, 1987). They found that mobility, followed by accessibility and surface exposure have high antigenic properties.

1.4.1.4. Sequence variability

Sequence variability in homologous proteins have mostly been associated with antigenic determinant regions (Crumpton, 1974; Reichlin, 1975). This method has been used to predict antigenicity of VP1 foot and mouth disease virus protein, which has high sequence variability among serotypes (Brown *et al.*, 1986).

1.4.2. Types of epitopes

Epitopes or antigenic regions are regions on a proteins (antigen) that bind specifically to a paratope of an antibody. Antibodies contain a paratopes that are made up of several hyper-variable loops of residues that make contact with the epitope of the antigen (Richard *et al.*, 1978; Stave and Lindpaintner, 2013). Epitopes have heterospecific binding capability and paratopes of an antibody has a polyfunctional

nature and can even bind stronger to other epitopes than the one it was raised (van Regenmortel, 1986). Epitopes can be linear (sequential or continuous) or conformational (discontinuous) depending on their structure (Li *et al.*, 2013). Monoclonal antibodies (MAbs) are usually used to delineate antigenic regions of viral proteins.

1.4.2.1. B and T cell epitopes

B-cell epitopes are recognised by B-cell receptors which recognise native tertiary and quaternary structures and T-cell epitopes are recognised by T-cell receptors which recognise peptide fragments (van Regenmortel, 2008). B-cell epitopes are mostly conformational configured and majority of T-cell epitopes are linear (Bentley, 1996).

1.4.2.2. Sequential and conformational epitopes

Sequential epitopes bind to a paratope in their primary structure while conformational epitopes, which consist of amino acids normally not contiguous in the primary sequence, but require a specific folding for binding to antibody paratope (Sela, 1969; van Regenmortel *et al.*, 1988).

1.4.2.3. Cryptotopes, Mimotopes and Neotopes

Cryptotopes are epitopes that are normally hidden in an intact viral particle, usually buried when the subunits associate into a capsid (van Regenmortel, 2008). They are exposed only after fragmentation, depolymerisation or denaturing of the antigen (Jerne, 1960). Antibodies raised to denatured protein will not necessarily react with the corresponding intact molecule (van Regenmortel *et al.*, 1988). Mimotopes are

those epitopes which mimic a complex epitope structure thus causing a similar response to the correct antibody but the primary sequence is different (Geysen *et al.*, 1986). Neotopes are those that are only active through conformational changes in the monomers and are specific for quaternary structure. They are not found on the constituent monomeric subunits of viral capsids (van Regenmortel, 1966; Neurath and Rubin, 1971). Conformational changes in the monomer induced by intersubunit bonds or by juxtaposition of residues from neighbouring subunits give rise to neotopes (van Regenmortel *et al.*, 1988).

1.4.2.4. Neutralising epitopes

These epitopes are recognised by antibodies that are able to neutralise viral infectivity. The antibody binding to the epitope interfere with the ability of the virus to attach to receptors of the host cell (van Regenmortel *et al.*, 1988). These are the epitopes found on VP2 in both BTV and Palyam serogroup orbiviruses (Huisman and Erasmus, 1981; Grubman *et al.*, 1983; Mertens *et al.*, 1989; White and Eaton, 1990), AHSV VP2 (Burrage *et al.*, 1993) and on VP5 of Kemerovo serogroup orbiviruses (Moss *et al.*, 1987; Nuttall *et al.*, 1990). These epitopes were identified using monoclonal antibodies and Immunoprecipitation.

This confirms the immunogenic nature of these proteins. Prediction of antigenic regions on these proteins can thus serve as a base for the identification of epitopes but to locate them by experimental means is probably a more reliable approach (Bentley *et al.*, 2000). Although there are several methods used to identify antigenic regions on proteins, such as X-ray crystallography and nuclear magnetic resonance, the experimental method of interest in this study is phage display.

1.4.3. Mapping of epitopes using phage display

Phage display (Smith, 1985) is one method that has been used experimentally to map antigenic regions. It works by expressing foreign peptides, proteins or antibodies on the surface of phage particles (Smith, 1985; Kay, 1994; Kay and Hoess, 1996; Ngubane *et al.*, 2013; Rebollo *et al.*, 2014; Christiansen *et al.*, 2015). To achieve expression, a gene encoding the protein of interest is incorporated into a phage genome or phagemid as a fusion to a gene encoding a phage surface protein (Hoogenboom *et al.*, 1998). The gene is then expressed during the normal phage coat protein expression processes and displayed along with the phage surface protein. This creates a physical link between the genotype and phenotype of the protein (Smith, 1985; Smith and Scott, 1992). Phage display enables easy screening of target peptides or proteins with specific binding properties from a large pool of variants (Willats, 2002). Screening is by affinity selection (bio-panning) and involves exposing the phage library to immobilised target molecules, washing away non-binders and elution of binding phage (Parmley and Smith, 1988; Willats, 2002). The binding phage with the peptides or proteins with the desired properties are amplified by infecting bacterial cells (Hoogenboom *et al.*, 1998; Ngubane *et al.*, 2013; Rebollo *et al.*, 2014; Christiansen *et al.*, 2015). Epitopes are identified by sequencing the phage DNA of the binding phages and subjecting them to *in silico* analysis programs (Bentley *et al.*, 2000). Phage display and epitope mapping has been used in analysis of protein to protein interaction and also in new drug design and signal transduction studies (Zhao *et al.*, 2012). The method has been used to identify epitopes amongst others for hepatitis B virus (Xin *et al.*, 2003), hepatitis C virus (Ferrieu-Weisbuch *et al.*, 2006) and *S. pneumoniae* (Buchwald *et al.*, 2005).

1.4.3.1. Advantages of phage display systems

The major advantage of phage display systems is that it offers a direct link between the phenotype and genotype of a phage particle. This makes it easier to select and identify positive clones and sequencing the genome reveals the sequence encoding the expressed peptide (Smith, 1985). It also allows isolation of positive clones by selection procedure (biopanning) and not by screening filters with plaques or clones, thus allowing access to larger libraries than before (Lane and Stephen, 1993). Amplification of library size or reactive clones is achieved with small culture volumes because filamentous phage do not lyse their host, but rather extrude virions out of viable cell thus one growing cells produces thousands of phage (Kauffman, 1992; Joyce, 1992; Schatz, 1994). Phage display systems have been used to identify epitopes, discover cell surface receptors, and define substrate specificity of proteases, kinases and protein modifying enzymes (Kay, 1994; Ngubane *et al.*, 2013; Rebollo *et al.*, 2014; Christiansen *et al.*, 2015).

1.4.3.2. Phage display in orbiviruses

In Orbivirus studies, phage display has been used to map several antigenic regions. Epitopes on VP7 (Du Plessis *et al.*, 1994), VP5 (Wang *et al.*, 1995; Martinez-Torrecedrada *et al.*, 1996), NS1 (Du Plessis *et al.*, 1995) of BTV and VP2 (Bentley *et al.*, 2000) of AHSV were identified and mapped. These epitopes were identified using antibodies raised in mice, rabbits, chickens and horses. More epitopes were mapped with mice, rabbit and chicken antibodies than when horse antibodies were

used. This is a concern as horses are the target hosts for AHSV. Epitopes on VP2 (between amino acid residues 253 to 414) have been identified using an overlapping AHSV-4 VP2 fragment expressed in *E. coli* and tested in mice and rabbits using ELISA, immunoblotting and neutralisation tests. Although they induced neutralising antibodies *in vivo*, they could not completely remove neutralising antibodies in horse sera in virus neutralisation tests, indicating that these are not the immunodominant or neutralising epitopes in horses (Martinez-Torrecuadrada and Casal, 1995). Eight antigenic sites (between residues 48 and 265) have been identified when AHSV-4 recombinant VP5 protein, expressed in insect cells, was tested against antibodies raised in mice and rabbits (Martinez-Torrecuadrada *et al.*, 1999). However, insignificant level of binding was observed when these epitopes were tested with horse sera (Martinez-Torrecuadrada *et al.*, 1999, Martinez-Torrecuadrada *et al.*, 2001). Fifteen neutralising epitopes on AHSV-4 VP2 (residues 199 to 689) were identified when bacterially expressed VP2 and baculovirus-expressed VP2 fragments, alone or in combination with VP5 were tested using *in vitro* neutralisation assays in mice, rabbit and horse sera. Combining two epitopes on VP2 (321-339 and 377-400) induced higher reactivity in rabbits when analysed by PEPSCAN (Martinez-Torrecuadrada *et al.*, 2001).

1.4.3.3. Other epitope mapping methods

Other epitope mapping methods involve using consecutive overlapping synthetic peptides covering the region of interest or the entire protein primary sequence (Kazim and Atassi, 1981). Epitopes are identified by screening antibody binding in immunoassays like ELISA (Otvos *et al.*, 2000; Hjelm *et al.*, 2010; Winkler, 2011). The method's limitation include short peptide length preventing formation of secondary

structures and thus can be used to detect linear epitopes only (Hudson *et al.*, 2012). Structure determination of the antigen-antibody complex using X-ray crystallography (Du *et al.*, 2009; Cho *et al.*, 2003) and nuclear magnetic resonance (Rosen and Anglister, 2009; Hoyer *et al.*, 2008) are rarely used methods. These methods have great success particularly in mapping of conformational epitopes. Limitations include that they require a high level of technical skills, are labour intensive, work best with monoclonal antibodies and may not be methods of choice when using polyclonal antibodies (Hudson *et al.*, 2012).

1.4.4. Sequencing of clones

All sequencing techniques rely on sufficient and appropriate collecting and processing of samples. Sequencing techniques are all developed from a method by Sanger and colleagues (1977) which was based on selective random incorporation of chain-termination dideoxynucleotides. This method has been adapted several times from manual to automation and was instrumental in the Human Genome Project in 2001 (Collins *et al.*, 2003). Though successful, the method was laborious, produced low data output, took long to complete and could not be used to sequence more than a hundred individual DNA clones (Matochko *et al.*, 2012). Advances in this technique led to high-throughput or next generation sequencing which increased the data or sequence output by millions.

1.4.4.1. High throughput sequencing

Next generation sequencing techniques are fast and reliable and cheap because samples can be multiplexed and produce high data output in a short space of time (Ravn *et al.*, 2013). Platforms of next-generation sequencing include the Illumina

HiSeq2000 (Fedurco *et al.*, 2006; Turcatti *et al.*, 2008), Roche 454 (Petrosino *et al.*, 2009; Ronaghi, 2001), and Life technologies/Applied Biosystems' SOLiD system. In all platforms the DNA sample is fragmented and ligated to adaptors and fragments amplified by PCR. The platforms differ in chemistry, read length of base pairs produced, run time, throughput per run and reads produced per run (Di Bella *et al.*, 2013). The choice of a sequencing method depends on certain factors which may include size of the repertoire to be sequenced, read length, complexity, accuracy and depth of coverage of resultant sequences (Loman *et al.*, 2012).

1.4.4.2. Illumina MiSeq2000

In this platform, libraries are made by fragmenting DNA, blunt ended and dephosphorylated, ends are ligated to adapters and amplified by bridge PCR (Adessi *et al.*, 2000). Amplified DNA is denatured to single strands. This is then sequenced with sequencing primers incorporating nucleotides tagged with fluorescent dyes and a cleavable blocking group i.e. reversible terminators. When the tagged nucleotides are washed through a flow-cell, each nucleotide fluoresce at a different wavelength which is captured by a sensor attached to a computer with a program to visualise data. The dye and blocking group attached to the nucleotide are cleaved to allow further incorporation of another tagged nucleotide in each cycle (Turcatti *et al.*, 2008). This system has high accuracy, low error rate with more substitution rather than insertions or deletions, employs reduced hands-on time and is cheaper with higher reads output per run. However, the major disadvantage of the system is the short read length produced and the long run time (Turcatti *et al.*, 2008). These advantages i.e. low error rate, large data output and less technical made this system desirable for this project. Also, the ability of the system to substitute rather than insert or delete nucleotide

makes it easy to detect and trace and correct during data analysis, in particular amino acids, for the purpose of this study.

1.4.4.3. Roche 454

This method was the first to be commercialised and used as a sequencing platform (Margulies *et al.*, 2005). It used pyrosequencing, which means it depends on the detection of pyrophosphate released during the sequencing by DNA synthesis process rather than chain termination with dideoxynucleotides in Sanger sequencing (Ronaghi *et al.*, 1996). In this method libraries are constructed by fragmenting DNA and ligating with adaptors, denatured to single strands, captured by amplification beads and amplified by emulsion PCR (Dressman *et al.*, 2003; Berka *et al.*, 2010). Beads are washed on a picotiter plate bearing the universal sequencing primers and sequencing is by pyrosequencing (Ronaghi *et al.*, 1996; Froehlich *et al.*, 2010). This results in long read lengths and high speed with short run time. Disadvantages of the method include low throughput, automation high error rate especially in 40 homopolymers (consecutive repeating of same base) and high cost per-base i.e. 10 times more than Illumina's HiSeq 2000. The dominant error type is insertion and deletion rather than substitutions (Bennet, 2004; Huse *et al.*, 2007; Shendure and Hanlee, 2008; Quince *et al.*, 2009; Gilles *et al.*, 2011, Wang *et al.*, 2011; Luo *et al.*, 2012).

1.4.4.4. Applied Biosystems' SOLiD system (Life technologies)

In this method, libraries are constructed by DNA fragmentation, adaptor flanking of fragments to P1 adaptor, attach next to magnetic beads and amplifying by emulsion PCR (Dressman *et al.*, 2003). PCR product bearing beads are then covalently bound to glass slides and sequenced by hybridisation and ligation with fluorescently labelled

DNA ligase di-base probes (Housby and Southern, 1998; Macevicz, 1998; Shendure *et al.*, 2005; Margulies *et al.*, 2005; McKernan *et al.*, 2006). Method produces short reads, involves an error-correction scheme using two-base encoding leading to high accuracy (99.99%) (McKernan *et al.*, 2006; Liu *et al.*, 2012). Disadvantages include short read length, long run time and technically challenging emulsion PCR (Shendure and Hanlee, 2008; Di Bella *et al.*, 2013).

1.4.5. High throughput sequencing of phage display libraries

Phage display give rise to a large number of individual clones after selection, which is necessary for diversity. To evaluate this diversity, quality control and to identify all binding clones, a large number of random library members should be sequenced and analysed (Ravn *et al.*, 2010; Glanville *et al.*, 2009; 'T Hoen *et al.*, 2012; Matochko *et al.*, 2012). High throughput sequencing produces millions of reads to obtain sufficient coverage representation (Ravn *et al.*, 2013) and therefore is desirable for this purpose. Handling and analysis of data from high throughput sequencing remains a problem as development of software continues as technology is relatively new (Zhang *et al.*, 2011). Deep sequencing also allows for the detection of defects and biases and gives in-depth sequence information about the library. This is useful because it assists in identifying those sequences that could not be detected by screening but are enriched during the selection process and have excellent binding capacity (Rvan *et al.*, 2010). However, care should be taken not to select and sequence other components of the panning system, the target unrelated peptides (TUP). These are peptides that will bind to the blocking agent, plastic binders or impurities found in antibody preparations and those with propagation advantage (Thomas *et al.*, 2010). Although it is almost impossible to completely rid the panning process from TUP, they can drastically be

reduced by incubating the phage pool with the blocking agent before selection (de Kruif *et al.*, 1995; Vodnick *et al.*, 2011), or compare the selected peptides to the database server of known TUPs (Huang *et al.*, 2010; Matochko *et al.*, 2014).

Illumina high throughput sequencing was used to characterise and confirm the quality of complex phage displayed antibody libraries (Ravn *et al.*, 2010; Ravn *et al.*, 2013). It also enabled characterisation and selection of peptides from libraries of proteins displayed on T7 phage even after one round of selection against cell surface receptors (Fowler *et al.*, 2010; 'T Hoen *et al.*, 2012). Illumina high throughput sequencing assisted in examining changes in the diversity of peptides as a result of iterative selection and amplification in bacteria, which was found to collapse to ~20% (Matochko *et al.*, 2012). Peptides that bind to *Mycobacterium tuberculosis* have also been identified (Ngubane *et al.*, 2013). In phage display libraries, the Roche 454 method was used to identify peptides emerging from *in vivo* panning against skin, fat-tissue, bone marrow and skeletal muscles (Dias-Neto *et al.*, 2009). It also aided in finding antibodies that bind to various proteins displayed on a bacterial surface (Zhang *et al.*, 2011). High throughput sequencing enhanced the identification of patient-specific motifs in a polyclonal serum using phage display (Christiansen *et al.*, 2015).

High throughput sequencing has been applied to orbiviruses. The complete genomes of BTV, AHSV, EEV and human rotavirus have been sequenced using this method (Potgieter *et al.*, 2009; Minakshi *et al.*, 2012; Lorusso *et al.*, 2013; Rao *et al.*, 2013; Caporale *et al.*, 2014; Potgieter *et al.*, 2015). Sequence variation within geographically distinct regions has enabled primer design of PCR-based amplification techniques (Maan *et al.*, 2012; Susmitha *et al.*, 2012; Zientara *et al.*, 2012). With an increase in incidence of BT in temperate regions, high throughput sequencing has recently been used to serotype BTV in India (Rao *et al.*, 2013).

Limited information is currently available for epitope mapping using high throughput sequencing in orbiviruses. Although epitopes on some of the AHSV-4 proteins have been mapped, no comprehensive study has been done on all the structural and non-structural viral protein epitopes. Especially their interaction with antibodies of immunised horses, and the effect the epitope combination will have on the neutralising activity. This project aims to identify B-cell epitopes of all the structural and non-structural AHSV-4 viral proteins recognised by the horse's immune system, which is the naturally infected host. This will be done using phage display which allows testing of peptides longer than those identified by PEPSCAN, increasing the chances of peptides adopting a secondary structure, thus allowing more antibodies to bind. This is part of a bigger project that is attempting to develop a novel vaccine using parts of the African horse sickness virus serotype 4, with the possibility of extending to other serotypes, to give a long-lived protective immunity.

CHAPTER 2: MATERIALS AND METHODS

2.1. AHSV-4 clones and horse sera

As source of the AHSV-4 genome, cDNA copies of each genome fragment cloned in pET102/D-TOPO (Invitrogen) or pGEM-T (Promega) (Table 2.1) were obtained from Ms Erika Faber, ARC-OVI, Pretoria, South Africa. The sera were obtained from a horse vaccine trial where five horses were vaccinated twice subcutaneously with a monovalent AHSV-4 live attenuated vaccine using 5×10^4 viable virus in 2 ml DMEM medium (Lonza) prepared at the Agriculture Research Council–Onderstepoort Veterinary Institute. The horses were first vaccinated on day 0 and boosted on day 21. Serum samples were collected in separate tubes three days prior to the first vaccination, 28 days and 52 days after first vaccination and stored at $-20\text{ }^{\circ}\text{C}$ until needed. This pre-immunisation sera will be referred to as day 0 in the study. Temperature and overall condition were monitored in horses before and after vaccination.

Animal research protocols were approved by both the animal ethics committees at the ARC-OVI and OBP Ltd and the AEC of UP. Section 20 approval for work with AHSV was also obtained from the South African department of agriculture, forestry and fisheries (DAFF) project number 01/20/JT01.

2.2. AHSV-4 cDNA genome preparation

In order to construct a genome-targeted phage displayed library, the plasmids containing AHSV-4 segments were amplified by transformation in bacterial cells (Table 2.1). As indicated in Table 2.1, the larger fragments S1 (VP1), S2 (VP2) and S3 (VP3)

were divided into two equal fragments for easier cloning. The rest of the segments were cloned as full length.

Table 2.1: Details of the clones of the AHSV-4 genomic segments.

Segment	Encoded protein	Size (bp)	Vector	Host cell
S1-1	VP1-1	~1950	pET102/D-TOPO*	E. cloni ^{\$}
S1-2	VP1-2	~1950	pET102/D-TOPO*	E. cloni ^{\$}
S2-1	VP2-1	~1600	pGEM-T [#]	JM109 [@]
S2-2	VP2-2	~1600	pET102/D-TOPO*	E. cloni ^{\$}
S3-1	VP3-1	~1350	pET102/D-TOPO*	E. cloni ^{\$}
S3-2	VP3-2	~1350	pET102/D-TOPO*	E. cloni ^{\$}
S4	VP4	~1900	pET102/D-TOPO*	E. cloni ^{\$}
S6	VP5	~1500	pET102/D-TOPO*	E. cloni ^{\$}
S9	VP6	~1100	pET102/D-TOPO*	E. cloni ^{\$}
S7	VP7	~1000	pET102/D-TOPO*	E. cloni ^{i\$}
S5	NS1	~1700	pGEM-T [#]	JM109 [@]
S8	NS2	~1100	pET102/D-TOPO*	E. cloni ^{\$}
S10	NS3	~600	pGEM-T [#]	JM109 [@]
Total		~18700		

* Life technologies

Promega

^{\$} Lucigen

@ Sigma-Aldrich

2.3. Transformation of chemically competent *E. coli* with plasmid

The plasmids shown in Table 2.1 with the appropriate size insert were amplified by transformation in chemically competent *Escherichia coli* genotypes E. cloni (Lucigen) or JM109 (Sigma-Aldrich). The bacterial cells were transformed in a reaction where approximately 50 ng of DNA was added to 20 µl of competent cells (Table 2.1). This was mixed gently and incubated on ice for 30 min. The tubes were moved to a 42 °C water bath (heat shock) for 45s to encourage the cells to take up DNA and placed

back on ice for a further 2 min. Luria Bertani (LB) medium (960 μ l) was added to the tube and incubated for an hour at 37 °C shaking at 240 rpm. Afterwards, 50 μ l was plated on pre-warmed LB with 100 μ g/ml ampicillin and 2% glucose (LB/Amp) agar plates. The plates were incubated overnight at 37 °C. The next morning, randomly selected colonies from each plate were picked and prepared for PCR to confirm the insert size. The clones showing correct sizes were then grown in 25 ml of LB/Amp medium and incubated overnight at 37 °C shaking at 240 rpm for plasmid purification.

2.4. Plasmid purification

The clones were divided into different tubes and plasmids extracted from bacterial cells for further manipulation using the QIAGEN miniprep kit following the manufacturer's protocol (May 2012). The bacterial cell culture was centrifuged for 10 min at 3000 xg (KUBOTA 8100). The supernatant was discarded and excess media removed. The pellet was subjected to QIAGEN miniprep protocol and reagents. Plasmid DNA was eluted with 50 μ l nuclease free water (BDH chemicals) and concentrations determined by measuring the absorbance at 260 nm of 1 μ l in a NanoDropND-1000 spectrophotometer (Inqaba Biotechnology). These plasmids were the source for insert PCR.

2.5. Insert amplification by PCR

Polymerase chain reaction was used to confirm the presence and the size of inserts after cloning and transformation using bacterial cell lysate as template DNA. To do this, randomly selected colonies were picked using a pipette tip and added to 50 μ l nuclease free water (BDH chemicals). After 5 min at 100 °C in a digital dry bath

(Labnet), the tubes were placed on ice to cool. A PCR cocktail was prepared by adding 12, 5 µl of (2x) GoTaq® green master mix (Promega), 1 µl (10 pmol/µl) of each primer pair (Table 2.2) and 10.5 µl of cell lysate to make a 25 µl reaction.

In order to amplify DNA for library construction and sequencing, a proofreading polymerase was used. The reaction cocktail (50 µl) comprised of 5 U TaKaRa Ex Taq enzyme, 1x Ex Taq buffer (TaKaRa Clontech), 4 µl (2.5 mM each) dNTPs (all from TaKaRa Clontech), 1 µl (10 pmol/µl) of each primer pair (Table 2.2), 500 ng DNA and water.

As PCR control, a reaction with the appropriate vector without insert was included. PCR reactions were set at 92 °C for 1 min for denaturing the DNA, melting temperature as per Table 2.2 for 30s for primers to anneal and 72 °C for 4 min for extension for 35 cycles (Table 2.2). The PCR products were analysed by agarose gel electrophoresis (2.6).

Table 2.2: Primer sets used for colony PCR.

Vector	Primers pair	Annealing temperature (°C)	Cycles
pET102/D-TOPO	T7 5' TAGTTATTGCTCAGCGGTGG 3' TrxFus 5' TTCCTCGACGCTAACCTG 3'	56	35
pGEM-T	T7 5' TAATACGACTCACTATAGGG 3' M13 5' CAGGAAACAGCTATGAC 3'	54	35
pCVEPI585042	M13 Ff 5' GTAAAACGACGGCCAG 3' M13 Rev 5' CAGGAAACAGCTATGAC 3'	56	35

2.6. Agarose gel electrophoresis

DNA was separated using either 2% agarose gel electrophoresis for smaller (<600 bp) fragments and 1% for bigger (>600 bp) DNA fragments depending on the expected sizes. Sizes were determined by comparing to a 1 Kb Hyperladder maker (Bioline). Agarose gels were prepared using 1x Tris-Acetate-EDTA (1xTAE; Appendix A) buffer and stained with SYBR® Safe (Invitrogen) to visualise the DNA. The gels were visualised under UV light (Auto Chemi® system, UVP Bioimaging systems) and a picture taken. Photos were printed with the “invented” option to save on black ink.

2.7. DNA precipitation

To concentrate DNA, samples were precipitated and resuspended in smaller volumes. DNA was precipitated by adding an equal volume of isopropanol (Labchem) and mixing well. The mixture was incubated at 4 °C overnight. Next day the DNA was collected by centrifugation at 13 000 xg in a micro centrifuge (Merck Millipore) for 30 min. The supernatant was discarded without disturbing the pellet. The pellet was washed with 30 µl of 70% ethanol (Merck Millipore) to remove excess salt that may have precipitated with the DNA. Tube was centrifuged at 13 000 xg for 20 min. Ethanol was removed from the tube by carefully placing a pipet tip very close to the pellet but not touching it and aspirating the liquid. The pellet was air dried for 10 min at room temperature and then resuspended in desired volume of nuclease free water (BDH chemicals).

2.8. Crystal violet staining of agarose gel

DNA can be visualised using crystal violet but with lower sensitivity compared to SYBR® Safe (Rand, 1996). This is exploited when large amounts of DNA needs to be separated by electrophoresis and the bands cut out for library construction. Using this method, the agarose gel is prepared as in section 2.6 but 10 µg/ml crystal violet (BDH chemicals) is added instead of SYBR® Safe. The same concentration of crystal violet is added to the running buffer. The gel is viewed on a white light box (Concordia) and separation can be monitored during electrophoresis. The expected bands are then excised from the gel and DNA extracted as in section 2.9. DNA separated and extracted in this way may have increased cloning and transfection efficiency (Rand, 1996).

2.9. DNA gel extraction

After agarose gel electrophoresis the desired DNA fragments were cut out directly from the gel and extracted. The DNA from the portion of gel was purified with a QIAGEN gel extraction kit following manufacturer's protocol. DNA fragments were eluted with nuclease free water (BDH chemicals) and the concentration determined by measuring the absorbance of 1 µl in a NanoDropND-1000 spectrophotometer (Inqaba Biotechnology) at 260 nm.

2.10. Phagemid vector preparation

Phagemid vector pCVEPI585042 was provided by Gupta and Chaudhary (University of Delhi South Campus, India). It was used to construct an AHSV-4 genome-targeted phage display library. Glycerol stock of the vector in TG1 cells was scraped into 25 ml

of LB. This was incubated at 37 °C shaking at 240 rpm overnight. The cells were collected by centrifuging at 13 000 xg for 15 min. The collected pellet was subjected to plasmid purification as in section 2.4.

The purified vector was prepared for cloning by linearising with *PmeI* restriction enzyme (NEB) following the manufacture's protocol. The enzyme linearises the 3.4kb plasmid and forms blunt ends. The reaction comprised of 1 µg of vector DNA, 2µl (10x) CutSmart® buffer (NEB), 1µl (10 U/µl) of *PmeI* (NEB) and water to make up a 20 µl. The reaction was incubated at 37 °C overnight. The next morning the enzyme was heat inactivated by incubating at 65 °C for 20 min and the digestion was checked by agarose gel as in section 2.6. After it was observed that the enzyme cuts the plasmid, another reaction was prepared as before. However, 1x T4 ligase buffer (Promega) was used instead of the *PmeI* buffer to check if *PmeI* will cut the vector in 1x T4 ligase buffer. This was necessary because *PmeI* is included in the ligation reaction to re-cut self-ligated plasmid. This enhances ligation efficiency. The reaction was prepared as before and incubated at 25 °C overnight. The T4 DNA ligase (Promega) is optimal at 25 °C in T4 ligase buffer according to manufacturer's protocol. Reaction was inactivated and separated as before in agarose gel. This linearised plasmid was used for library construction.

2.11. Genome-targeted phage display library construction

To minimise the amount of vector DNA in the library, the AHSV-4 cDNA fragments were amplified by PCR from the plasmids as in section 2.5 using M13 primers listed in Table 2.2. Five PCR reactions were prepared for each of the 13 fragments to avoid biasness. After PCR, the five reactions were pooled and 5 µl of the PCR product was

analysed on an agarose gel. The remaining PCR products were precipitated with isopropanol as in section 2.7. The pellet was air dried for 10 min at room temperature and re-suspended in 20 µl of nuclease free water. To remove any remaining vector DNA, the PCR products were separated by crystal violet stained agarose gels as in section 2.8 and bands purified as in section 2.9. The fragments were the source of the AHSV “genome” for library construction.

2.11.1. Fragmentation by *DNase I* digestion

Digestion reactions were reduced from 13 reactions to only four reactions. This was done by combining fragments with similar sizes as listed in Table 2.3. This was irrespective of whether the protein is a major, minor or non-structural protein.

Table 2.3: *Combination of fragments of similar sizes.*

Groups	Contents
Combo 1	NS1, VP2-1, VP 2-2, VP5
Combo 2	NS2, VP3, VP6, VP7
Combo 3	VP1-1, VP1-2, VP4
Combo 4	NS3

DNA digestion with *DNase I* (Roche) was performed using methods previously described (Wang *et al.*, 1995; du Plessis and Jordan, 1996; Gupta and Chaudhary, 1999; Bentley *et al.*, 2000; Fehrsen *et al.*, 2005) with minor modifications. Digestion reactions were prepared on ice. Different concentrations of *DNase I* (Roche), 2.5 U/ml, 2 U/ml, 1.5 U/ml, 1.25 U/ml and 0.6 U/ml, were prepared in cold 1x *DNase I* buffer and preliminary digestion reactions performed. Five tubes containing 1 µg fragment DNA was diluted in cold *DNase I* buffer to make a 10 µl mixture. To each of the five tubes, 3.5 µl of the diluted *DNase I* enzyme was added and mixed. The tubes were placed

on a rack and incubated in a 15 °C water bath for 10 min. The reactions were stopped by adding 2 µl of stop solution and mixed gently. An aliquot (10 µl) of the digested DNA was separated on a 2% agarose gel (2.6). After the correct *DNase I* concentration to achieve the desired fragment sizes i.e. 50 bp - 600 bp was identified, reactions were up-scaled to five reactions for each combination of fragments. After DNase digestion the five reactions were pooled. An aliquot (5 µl) was analysed on agarose gel electrophoresis to confirm the size. Fragments were then concentrated using isopropanol as before, separated using crystal violet stained agarose gels (2.8), and DNA between 50 bp to 600 bp extracted as in section 2.9. These fragments were then prepared for cloning into the phagemid vector.

2.11.2. DNA fragment termini modification

To be able to clone into the vector pCVEPI585042, the fragment ends had to be made blunt. Blunt ending of DNA fragments was performed using the method of Du Plessis and Jordaan (1996) with minor adjustments. In this method, 1 µg of DNA was mixed with 5 µl (10x) T4 polymerase buffer (Promega), 2 µl of 100 µM dNTPs (Promega), 5 U of T4 DNA polymerase enzyme (Promega) and water to make a 50 µl reaction. The reaction was incubated at 15 °C for 1 hour after which 1 U of Klenow fragment (Promega) was added to the reaction and incubated at 37 °C for a further 30 min. To prevent the fragments from ligating to each other and forming concatemers they were dephosphorylated. After blunt-ending, dephosphorylation continued directly by adding 6 µl (10x) Antarctic phosphatase buffer (NEB), 5 U Antarctic phosphatase (NEB) and 3 µl of water to 50 µl of the previous reaction to make it a 60 µl reaction. This was then incubated at 37 °C for 15 min. The reaction was heat inactivated at 65 °C for 5 min and placed at room temperature to cool. DNA was purified with a QIAGEN PCR

purification kit following the manufacturer's protocol and eluted in 30 μ l of nuclease free water (BDH chemicals).

2.11.3. DNA ligation

The blunt ended, dephosphorylated AHSV fragments were ligated in the *PmeI* digested vector (2.10). Ligation was performed at a high vector to insert ratio i.e. 10:1 using the T4 ligase manufacturer's protocol. Each ligation reaction contained approximately 50 ng blunt DNA fragments of each combo separately, 500 ng linearised pCVEPI585042, 2 μ l (10x) T4 DNA ligase buffer (Promega), 1 mM ATP (Thermo scientific), 5 U *PmeI* (NEB), 2 μ l 50% PEG 4000 (Thermo scientific), 5 U T4 DNA ligase enzyme (Promega) and nuclease free water (BDH chemicals) up to 20 μ l. The reaction was mixed gently and incubated at 25 °C overnight. The following morning the reaction was incubated at 16 °C for two hours and inactivated by placing at 65 °C for 5 min. This ligated DNA was then precipitated using isopropanol and the pellet resuspended in 10 μ l nuclease free water.

2.11.4. Electroporation

The ligation reactions of each combo were electroporated separately into *E. coli* TG1 cells. Electroporation cuvettes (0.1 cm gap, VWR, Europe) were pre-chilled on ice for about 10 - 15 min. Each ligation was divided into two tubes of 5 μ l to which 40 μ l of *E. coli* TG1 electroporation-competent cells (Agilent technologies) were added. This was quickly transferred to the cold electroporation cuvette and levelled by tapping on a hard surface. The cells were electroporated using a Biorad Gene Pulser II (BIO RAD), set at 200 Ω , 1700 V and 2.5 μ F and immediately transferred to 1 ml LB growth media

which was pre-warmed at 37°C. The cells were then incubated at 37 °C for an hour shaking at 240 rpm. The two tubes per combo were pooled and 10 fold dilutions were prepared in fresh LB. One hundred microliters of each dilution was plated on pre-warmed LB/Amp small plates (9 cm). The remaining sample was centrifuged at 13 000 xg for 10 min and resuspended in 400 µl fresh LB and 200 µl plated on each of the two big plates (16 cm). All plates were incubated overnight at 30°C. The following morning colonies were counted. An average colony forming unit (CFU) was used to calculate the library size using the formula: CFU x dilution factor x plated fraction. Colonies for each combo from the dilution plates were randomly picked and fragment size confirmed by colony PCR as in 2.5. The remaining colonies were scraped off and stored in 2x TY containing 15% (v/v) glycerol at -70°C.

2.11.5. Phage rescue

The primary library consists of phagemids in the bacterial cells. Phages are then rescued for panning with the aid of a helper phage such as M13K07 (NEB). One tube of each of the combos was removed from the -70 °C and allowed to thaw completely. This was mixed well. An aliquot of each combo was pooled proportionally into 100 ml of 2x TY with ampicillin and glucose (2x TY/Amp), making sure the starting optical density at 600 nm (OD_{600}), was between 0.05 - 0.06 but sufficient bacteria to represent each combo. The cells were incubated at 37 °C shaking at 240 rpm and periodically checked until it reached exponential growth where OD_{600} is 0.5-0.6. Forty millilitres (the rest were kept, see below) of the cells were transferred to an Erlenmeyer flask and M13K07 helper phage (NEB) which assists in phage packaging and release, was added to the cells at a multiplicity of infection (MOI) of 1:20 (bacterial cells = 8×10^8 CFU/ml when OD_{600} is equal to 1). The cells were then incubated at 37 °C for 30 min

without shaking and another 30 min shaking at 100 rpm. The cells were centrifuged for 15 min at 31 000 xg (KUBOTA 8100) and the supernatant discarded. The pellet was resuspended in 25 ml of 2x TY with 100 µg/ml ampicillin and 25 µg/ml kanamycin (2x TY Amp/Kan) but no glucose and transferred to another Erlenmeyer flask. The flask was incubated overnight at 30 °C shaking at 240 rpm. The next day the cells were centrifuged at 31000 xg (KUBOTA 8100) for 15 min. This time the supernatant was transferred to a sterile tube and the pellet discarded. Phage was precipitated by adding 1/4 volume of 20% polyethylene glycol 6000 (PEG) with 2.5 M NaCl (PEG/NaCl) to the supernatant. This was mixed well and incubated on ice for an hour. Phages were collected at 31000 xg (KUBOTA 8100) for 15 min and the supernatant decanted. Phage pellet was resuspended with 500 µl 1x PBS and stored in 50 µl aliquots in glycerol at -70°C. These phages were used in panning.

The remaining cells (60 ml at 0.5 - 0.6 OD₆₀₀) were centrifuged at 31000 xg for 10 min, the pellet resuspended in 1 ml 2x TY of 15% (v/v) glycerol stocks and aliquots of 200 µl were prepared and stored at -70 °C until needed. Plasmid was isolated from half of this remaining library from which DNA was prepared for high throughput sequencing to determine whether the whole genome was covered in the library. The plasmid was purified as in 2.4 and inserts were amplified by PCR in five reactions as in 2.5 using the M13 primers. The PCR products were precipitated as in 2.7, separated by crystal violet gel as in 2.8 and band extracted as in 2.9. The purified DNA library was sent for sequencing at ARC-OVI Biotechnology Platform for Illumina high throughput sequencing.

2.11.6. Determining phage titre

Phage titring determines the number of infectious particles in a sample. To determine the titre of phage stocks, midlog phase TG1 cells are required. Firstly an overnight culture of the TG1 cells from a glycerol stock was made. This was then diluted 1/100 in fresh 2x TY medium and incubated at 37 °C until the OD₆₀₀ is 0.5-0.6. The phage stock was diluted tenfold in 2x TY. Equal volumes of phages and exponential growing TG1 cells were mixed and incubated at 37 °C for 30 min without shaking. Afterwards, the tubes were mixed well and 100 µl of each dilution was plated on tryptone yeast extract (TYE) agar plates with 100 µg/ml ampicillin and 2% (w/v) glucose. As a control, 100 µl of exponentially growing TG1 was also plated. Plates were incubated overnight at 30 °C. The titre was calculated by using formula: number of colonies per plate x dilution x 1 ml / volume plated.

2.12. AHSV-4 enzyme-linked immunosorbent assay (ELISA)

To confirm the presence of antibodies to AHSV-4 in the horse sera (2.1) to be used for panning, they were tested on AHSV-4 in ELISA. All samples were tested in duplicate to ensure reproducibility. A 96 well Polysorb microtiter plate (Nunc) was coated with 50 µl of 10 µg/ml of AHSV-4 virus particles (gift from W van Wyngaardt) diluted in 1x PBS, pH 7.4. Milk powder was used as negative control. The plate was incubated at 4 °C overnight in a closed, moist container. The next morning, plates were emptied and each well filled to the brim with 2% (w/v) milk powder in 1x PBS (MPBS). Plates were incubated for an hour at room temperature then washed three times with 1x PBS containing 0.05% (v/v) Tween-20 (Saarchem) (PBST). Horse serum was diluted 200x and 500x in 2% (w/v) MPBST and 50 µl of each dilution was added to the wells and incubated at 37 °C for an hour. Plate was washed three times with 1x

PBS and three times with PBST. After washing, 50 µl Protein A/G peroxidase (Thermo Scientific) diluted 1:10 000 in PBST was added to each well. This was then incubated at 37 °C for 45 min. After washing plate three times with PBST and one time with 1x PBS, 50 µl of substrate solution which contains one o-Phenylenediamine dihydrochloride (OPD) tablet (Sigma) completely dissolved in 5 ml 0.1 M citrate buffer pH 4.5 (0.1 M citric monohydrate, 0.1 M trisodium citrate) and mixed with 2.5 µl of hydrogen peroxide (30%) (Saarchem) (H₂O₂), was added to each well on the plate. This was incubated at room temperature for 10 min. The reaction was stopped by adding 50 µl of 2N sulphuric acid (Associated Chemical Enterprises) and plate was read at 492 nm in a Multiscan EX reader (Thermo Electron Corporation) and analysed with Microsoft excel[®] 2013.

2.13. Horse IgG purification

To be able to perform biopanning, total horse IgG had to be purified from the serum. The horse serum (section 2.1) was diluted 200x in 1x PBS and total IgG was purified using the protein G HP spin trap / AB spin trap kit (GE healthcare life sciences) following the manufacturer's protocol. Eluted IgG concentrations were measured using a spectrophotometer (UV-1800, Shimadzu) at absorbance of 280 nm. The absorbance of a 1 mg/ml of IgG at 280 nm is 1.35, therefore the concentration was calculated using the formula: $(1 \text{ mg/ml} \times A_{280}) / 1.35$ (Bialuk *et al.*, 2011). The IgG was stored in 50 µl aliquots at -20 °C until needed. This IgG was used to coat the plate during biopanning.

2.14. Affinity selection by biopanning

Affinity selection or biopanning was used to select peptides from the library that are recognised and bound by antibodies from immune horses. Panning was performed as previously described (van Wyngaardt and du Plessis, 1998) for each serum sample from all five horses from each of the three time points. The first round of panning was performed by coating two wells, per serum sample, of a polysorp microtiter plate (Nunc) with 100 μ l of 20 μ g/ml of IgG diluted in 1x PBS. The plate was incubated at 4 °C overnight. The next day, unbound IgG was discarded and 300 μ l of 2% (w/v) MPBS was added to each well and incubated at room temperature for an hour. The wells were emptied and washed three times with 1x PBS. The rescued library phages (10 μ l) were pre-incubated for 20 min in 1 ml of 2% MPBS with 0.1% Tween-20 (2% MPBS 0.1%T) and 100 μ l was added to each well. The plate was incubated at 37 °C for an hour. Unbound and non-specifically bound phages were removed by emptying the plate and washing 10 times with 0.1% (v/v) PBST and 10 times with 1x PBS. Afterwards, 100 μ l of elution buffer (0.1 M Glycine-HCl, pH 2.2) was added to each well and let to stand at room temperature for 10 min. The released phages were transferred to a tube containing 50 μ l of 1 M Tris-HCl pH 9.0 neutralising buffer. The phages were used to infect 5 ml of exponentially growing TG1 cells with OD₆₀₀ of 0.5 to 0.6. This was incubated at 37 °C for 30 min and another 30 min shaking at 100 rpm. The infected cells were diluted 10x in four tubes and 100 μ l of each dilution plated on TYE with 100 μ g/ml ampicillin and 2% glucose plates. The remaining cells were centrifuged at 3100 xg for 15 min and pellet resuspended in 400 μ l 1x PBS and 200 μ l plated on similar big TYE. The plates were incubated at 30 °C overnight. Next morning, after colonies on titre plates were counted, all cells were scraped off from plates and suspended in 1 ml 1x PBS and 15% (v/v) glycerol and 100 μ l aliquots stored at -70 °C. Phages were

rescued from cells as in section 2.11.5. Four rounds of panning were done for each of the serum samples following the same protocol. After each round the output phage increase was determined as the output divided by the input. Half the phages rescued after the first round were used as input phages for the second round of panning. The remainder of the phages were kept at -70 °C and used in ELISA to monitor panning enrichment.

2.15. Polyclonal phage ELISA

The degree of enrichment of binding phages during screening was tested by ELISA. This increase in “recognised” peptides displayed on the phage will be indicated by an increase in ELISA signal. A polysorp microtiter plate (Nunc) was coated with 50 µl of 20 µg/ml purified IgG in 1x PBS in duplicate for all horse serum samples to ensure reproducibility. This was incubated at 4 °C overnight. The plate was washed three times with 0.05% PBST and blocked with 350 µl of 2% (w/v) MPBS and incubated at room temperature for an hour. The plate was emptied and 50 µl of phage dilution [2 µl in 200 µl of 2% (w/v) MP/PBST] was added and incubated at 37 °C for another hour. The wells were then washed five times with 0.1% (w/v) PBST and 50 µl of mouse monoclonal B62-FE2 (PROGEN Biotechnik), which binds to pVIII, the major coat protein of filamentous phage M13, in 2% MPBS 0.1%T (1:1000) was added and incubated for an hour. The wells were again washed five times with 0.1% PBST and 50 µl of polyclonal rabbit anti-mouse immunoglobulins / HRP (Dako, Denmark) in 2% (w/v) MPBS 0.1%T (1:1000) added and incubated for an hour. After washing, 50 µl per well of substrate was added and treated as in section 2.12.

2.16. Next generation sequencing (NGS)

To analyse the repertoire of AHSV-4 peptides selected by panning, the phages after round three were chosen for high throughput sequencing although four rounds were performed. The glycerol stock of cells containing the phagemid after round three of panning were inoculated into 25 ml of 2x TY/Amp and incubated at 37 °C for an hour. Afterwards, the cells were collected by centrifugation at 13 000 xg. The cells were divided into different tubes and plasmid DNA was extracted using a miniprep kit as described in section 2.4. The inserts fused to plasmids were amplified by PCR amplified as in section 2.5 using CVEP forward and reverse primers (Table 2.4) with annealing temperature of 56°C. The primers are designed to be close to the insert and flanked by Illumina platform adapters at the ends. An aliquot of the PCR product was analysed by agarose gel electrophoresis and the rest of the sample was precipitated as in section 2.7. The concentrated DNA was separated by crystal violet agarose gel electrophoreses as in section 2.8 and the DNA bands between 50 bp and 600 bp extracted as described in section 2.9. This DNA sample was sent for high throughput sequencing at Inqaba Biotech using the Illumina MiSEQ v3 (150 cycles) platform.

Table 2.4: Primer set used for Illumina MiSEQ v3 sequencing platform. Adapters are in italics.

Vector	Primers
pCVEPI585042	Ff <i>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGGCTAGCAACGCGTCCG</i>
	Rev <i>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCAGGCGCGCCG</i>

2.17. Data analysis

The data received from Illumina MiSEQ sequencing was analysed using CLC genomics workbench version v7.5 (<http://www.clcbio.com/products/clc-genomics-workbench/>) and Microsoft® Excel 2013. The sequence data was received as a set of

paired-end reads sequenced from both directions. The two reads are one fragment that has been sequenced from both ends. The sequences were imported as paired-end reads in CLC genomics workbench. Some of the sequences existed in the pool as one sequence, for example forward read with no reverse read or vice versa. This usually occurs when there has been a problem with DNA fragment sequencing of either read. In this case the forward or reverse read in the pool represented the DNA fragment.

2.17.1. Sequence trimming

The imported sequences still had the primer sequences at each respective end which could interfere with mapping to the reference sequence, thus skewing results. The primer sequences were removed from each paired-end read in both forward and reverse directions and from those reads that existed as single reads. Trimming of the pool of reads was done using the “trim sequence” function which uses a trim adapter list comprised of both forward and reverse primer sequences flanked by an adapter specific for Illumina NGS platform (Table 2.4). After removing the primer sequences of each read, low quality reads and sequences less than 50 bp, the sequences were ready to be mapped against the reference sequence.

2.17.2. Sequence mapping

In order to determine the identity of each read and to check if the whole genome is covered, the trimmed unpanned reads were mapped to the AHSV-4 reference genome. Since AHSV has a segmented genome which also contains untranslated regions which would not produce a functional peptide, a sequence list of full nucleic

acid sequence for all ten open reading frames (ORF) of AHSV-4 segments was created. The trimmed reads were then mapped to the ORF sequence lists using the “map reads to reference” function. A similar process was followed with sequences from the panning process (round three of all three time points), to determine which regions of the segments recognised and bound the purified immune horse IgG antibody repertoire. The mapping output showed the total number of reads that mapped to each of the ORF of the ten segments in the reference sequence list. Each segment’s mapping profile was analysed individually for each horse. Colour schemes were used (see Fig. 3.5) to differentiate paired, reverse or forward reads. The reads that map as paired reads appear as blue, reads that map as forward read only appear green and those that map as reverse read appear red on the mapping output.

2.17.3. Sequence distribution

The mapping data was further analysed using Microsoft® Office Excel 2013 program. The total read count data obtained after mapping the reads to the ORF for each segment was converted to percentages by using the formula: (number of reads mapping to a segment / total number of reads mapping the entire genome) x 100. The sequence distribution for the respective segments for all samples were illustrated by means of pie charts.

The mapping sequences were further analysed to check the positions where they matched on each segment. For this purpose, the total number of mapping sequences for each segment were exported to Microsoft® Excel without gaps. A proportion of the total number was determined using formula: total number of base matches per position / total number of base matches on the segment. Resulting data was illustrated by a

mapping graph showing the number of times each base mapped on the reference segment sequence. Day 0 data was assumed to be baseline and was used to normalise the data of day 28 and day 52. This was done by subtracting the proportion of day 0 from day 28 and day 52.

The mapping observed on the AHSV-4 segments were also compared to see if the five horses identify and bind similar regions on the segments. For example, the VP2 data from day 28 of all the horses was now illustrated on the same graph. The peaks were numbered in ascending order according to the number of peaks and the number of horses recognising a region on the graph. For example, on a graph with four peaks, a region recognised by all horses will be number 1.

2.17.4. Sequence translation and alignment

The sequences were further investigated to check if they are in frame with the vector gene encoding pVIII protein, which is necessary to produce a functional peptide. It was also done to establish if data was “lost” during the translation and if the amino acid sequence mapping resulted in any notable change when compared to the nucleotide mapping pattern. To do this, the untrimmed nucleotide sequences were first translated to amino acid sequences using CLC. To translate the sequences for this purpose, the unpaired “raw” reads were imported in a FASTA format instead of the Illumina sequence format. The forward reads were translated in all three forward reading frames and the reverse reads were translated in all three reverse reading frames. The amino acid sequences that produced a functional peptide were filtered from the pool by searching for the vector sequence on each peptide end. The resultant forward and reverse translation data were grouped to form a new sequence list which was used to

create a BLAST database. The ORF amino acid sequences of the ten AHSV proteins were used as reference and blasted against new peptide sequence list data.

Each peptide that matched was illustrated on a graph by manually counting the number of times an amino acid aligned at a position on the respective proteins. This was done by checking the alignment table in CLC which shows where each peptide matches on the reference protein. The data collected was then used to draw a “per amino acid” alignment graph in Microsoft® Excel. Peptide matches were illustrated as peaks.

To show the mapping profile of peptides on a single peak, a representative of each overlapping clone that matched was selected and used as a query in the peptide list. After obtaining the original sequence with contains the vector sequence, a list was created of representative sequences and then blasted against a single protein from which the peptides were derived from.

Due to the cumbersomeness of this manual approach, it was only done for day 28 horse 2 sequences. It was assumed that if the mapping patterns of the nucleic acid and amino acid matches were the same for a small subset of samples, then it should follow a similar pattern for all the samples if treated the same. The peptide alignment was compared to the DNA mapping in 2.17.3.

2.18. Peptide ELISA

The antigenicity of the selected regions on the segments was further analysed using synthetic peptides in ELISA. Because of the costs associated with synthesising peptides, only peptides of four proteins could be tested as they were already available.

The peptides were obtained from Dr Alri Pretorius, ARC-OVI, Pretoria, South Africa. The peptides were 16-mer with 8 amino acid overlaps covering VP2, VP4, VP7 and NS3. All peptides were dissolved separately in 200 µl dimethyl sulfoxide (DMSO) with 800 µl 1x PBS. The peptide sequences and positions are presented in appendix (Table AC2). Depending on the peptide stock solution, small volumes (~1 µl) were diluted to a final volume of 10 µg/ml in 0.1M carbonate buffer, pH 9.6 to minimise the possible effect of DMSO on ELISA. No other coating buffer was attempted for coating. Fifty microliters of the dilutions were used to coat 96 polysorb microtiter plate wells (Nunc) in duplicate and incubated overnight at 4 °C. The plate was washed three times with 0.05% PBST after every incubation step. The next day unbound peptides were discarded and plate was blocked with 2% MPBS. The plate was incubated at room temperature for an hour. About 20 minutes to incubation completion, day 28 and day 0 horse serum were diluted 100x in MPBST and incubated at room temperature. The plates were emptied of the blocking agent and washed. The wells were filled with 50 µl of the diluted serum and incubated at 37 °C for an hour. The plate was then emptied and washed. Each well was filled with 50 µl of protein A/G peroxidase (Thermo Scientific) diluted 1:10 000 in 0.05% PBST which was pre-incubated for 10 min at room temperature. This was incubated at 37 °C for an hour. The plate was emptied, washed and wells were filled with 50 µl Tetramethyl benzidine (TMB) (Life technologies) chromogen solution. The plate was incubated at room temperature while monitoring for colour development. The reaction was stopped and immediately read as in 2.12 at 450 nm. Results were analysed in Microsoft® Excel.

CHAPTER 3: RESULTS:

3.1. Genome-targeted phage display library construction

3.1.1. AHSV-4 DNA fragment preparation

To make the genome-targeted phage display library, the AHSV genome was used in the form of already cloned cDNA. The cloned fragments were amplified from the vector by PCR (Figure 3.1). The sizes are slightly bigger than stated in Table 2.1 because of the position where the primers bound on the vector, which adds an extra ~400 bp in total. Segment 2-1 yielded a faint band but similar size to segment 2-2 as expected. The PCR was repeated with more template DNA and PCR yield increased (not shown). These fragments were gel purified separately and concentrated for fragmentation.

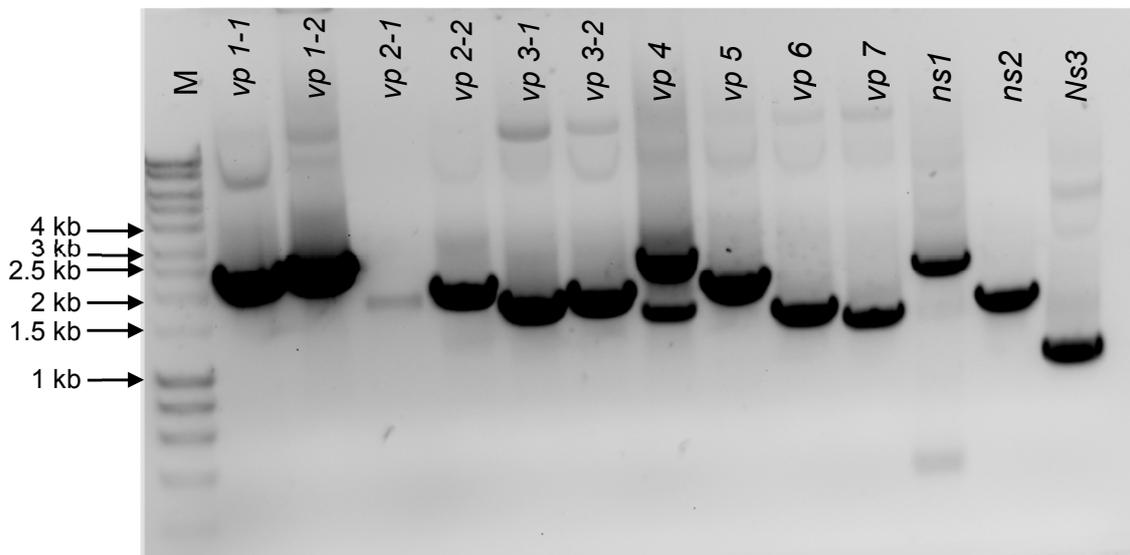


Figure 3.1: Gel showing PCR products of the cloned AHSV-4 genes to be used for library construction separated by 1% agarose gel electrophoresis and stained with SYBR Safe. M represents the 1Kb hyperladder marker (Bioline). The proteins coded by the respective AHSV-4 DNA segment is indicated at the top of the picture and the position of the markers in kilobase (kb) are indicated. *ns is segment encoding non-structural. vp is segment encoding viral protein.

The DNA fragments were amplified by PCR using a proofreading polymerase on a large scale, separated by electrophoresis, cut from the gel and purified. The fragments with similar sizes were pooled into four groups. The PCR fragments were digested with *DNase I* to desired sizes between 50 bp to 600 bp to allow cloning in the phagemid vector. The concentration of *DNase I* to obtain the desired fragment sizes was determined empirically by pilot digestions. Figure 3.2 shows the digestion of 1 µg combo 1 DNA in different *DNase I* dilutions of 1.25 U/ml, 1 U/ml, 0.6 U/ml, 0.3 U/ml and the control. An aliquot was analysed by 2% agarose gel electrophoresis. The desired fragment sizes of 50 bp to 600 bp were obtained when 1µg DNA was digested with a dilution of 1.25 U/ml *DNase I*.

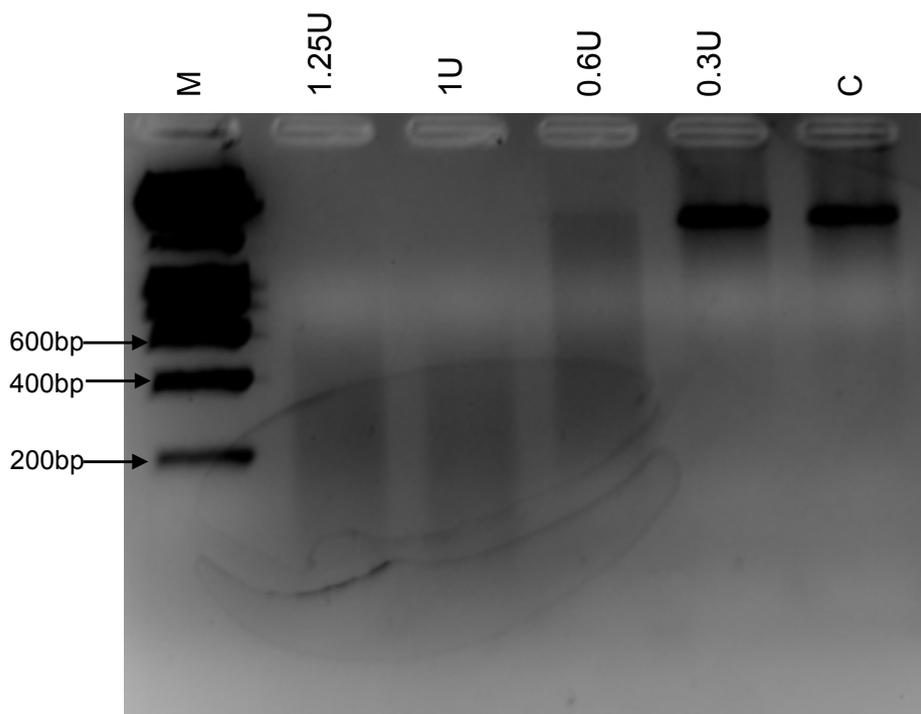


Figure 3.2: Pilot *DNase I* digestion of DNA combo 1 separated by 2% agarose gel electrophoresis, stained with SYBR Safe. Desired sizes of 50 bp to 600 bp were observed when DNA was digested with 3.5 µl of 1.25 U/ml *DNase I*. M is the 1Kb marker (Bioline) and each lane represents the digestion of 1 µg DNA with indicated concentration of *DNase I* indicated at the top of each lane. C is undigested DNA as negative control. The sizes of the markers are indicated as base pairs.

An upscale digestion was prepared using the same conditions as the pilot digestion to digest 5 µg DNA for each combo. The *DNase I* digested fragments were blunt ended and dephosphorylated in preparation to cloning in the phagemid vector pCVEPI585042. The DNA fragments were precipitated and ready for ligation.

3.1.2. Phagemid vector preparation

The phagemid vector pCVEPI585042 was digested with *PmeI* which yields blunt ends, allowing cloning of blunt-ended fragments. The site on the vector cut with *PmeI* allow for cloning of foreign DNA fragments and display of a fusion peptide on the major protein coat (pVIII) coded by *gene viii* of the M13 filamentous phage. Since the ligation reactions are prepared in the presence of *PmeI*, digestion at temperatures which allows T4 ligase to work was tested. The digestion reactions were incubated overnight at 25 °C and 37 °C and aliquots separated by 1% agarose gel electrophoresis. *PmeI* linearises 1 µg DNA at the recommended 37 °C as well as at the lower temperature of 25 °C where T4 ligase works (Figure 3.3). This meant that ligating blunt-ended *DNase I* digested DNA fragments in the vector using both enzymes at 25 °C may produce higher cloning efficiency since the re-ligated “empty vector” will be linearised again with the *PmeI*.

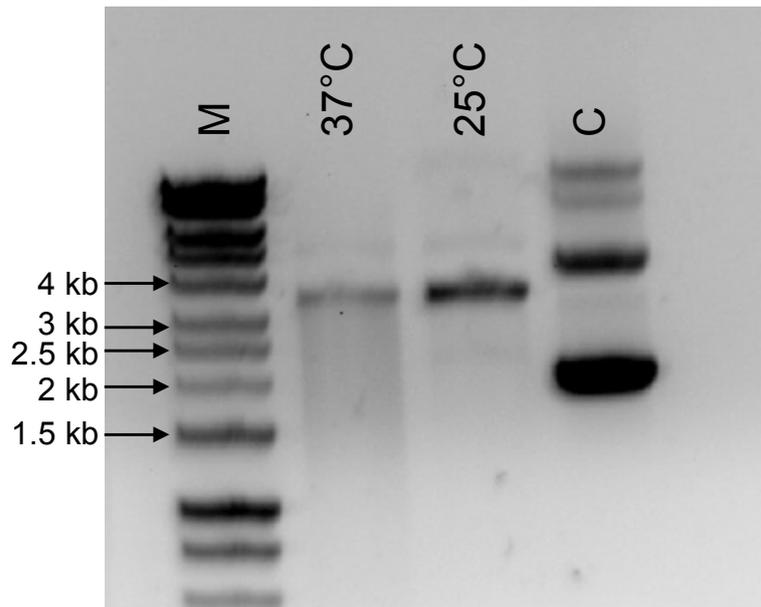


Figure 3.3: Vector pCVEPI585042 cut with *PmeI* at 37 °C and 25 °C separated by 1% agarose gel electrophoresis and stained with SYBR Safe. M represents the 1Kb marker (Bioline) and C is the undigested vector as negative control. The temperature used to digest 1 µg of the plasmid indicated for each lane. The size markers are indicated in kilobase (kb).

3.1.3. DNA ligation and combo formation

The blunt-ended, dephosphorylated fragments were ligated in the linearised phagemid vector (3.1.1) and electroporated into TG1 cells to make the library. Each combo was done separately. Table 3.1 shows the library sizes of each combo which was determined using the formula: CFU x dilution factor x fraction plated. The total size of the pooled primary library was 5.63×10^5 CFU.

Table 3.1: Library size of each combo compared to the theoretically required size to represent the genome with 99% probability.

Combo	Clones required	Actual library size (CFU)	Genome coverage
Combo1	5.9×10^3	1.35×10^5	22x
Combo2	5.6×10^3	2.83×10^5	50x
Combo3	5.6×10^3	1.24×10^5	21x
Combo4	5.0×10^2	2.08×10^4	41x
Total	1.7×10^4	5.63×10^5	33x

* Genome coverage = actual library size / minimum theoretical clones required

To confirm that the fragments were cloned in the vector and to determine the size range, the inserts of randomly selected colonies were amplified using PCR (Figure 3.4). In order for the whole genome to be represented in the library with a probability of 99%, the formula $N = \ln(1-P) / \ln(1-I/G)$ is used where N is the number of recombinants required to cover the genome, P is the desired probability, I is the average size of the cloned fragments (100 to 600 bp) and G is the size of the target sequence in bp (Clarke and Carbon, 1976). To cover the AHSV-4 genome a library should contain 1.7×10^4 CFU clones. Since the library was 5.63×10^5 CFU with an average fragment size of ~250 bp, this meant that the library was 33 times ($5.63 \times 10^5 / 1.7 \times 10^4$) more than the minimum number of clones required to cover the whole AHSV-4 genome.

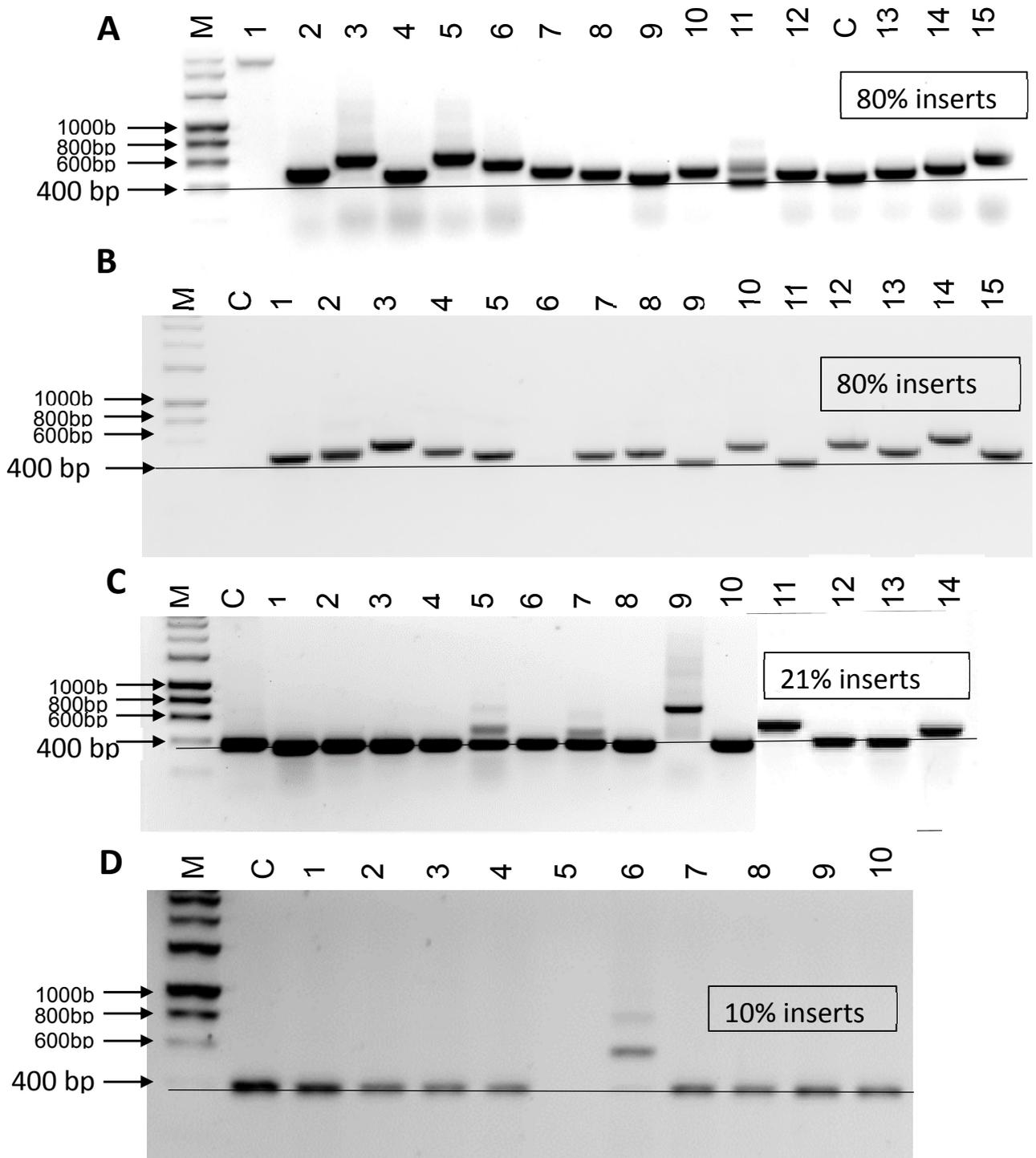


Figure 3.4: Colony PCR products of randomly selected clones separated by 1% agarose gel electrophoresis and stained with SYBR Safe. Clones bigger than 400 bp contains inserts. The box indicates the percentage of inserts per combo. The numbers represent lanes, C is the vector control and M is a 1Kb hyperladder maker (Bioline). A) Combo 1, B) Combo 2, C) Combo 3 and D) Combo 4. The size of the markers are indicated in base pairs (bp).

The control (vector with no insert) is approximately 400 bp when using M13 forward and reverse primers in PCR. The inserts from each combo were cloned with different efficiencies ranging from 10% to 80% (Figure 3.4). In all library combos, the size of the library was much bigger than the minimum required number of clones from 21x to 50x. Therefore, even combo 4 with only 1 in 10 clones with insert (10%) was not a limitation. The actual library for this group was 2.08×10^4 clones and required a minimum of 5.0×10^2 clones. This meant that the library was 41 times the clones required. Combo 3 with 3 in 14 clones with insert (21%) was 21x more than the minimum required. Clones 5 and 7 of combo 3 which looked like they had an insert but also a strong band at 400 bp were however not included in the percentage determination. Even combo 1 and 2 with 12 in 15 clones with inserts (80%) were 22x and 50x more than the minimum required. Table 3.1 shows the summarised library size results. This meant that libraries contained enough clones to continue and provided representation of each segment in the library.

The segments were randomly fragmented, which theoretically means that only 1 in 18 clones in a library will produce an insert in the correct orientation, in frame with the vector sequence to produce a functional peptide on the surface of the filamentous phage, coding for the native AHSV amino acid sequence. This is because a cloned fragment can be in 1 of 2 correct orientations, with three reading frames in either N- or C- terminal; $3 \times 3 \times 2 = 18$ (Clarke and Carbon, 1976; Jacobsson *et al.*, 2003). This means that the total “functional” primary library size is actually 3.12×10^4 clones (5.63×10^5 CFU / 18), which is still almost 2x times more than the minimum required size to represent the genome with 99% probability. Therefore the library was still big enough to represent each segment of AHSV genome and clones that may produce functional fusion peptides were still enough to cover whole genome. An aliquot of the library was

sequenced by high throughput sequencing. The DNA data aligned to the AHSV genome and even though the depth of coverage varies across the gene, there were no gaps, validating the theoretical calculations. Figure 3.5 shows an example of a genome coverage for one of the 10 segments. The next step was to continue with affinity selection of the library.

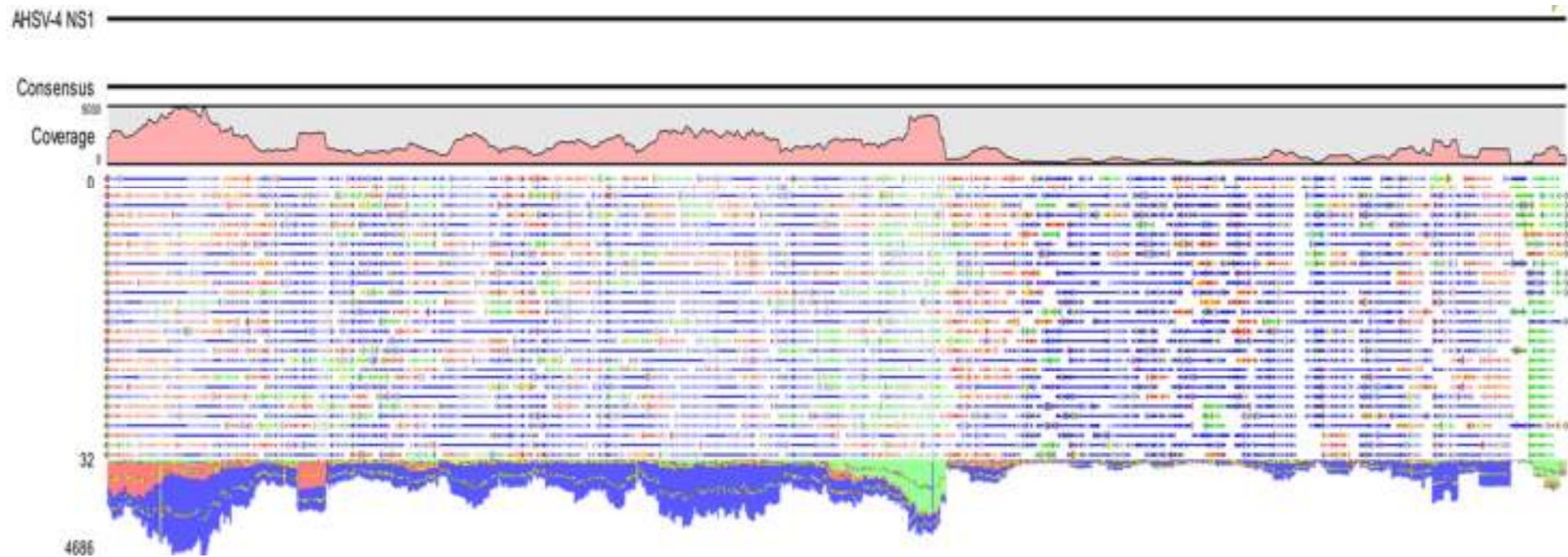


Figure 3.5: CLC Genomics Workbench read mapping representation of the unpanned library mapping against the AHSV-4 ORF segment encoding NS1. The sequences from the library covers the entire gene without any gap/s. The blue colour indicates a paired-end match, green is a forward read match and red is a reverse read match. Reference genome segment is indicated as a bold line at the top. Sequence depth is indicated by numbers on the left.

3.2. Horse serum characterisation and purification

One of the ways to affinity select a desired phage displayed peptide from a library is to immobilise an antibody on a solid surface. In this case the antibodies were from vaccinated horses which need to be purified. To confirm that the vaccinated animals indeed produced antibodies against AHSV-4, the sera were tested in ELISA. The sera of the three time points, day 0, 28, 52 were diluted 200x and tested for binding to whole virus. As expected, day 0 showed the lowest binding signal and day 28 showed the highest signal which decline by day 52. The horses had varying antibody levels against AHSV-4 with horse 1 having the most and horse 5 the least. Horse 4 was an outlier when compared to the other horses in both day 0 and day 52. It had a higher ELISA signal on day 0, and the signal was still higher than the other horses by day 52, which may indicate antibody persistence in this particular horse, although it did not have the highest signal in day 28. Horse 5 had the lowest signal in all three time points which may indicate poor immune response to the vaccine.

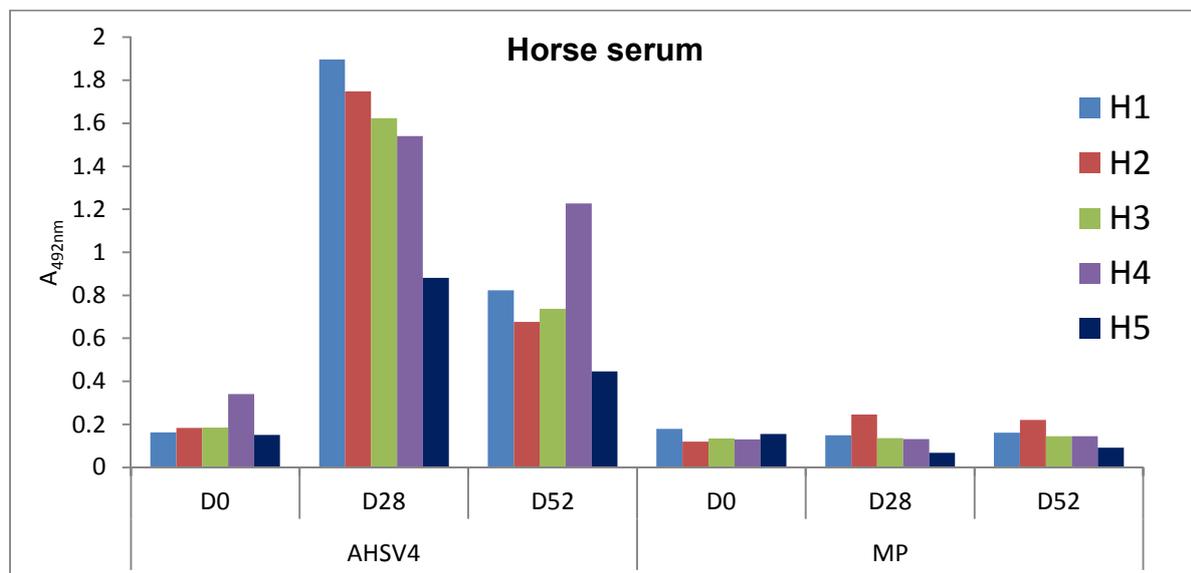


Figure 3.6: ELISA of antisera of the five horses collected pre-vaccination (day 0) and post-vaccination (day 28 and 52) reacting with purified AHSV-4. Each plotted value is an average of a duplicate reactions. Milk powder (MP) acts as the negative control.

The horse antisera samples were also sent to the Virology Department, OVI, for virus neutralisation tests (VNT). The samples showed presence of some neutralisation antibodies on day 28 and lower levels on day 52 (data not shown). Day 0 had no neutralising antibodies in all five samples (data not shown), validating its use as a negative control.

The immunoglobulin G (IgG) was purified from all horse serum samples and the concentration was determined to range between 1.4 to 7.9 mg/ml (Table 3.2). These IgG concentrations were lower than the average 13.4 mg/ml observed when horse sera was purified elsewhere (Perkins *et al.*, 2003). These IgGs were used to affinity select binders from the genome-targeted phage display library.

Table 3.2: Concentration of IgG after purification of antisera of the five horses collected before (day 0) and after (day 28 and day 52) vaccination with live attenuated monovalent AHVS-4.

Horse serum	IgG concentration (mg/ml)				
	H1	H2	H3	H4	H5
Day 0	5.2	5.9	5.5	5.8	7.9
Day 28	2	1.4	2.4	2.4	2.1
Day 52	6.1	5.8	5.5	5.3	6.2

3.3. Affinity selection by biopanning

The library was affinity selected for the presence of phage surface displayed binders using the purified horse IgGs. Selection of antigenic peptides entails incubating a phage library with an immobilised ligand, washing off unbound phage, eluting and neutralising the phage. The released pool of phages are amplified by infecting *E. coli*, rescued and used in a subsequent round of panning. In theory, only phage that displayed a functional fusion peptide identified by the ligand should be selected and amplified. To monitor the panning process, the total number of phages released after

each round was determined and an increase was an indication of enrichment of the binding phages. The output increase of each of the four rounds of panning with purified anti-AHSV-4 horse IgG is shown in Table 3.3. The input and output phage and also the percentage recovery data of each panning round is presented in Appendix C (Table AC1).

Table 3.3: Increase in phage output after each of the four rounds of panning with anti-AHSV-4-IgGs of horses of days 0, 28 and 52.

Library		OUTPUT INCREASE*			
		R 1	R 2	R 3	R 4
Day 0	H1	-	23x	22x	1x
	H2	-	1x	27x	1x
	H3	-	10x	100x	2x
	H4	-	85x	12x	3x
	H5	-	2x	18x	1x
Day 28	H1	-	20x	1x	1x
	H2	-	1x	1x	1x
	H3	-	3x	3x	1x
	H4	-	10x	0	1x
	H5	-	116x	0	1x
Day 52	H1	-	14x	1x	1x
	H2	-	1x	2x	1x
	H3	-	1x	15x	1x
	H4	-	5x	1x	1x
	H5	-	5x	20x	1x

*output increase = round (n)/round (n-1)

In theory, total phage output increase should follow a similar pattern already observed (Figure 3.6) where panning with day 0 IgG has no increases as the sera were from naïve horses and had no antibodies to bind the surface displayed peptides. Panning with day 28 IgG should have total phage output increases which declines with day 52 IgG. In general, output increase varied for the different time points and did not follow

the expected pattern. Phage output increases were highest when library was panned with day 0 IgG and the least with day 52 IgG. There was no panning phage output before round one and thus output increase for that round could not be determined. Phage output increase was observed with round two and three and dropped by round four with all IgG samples (Table 3.3). Phage output increases were also variable among the different horses within a time point. Horses 2, 3 and 5 performed better with day 0 IgG where there was an increase from rounds two and three followed by a drop in round four. The other horses 1 and 4 only had output increase in round two and which declined with subsequent rounds. When library was panned with day 28 IgG, all horses had an increased in phage output in round two and either remained the same (horse 2 and 3) or declined with the subsequent rounds. With day 52 IgG, horses 2, 3 and 5 performed better as they continued to have an increased phage output in round three which declined by round four. Horses 1 and 4 only had phage output increases in round 2 and declined with subsequent rounds.

Phage output increase or enrichment was further investigated using ELISA. It is assumed that an increase in binding signal in ELISA in subsequent rounds of panning with horse IgG will indicate enrichment of selected phages displaying functional fusion peptides. As expected, although there was phage output increase in day 0 (Table 3.3), there was generally no increase in binding signal of IgG to phages after four rounds of panning except for horse 2 (Figure 3.7). Horse 2 was an outlier and showed enrichment with every round of panning which drops in the fourth round. This was not expected since horse 2 showed very low phage output increases with day 0 IgG (Table 3.3). The signal of horse 2 IgG binding to unpanned library phages is similar to that of round three panning (Figure 3.7), which may suggest the possibility of anti-phage antibodies in the serum or non-specific binding. The signal decline after the first round

but the signal increases again in the subsequent two rounds and then drops in the fourth round of panning.

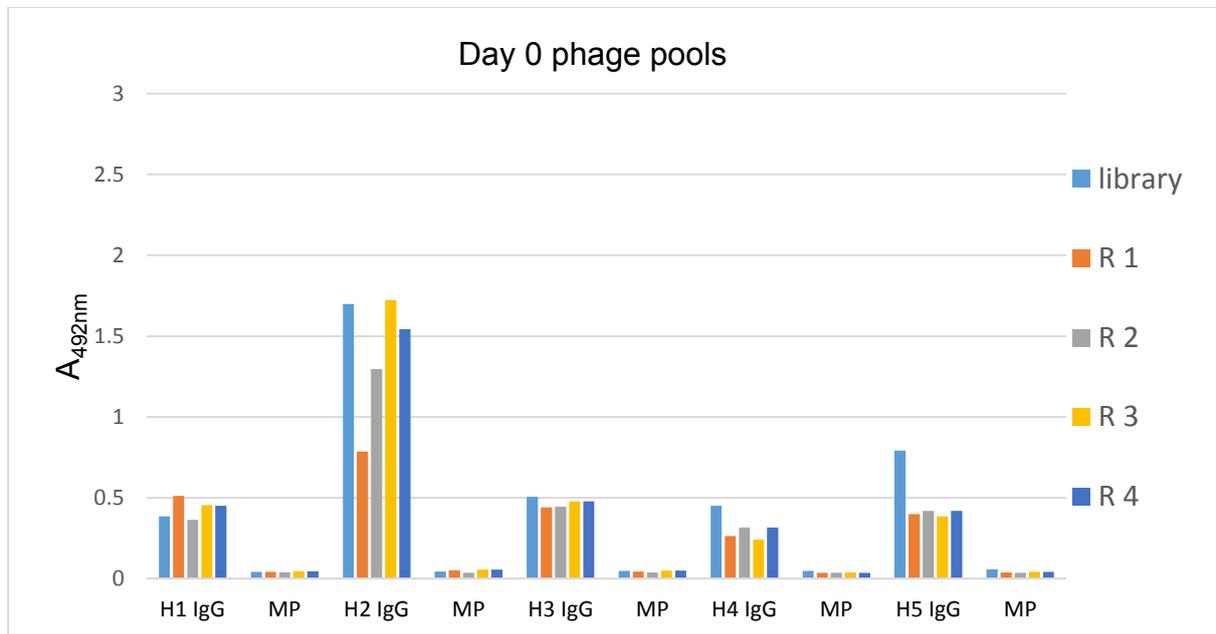


Figure 3.7: ELISA of the phage pools rescued from the unpanned library and phages selected during the four rounds of panning (R1 to R4) with day 0 IgG of all five horses, reacting with their respective IgGs. Each plotted value is an average of a duplicate reactions. MP is negative control

An increase in IgG binding signal with each round of panning was observed with day 28 IgG selected phages. In general, phages selected with day 28 IgG showed high antibody to phage, with signals exceeding 2.5. This was in accordance with whole virus ELISA which showed high binding signals with day 28 sera (Figure 3.6). Horses 2 and 5 had the highest signals (Figure 3.8). This is in contrast to the phage output increase which showed that these horses had the lowest output increases with day 28 IgG (Table 3.3). There was correlation between output increase and ELISA signals only for horse 3. The pattern varied for horse 1, 2, 4 and 5. Mostly there was no increase in binding signal after round three, which corresponds with the phage output increase results (Table 3.3)

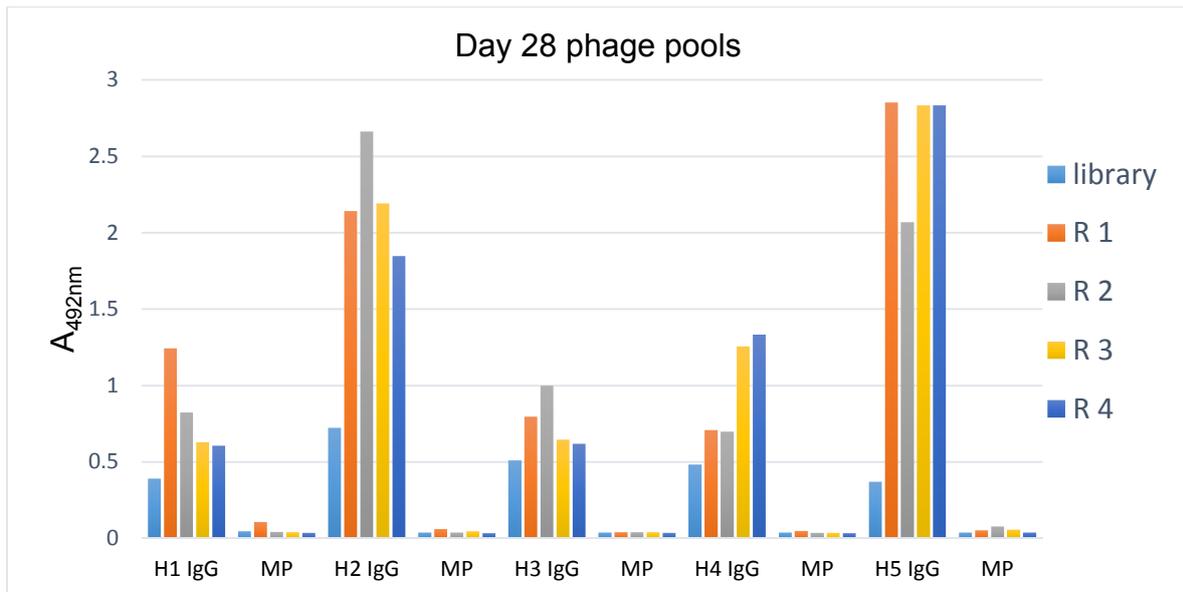


Figure 3.8: ELISA of the pool of phages rescued in the unpanned library and phages selected during the four rounds of panning (R1 to R4) with day 28 IgG of all five horses, reacting with their respective IgGs. Each plotted value is an average of a duplicate reactions. MP is negative control.

Interestingly, there was a big difference in the phage output increase and enrichment in day 52 (Figure 3.9). There was generally no difference in ELISA signal between the different phage pools except for horse 1 and 2 with signals up to 1.5. Horse 2 showed the same ELISA signal pattern as for day 0. Again there was no correlation between the phage output increase observed (Table 3.3) and binding signals (Figure 3.9). This was not expected as whole virus ELISA showed there were still antibodies against AHSV-4 in all horses. There was no marked difference between day 0 and day 52.

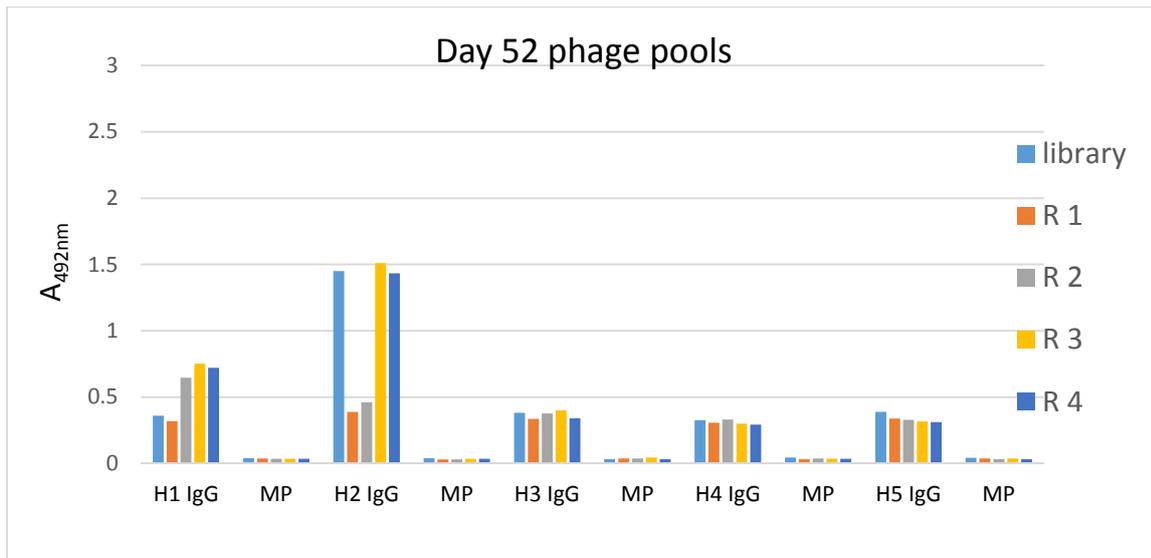


Figure 3.9: ELISA of the pool of phages rescued in the unpanned library and phages selected during the four rounds of panning (R1 to R4) with day 52 IgG of all five horses, reacting with their respective IgGs. Each plotted value is an average of a duplicate reactions. MP is negative control.

3.4. Characterisation of selected fragments

Phage display technology has the advantage of directly linking the surface fusion peptide to the gene encoding it and the sequence of the responsible fragment is identified by sequencing the insert DNA of the filamentous phage vector. This then allows further *in silico* analysis of the selected peptides. From the phage output increases and ELISA results, round three panning was chosen for further analysis as there was little or no enrichment after round three. Since the inserts are a maximum of 600 bp and the vector is 3.4 kb, the inserts were amplified by PCR to minimise the amount of vector sequences obtained. In addition, contrary to normal high throughput sequencing where the combination of reads forms the consensus sequence, in this case each read represents a clone. The sequence data was supplied as paired-end 2 x 75 bp reads for each DNA fragment sequenced.

The reads were imported into CLC genomics workbench program as paired-end sequences. The total number of sequences varied for the different time points and horses. This was not a big concern, a relative comparison was done for matches per nucleotide on a gene and not the total number of sequences. These were processed by trimming as described in 2.17.2. To confirm whether the sequence pools matched the AHSV genome, the trimmed reads were mapped to the AHSV segment sequences with each pair representing a clone. However, it was observed that some sequences mapped to the untranslated region of the segment and contained stop codons. These are an example of the non-specific binders or background phages inherent to phage display. This was worrying as it has potential to skew the mapping results with meaningless sequences that would not produce a functional fusion peptide. Therefore, the sequences were then mapped to only the ORF of the AHSV genes. In this way, only sequences mapping to regions that are capable of forming a native peptide would be selected. About 70% of the sequences could be mapped to the sequence list of the reference genome sequences. The remaining 30% either had sequence errors or was of unknown source (data not shown). In theory, if each portion of the genome is represented equally, then the proportion of the sequenced inserts mapping to each segment should be a factor of the segment size. The proportion of the total count of sequences received for each segment is illustrated (Figure 3.10).

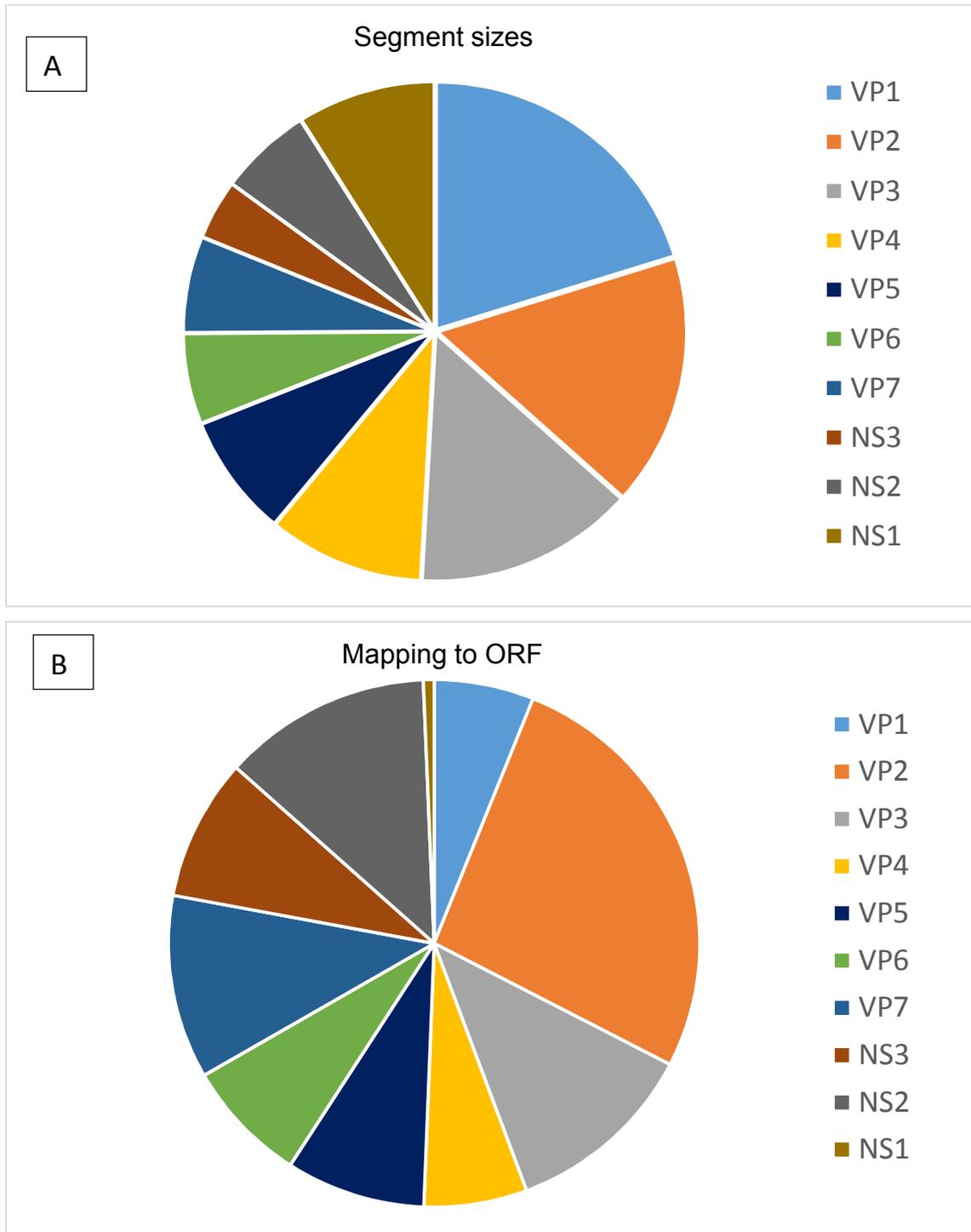


Figure 3.10: Proportional representation of AHSV gene segments. A) The size of each segment as a proportion of the total genome size. B) High throughput sequencing data of the unpanned library shown as a proportion of the number of reads mapping to each segment.

The sequences mapping to segments encoding VP2, VP7, NS2 and NS3 were represented at a higher frequency. This means that these segment sequences were at a higher proportion than the rest. The sequences mapping to segments encoding VP1, VP4 and NS1 were represented at lower frequencies than was expected. There was no substantial difference in the fragment size and frequencies representation of sequences mapping segments encoding VP3, VP5, and VP6. This pattern may have been caused by the readiness of a DNA fragment to be cloned and their ability to grow on both solid and liquid media. Biases in PCR can also play some role in skewing the sequencing results depending on the amplification efficiency of segments. The sequences that amplify better becomes better represented when compared to those that do not amplify as readily. However, when the sequences were analysed for coverage, the insert sequence pool from the unpanned library covered the entire AHSV-4 genome with no gaps, despite potential skewing of representation in the library. For example, NS1 was represented at a lower frequency than the fragment size in the library but was still fully covered (Figure 3.5), thus indicating that under representation of NS1 was not a limitation. The library could therefore be used for affinity selection of fusion peptide binders. Mapping to the other segments looked similar (data not shown).

The library was affinity selected for binders using purified total horse IgGs. Theoretically, when affinity selecting a library, the sequence distribution should change with each biopanning round and be biased towards the binding regions of each DNA segment. Figure 3.11 shows an example of a segment mapping after the panning process which shows that non-binders have been removed from the library. Although four rounds of panning were performed, round three affinity selected phage pool performed better in phage enrichment and thus was sent for high throughput

sequencing. Of interest was sequence data from day 28 as whole serum ELISA (Figure 3.6) and phage enrichment results (Figure 3.8) showed higher signals.

In order to identify the sequence of the linear, potentially antigenic segments, the round three sequence pools of peptides selected with day 28 IgG were mapped to the list of the reference genome sequences. Generally, as expected, there was a change in sequence mapping on the different segments when compared to the unpanned library. Instead of the whole segment area being covered, the sequence mapping with the panned phages showed a more selective clustering which was concentrated on certain regions of the segments. This was indicative of a successful biopanning process, meaning that binders were selected and enriched. There was a difference in sequence distribution per segment from day 0 to day 52 for all horses (Figure 3.12). Day 28 had the most notable change in proportional hits to each segment. It should be noted that Figure 3.12 only reflects the proportional sequence match per segment distribution and not the mapping pattern of the sequences. Although the distribution appear similar, the binding pattern of sequences was different as it will be clear later. As expected, a notable change of sequence distribution was observed with data from day 28. Analysis of the individual segments in the different time points indicated that the depth of sequences mapping to *ns3*, *vp5* and *vp6* were increased by day 28 and decrease to nearly day 0 levels by day 52 for all samples. However, the sequences mapping to *ns3* with day 28 data had a dominant increased depth compared to day 0 data which declined by day 52. Sequences mapping to *vp1*, *vp4* and *ns1* are very few by day 28 in all horses and resurfaces again by day 52 (Figure 3.12). Sequences mapping to *vp1*, *vp2*, *vp3* and *vp7* decreased from day 0 to day 28 and increased to almost starting levels by day 52.

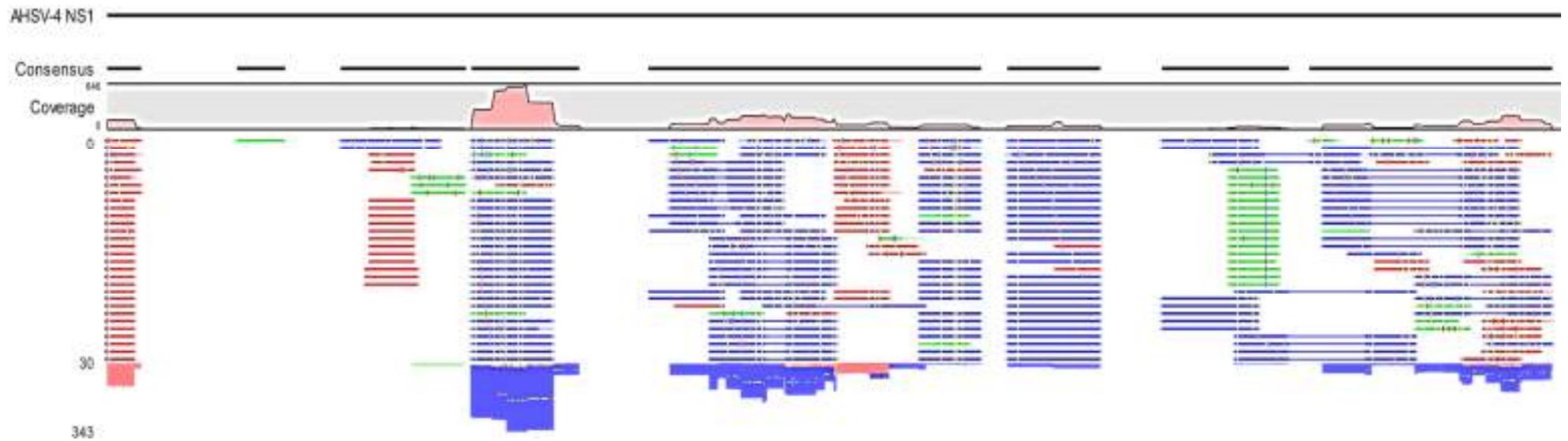


Figure 3.11: CLC Genomics Workbench read mapping representation of round three pool of sequences selected with day 28 IgGs and mapping against the AHSV-4 ORF segment encoding NS1. Gaps indicate that regions of interest have been selected. The blue colour indicates a paired-end match, green is a forward read match and red is reverse read match. Reference genome segment is indicated as bold line at the top. Sequence depth is indicated by numbers on the left

Further analysis of the mapping sequences per individual horse indicated that segments were recognised differently. A segment that has a consistently increased sequence depth in the immunised horses will indicate a potentially antigenic segment. This was determined with comparison of day 0 and day 28 sequences as it was with this data that the difference in depth per segment could be deciphered clearly. The sequences mapping to *ns3* were dominant and consistently enriched in all horses. Although the sequences mapping to *vp6* were increased in all horses, it was second in all horse but horse 2 and 4, where sequences mapping to *vp5* were more increased. All the other sequences mapping to *vp1*, *vp2*, *vp3*, *vp4*, *vp7*, *ns2* and *ns1* were consistently reduced in all horses.

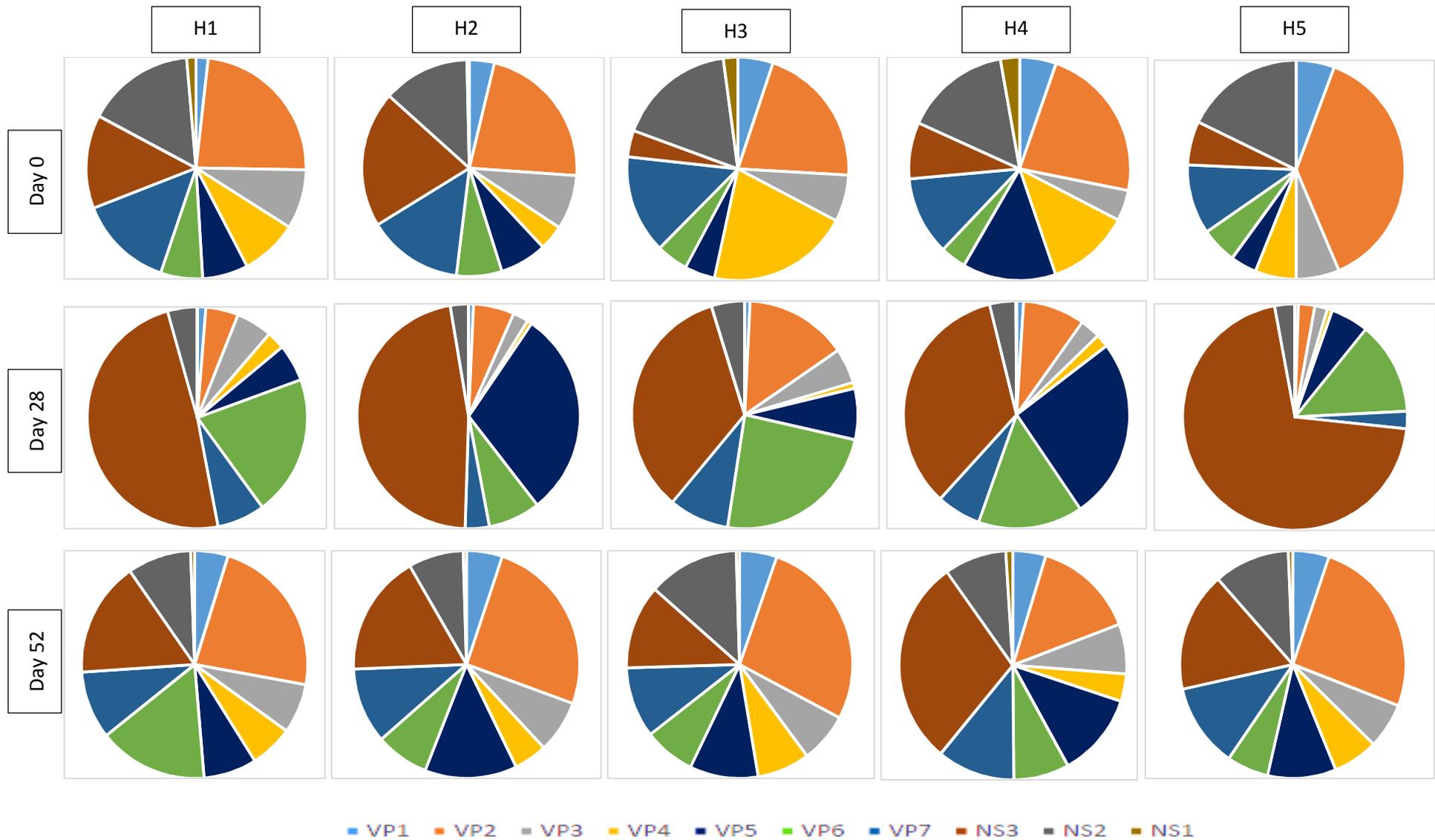


Figure 3.12: Proportions of sequence reads mapping to each segment after round three of library panning with anti-AHSV-4 IgG. Segments are colour coded in a clockwise fashion from VP1.

The sequences mapping to the segments were further analysed to investigate the regions with potential linear epitopes within the genes. These would be regions, on the segments, that have increased sequence depth of overlapping sequences which appear as a peak on the total sequence count proportionality graph (Figure 3.13). The size of the peak indicated the sequence depth on that region. Only day 28 data will be illustrated here because of the previous observed positive results (Figures 3.6, 3.8 and 3.12). Segment encoding NS1 had very few sequences mapped and thus was not selected for further characterisation. All other mapping data is presented in appendix (Figure AC1).

In order to visualise the results, the segments were compared on a single graph for all horses, for example VP2 data of each horse on one graph. This way it could be seen that some of the regions, mostly the dominant regions, were recognised by all horses (Figure 3.13). These regions were numbered in ascending order depending on the number of horses identifying a region in increasing order. Some of the regions were still identified by day 52, which may indicate that such regions provoke production of persistent antibodies with a longer half-life. Day 52 data is presented in appendix (Figure AC1).

One region was identified at the C-terminal half of *vp1* by anti-AHSV-IgG when the five horses were compared (Figure 3.13A). This region was still recognised by day 52 IgGs where almost the whole C-terminal half was recognised albeit at lower signal. Five regions on each segment were observed on *vp2*, *vp4*, *vp6* and *ns2*, however, only region marked 1 was dominantly identified by all horses (Figure 3.13 B, D, E, I). On *vp2*, the region identified by all horses was at the N-terminal end. This region was also still recognised by day 52 IgG. Regions 2 and 3 were also recognised by most horses with the exception of horse 4. Region 2 disappeared by day 52 and only region 3 was

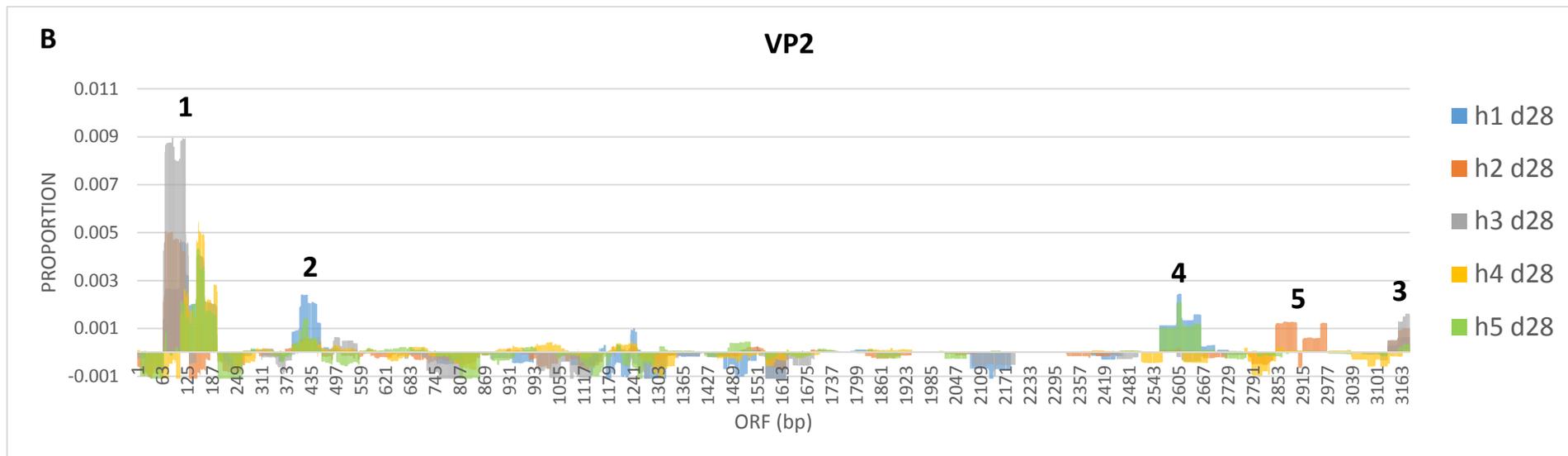
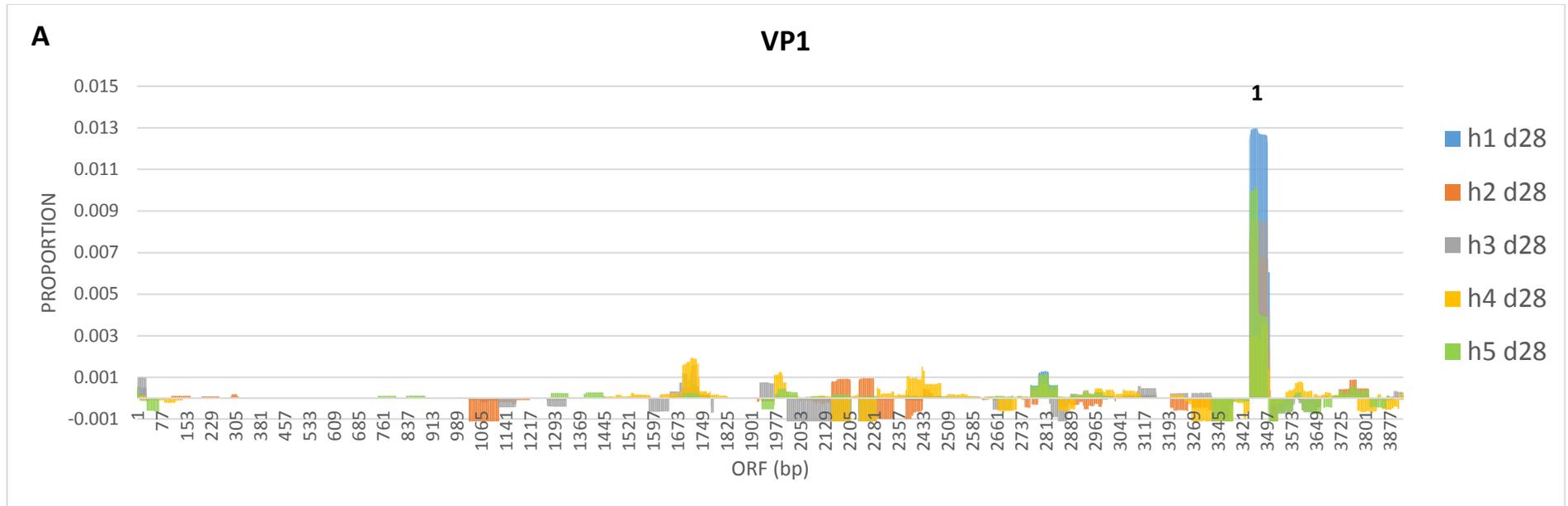
identified by horse 4 which did not recognise it in day 28. Region 4 was identified by horses 1 and 5 and by day 52 was identified by horse 3 and 4 although at very low levels. Region 5 was identified by horse 2 only (Figure 3.13B) and by day 52 all horses identified it at very low levels which may suggest that it may be an important region.

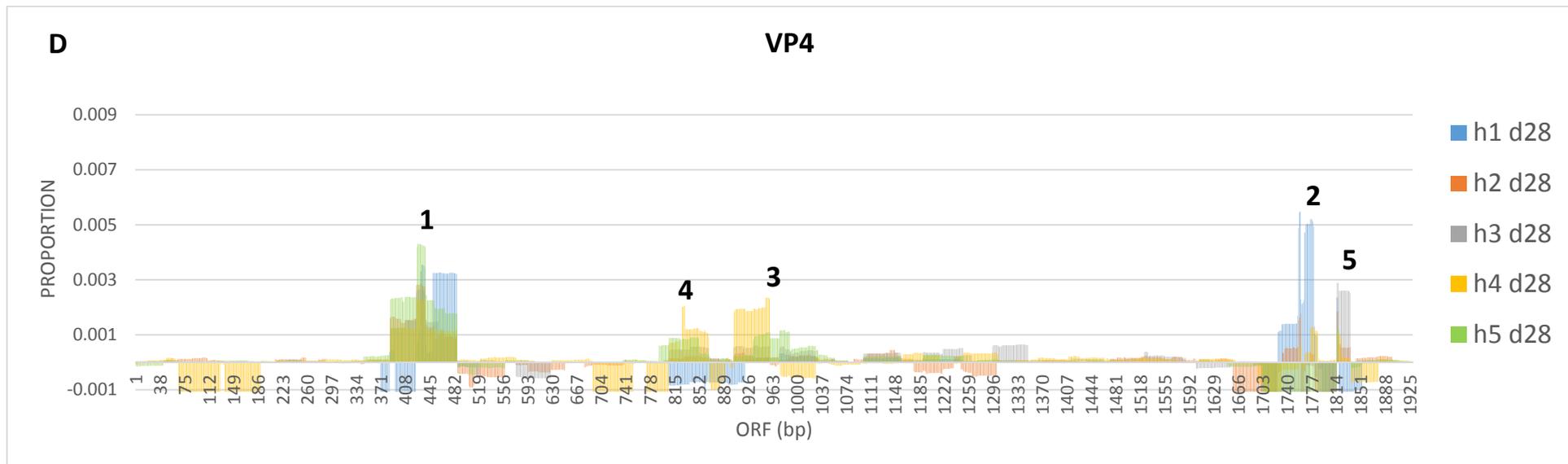
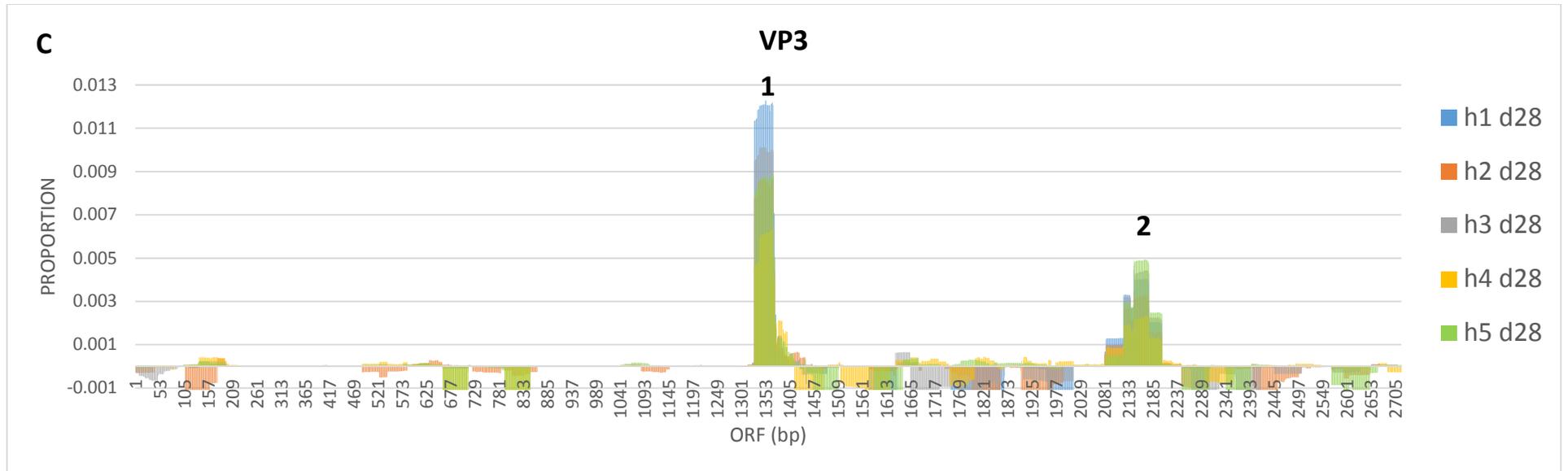
On *vp4*, the region identified by all horses was at the N-terminal half. This region was still reactive by day 52. Region 2 was identified by three horses with horse 1 giving the highest peak and by day 52 only was it recognised by horse 1 and 2. Regions 3, 4 and 5 were identified by horses 3, 4 and 5 (Figure 3.13D) which all disappear by day 52.

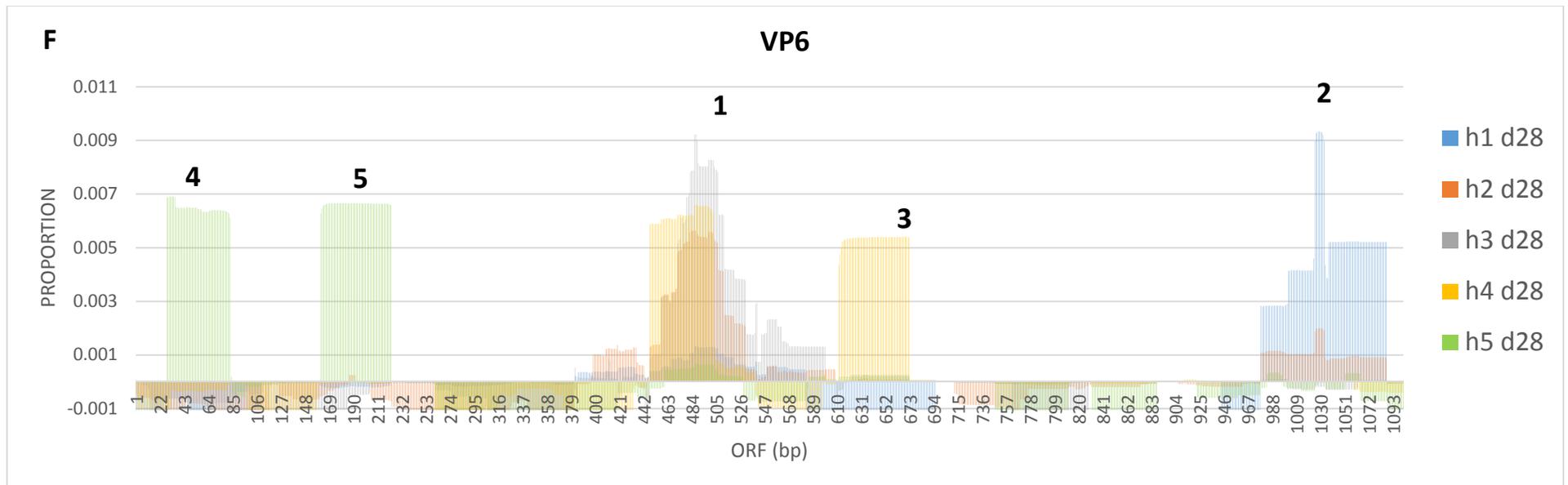
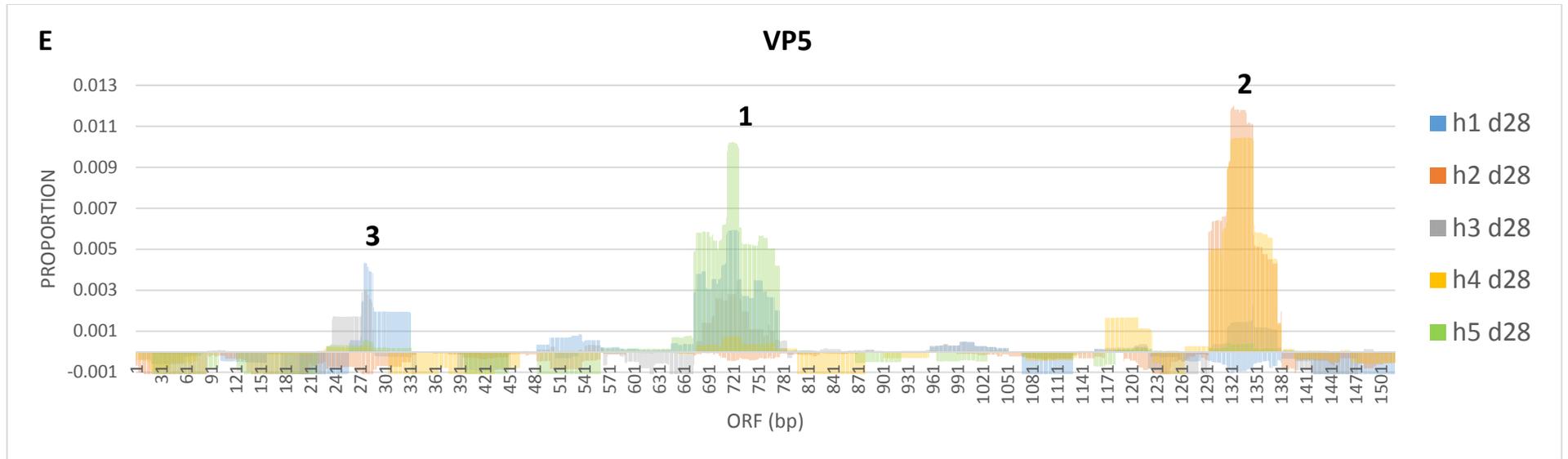
On *vp6*, the region identified by most horses was at the centre. Region 2 was identified by only two horses (1 and 2), region three by horse 4. Although horse 5 identified the dominant region (region 1) as low levels, it had higher peaks for regions 4 and 5 (Figure 3.13E). The region identified by all horses was at the centre for *ns2*. Region 2 on *ns2* was identified by all horses at very low levels, region 3 was identified by most horses except horse 5, region 4 identified by horses 2 and 3 and region marked 5 identified by horse 3. The whole *vp6* was reactive by day 52 albeit at lower levels.

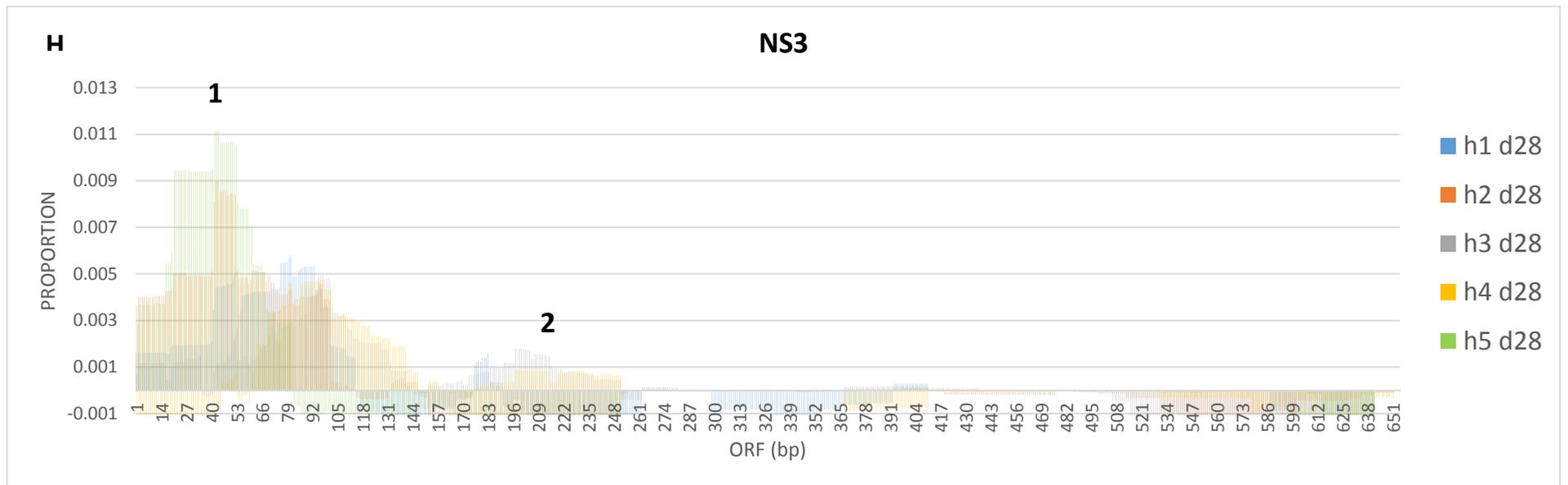
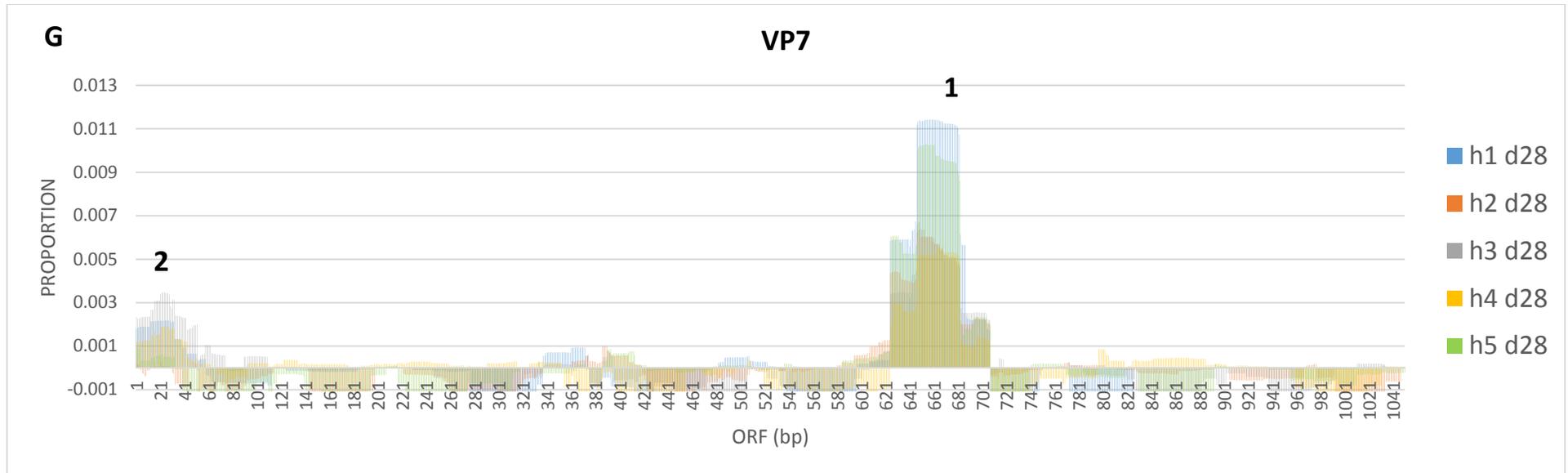
Two regions were identified on *vp3* and *vp7* and both regions were identified by all horses in both segments (Figures 3.13C, G) and were still identified by all horses at lower level by day 52. Three regions were identified on *vp5* where region 1 was recognised by all horses but horse 2, which gave higher signals in region marked 2 along with horse 4 which had lower signals in the region 1. The higher signals observed in region 2 disappeared by day 52. Horse 2 and 4 gave higher signals for region 1 by day 52. Horse 5 had higher peaks in region marked 1 but very low levels in region 2. Region 3 was identified by horses 1 and 3 and by day 52 it was identified by horses 3, 4 and 5.

Two regions were identified on *ns3* but only one region was identified by all horses on *ns3* (Figure 3.13H). Region marked 2 showed higher peaks for horse 4 by day 52.









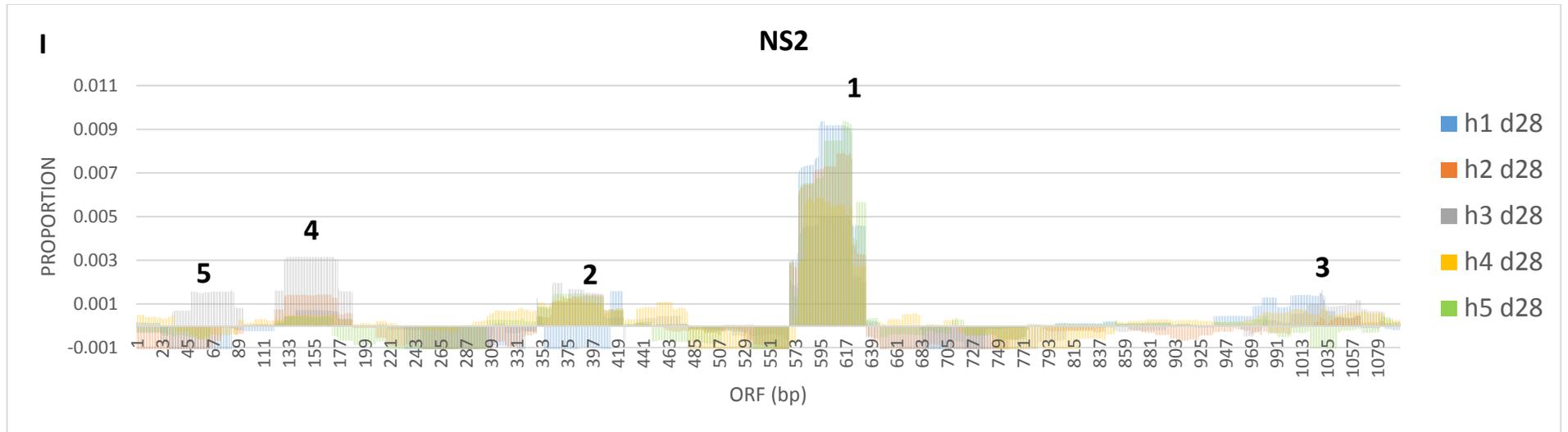
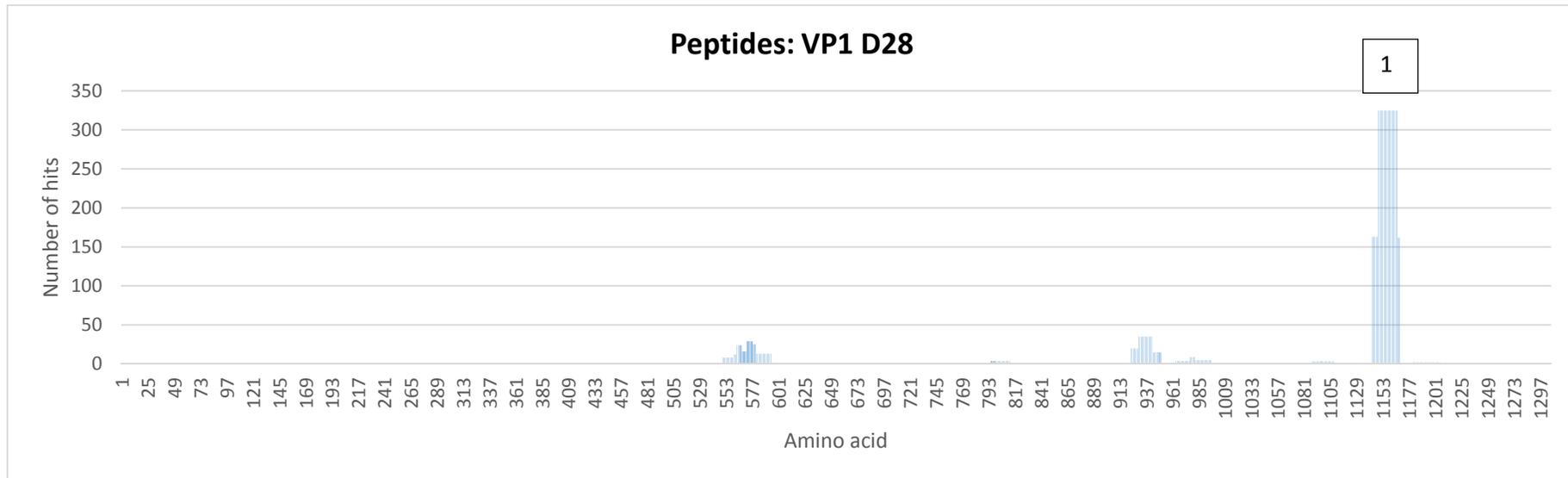
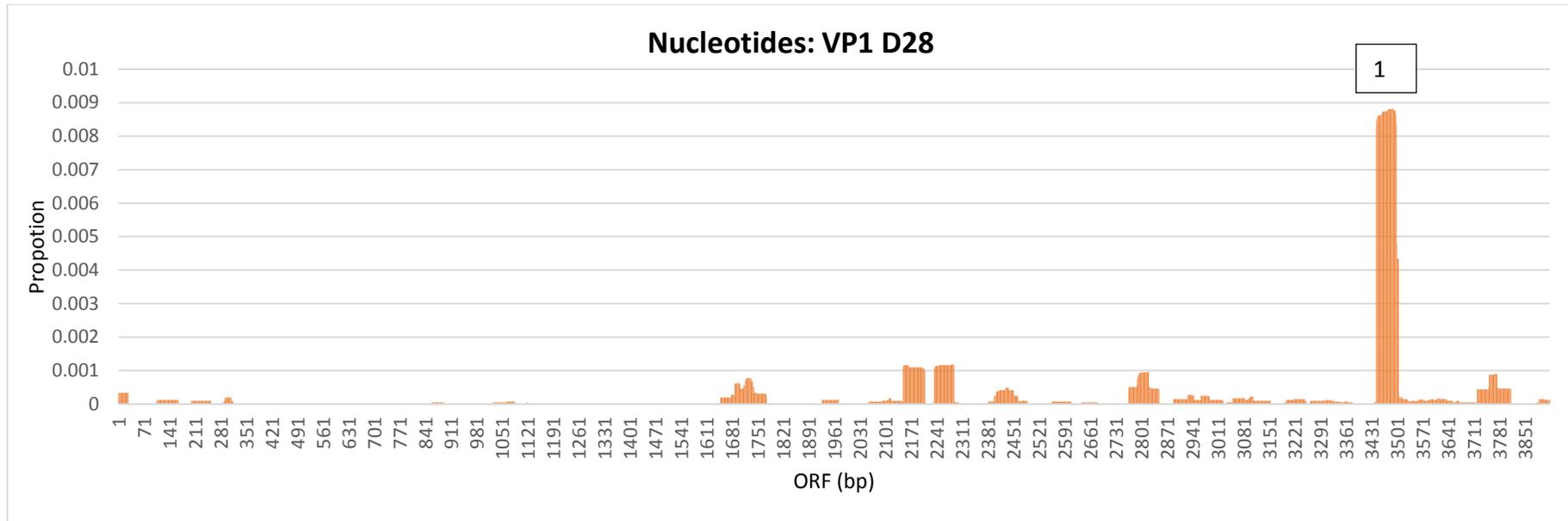
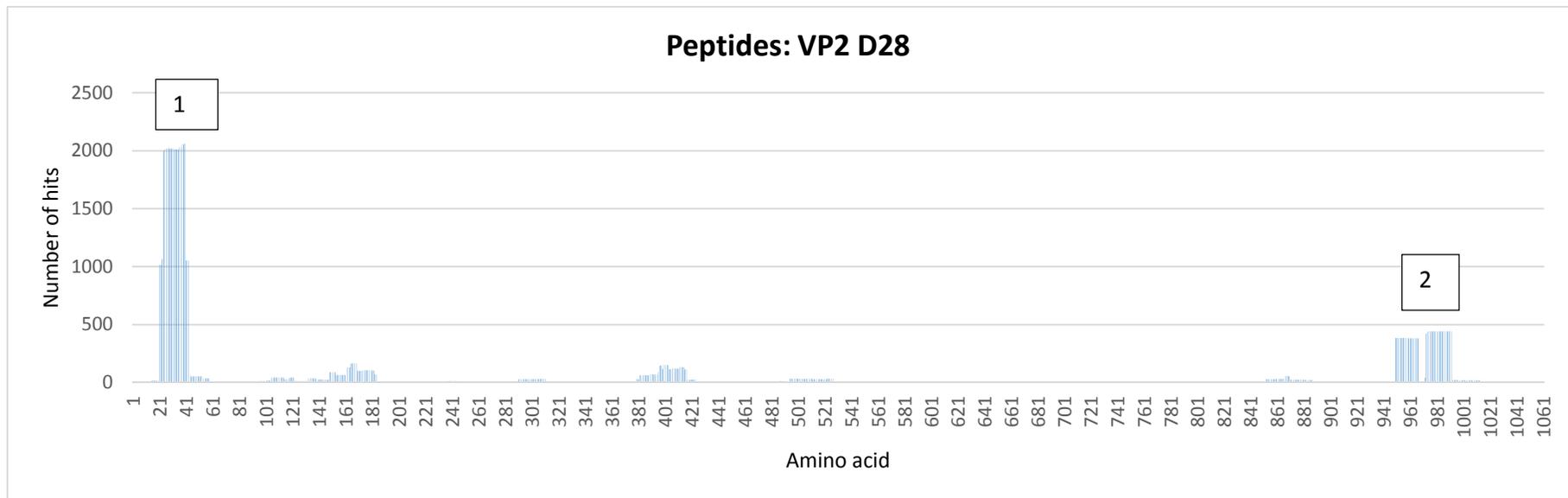
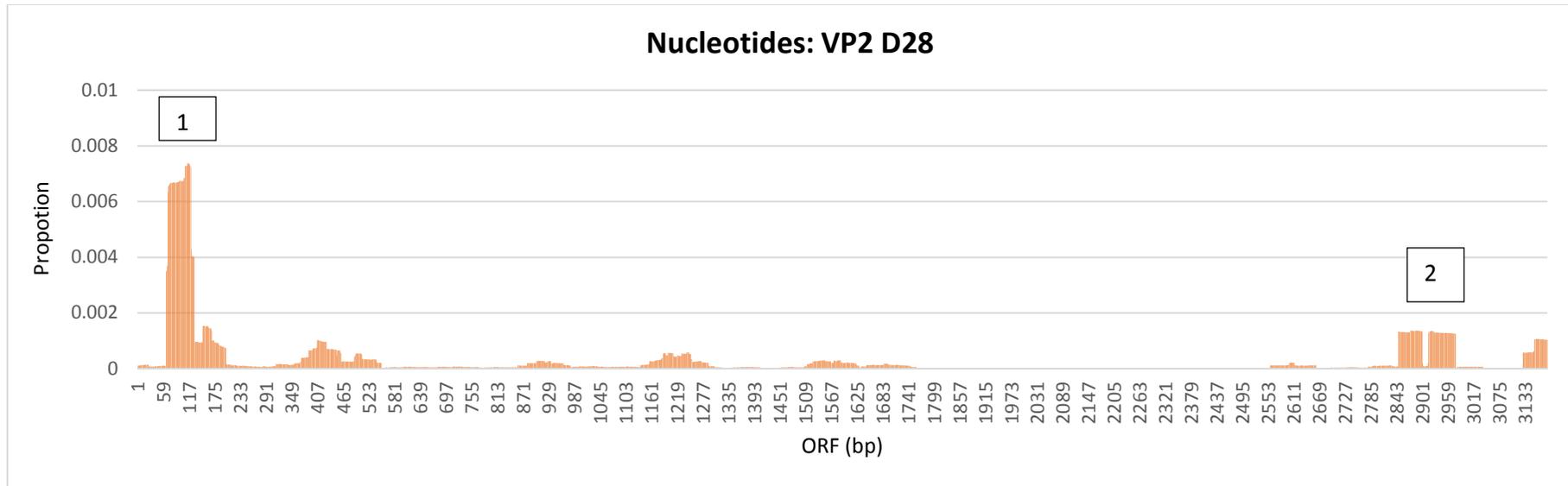
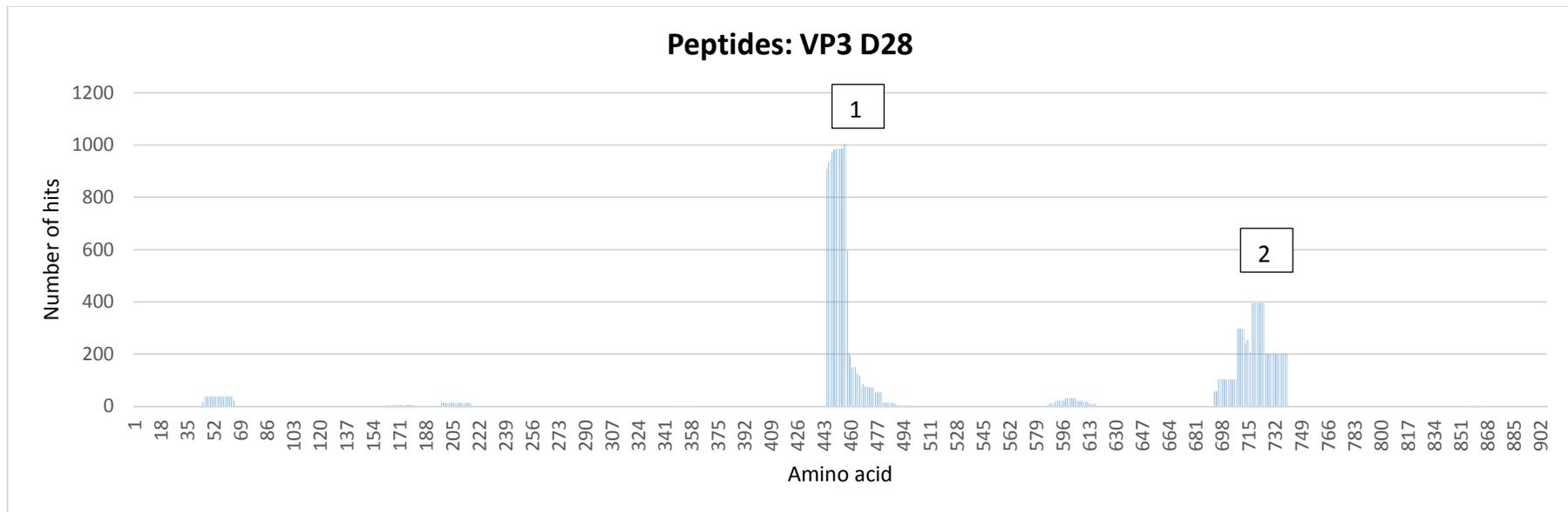
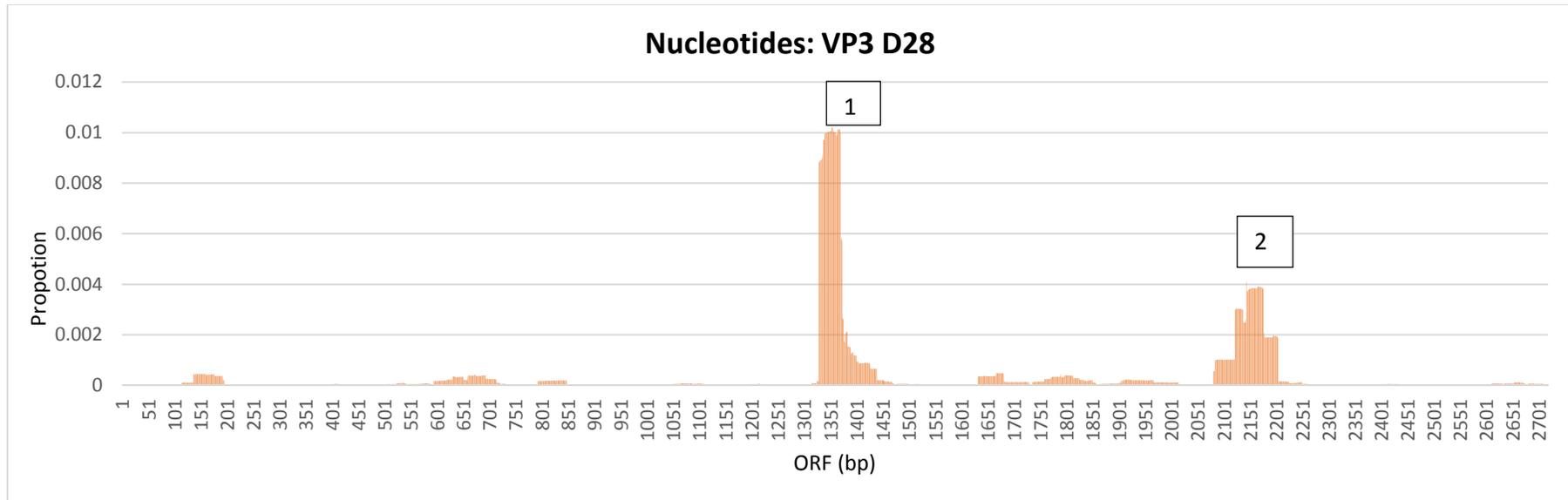


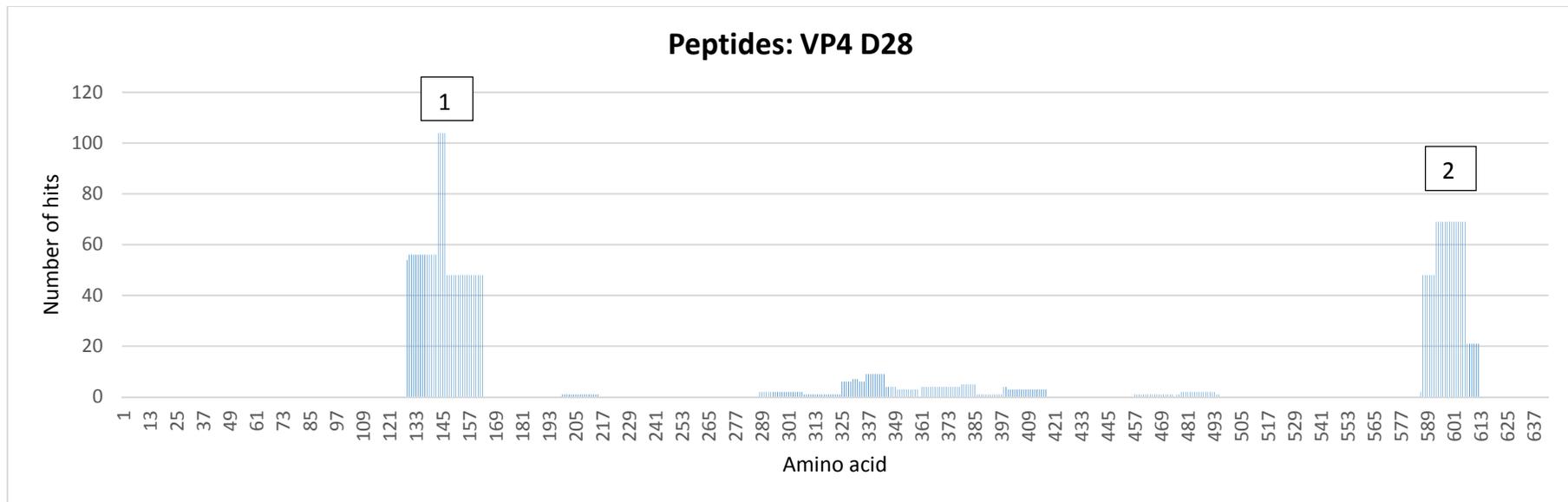
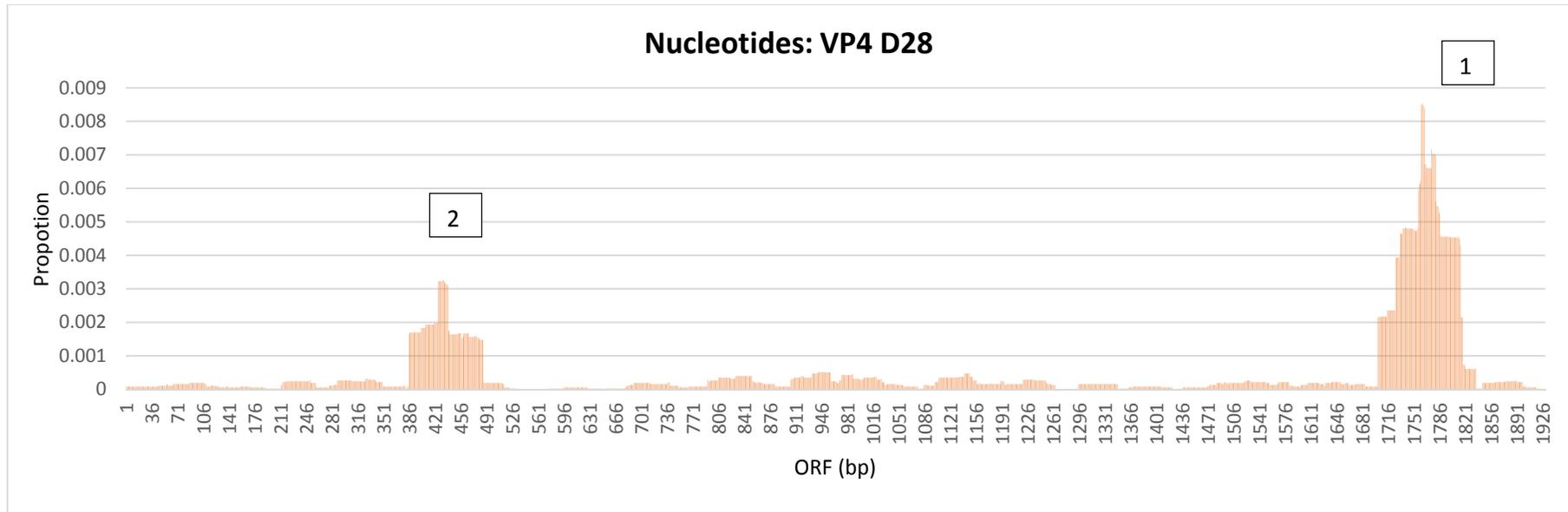
Figure 3.13. Day 28 IgG selected sequences converted to matches per nucleotide position as a proportion of the total nucleotide matches per gene segment. Data of all five horses visualised on one graph. Numbers represent the identified potentially antigenic regions. Data was normalised by subtraction of day 0 matches (baseline data).

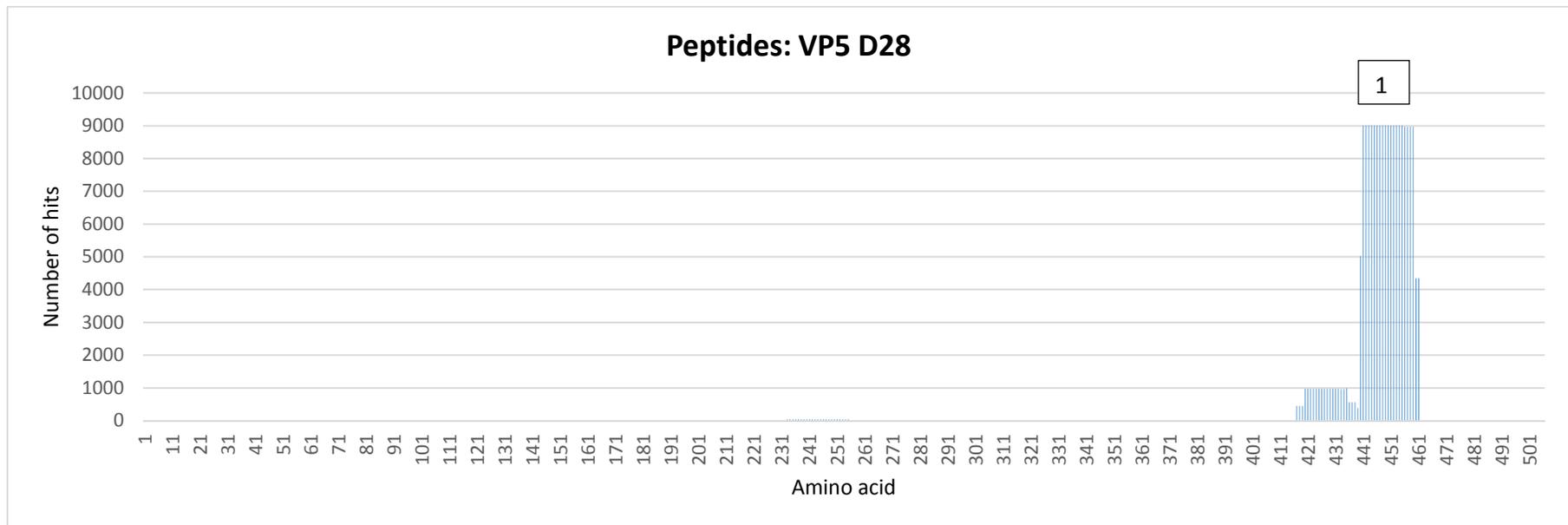
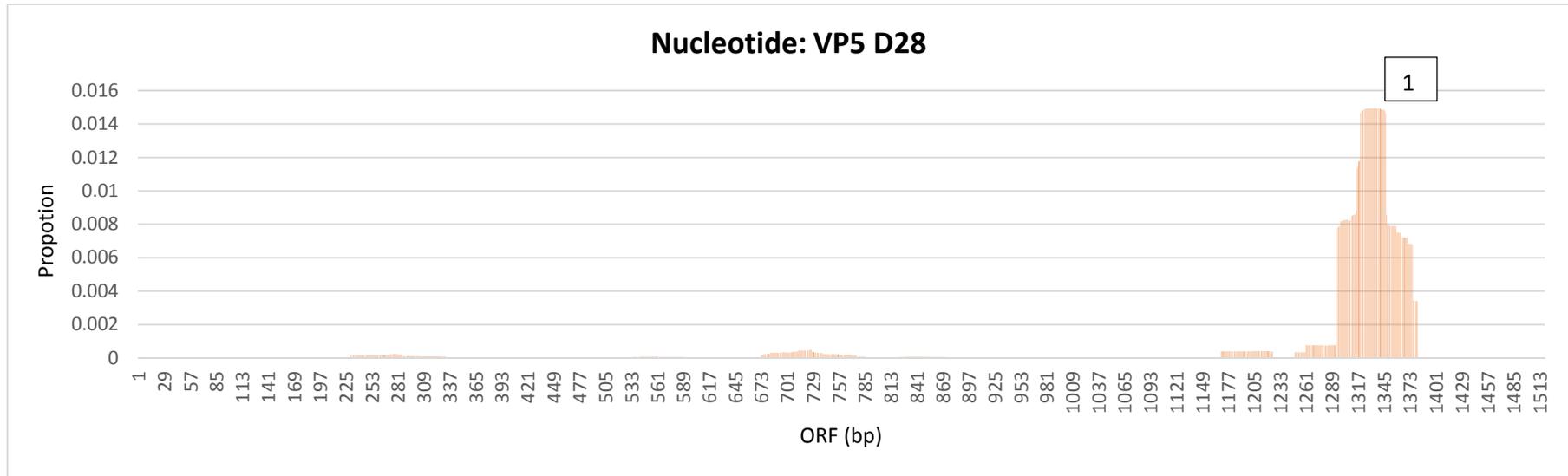
The mapped sequences were further analysed to check if the phage insert sequences do actually form a functional peptide. Traditionally, when the inserts of single clones are sequenced, they are first translated and then mapped to the amino acid sequence of the target protein. Sequencing errors such as insertions and deletions could affect the correct translation of reads and thus the matching pattern. This could cause discarding of reads/matches when the amino acids are filtered for correct reading frame orientation. How this data loss during translation impacts on the distribution pattern of mapping amino acids in comparison to nucleotides was investigated. The translated sequences mapped to the amino acid of the segments were compared to the nucleotide sequence mapping (Figure 3.14). Due to the cumbersomeness of this approach that leads to peptide alignment, only data for horse 2 is presented. However, it was assumed that the binding pattern shown by one horse will reflect on the other horses when using their data. Regions were numbered according to their corresponding peaks between the graphs. In general the mapping pattern of peptide alignment was similar to the pattern observed in nucleotide mapping of the selected horse, validating the use of the nucleotide sequences in mapping.

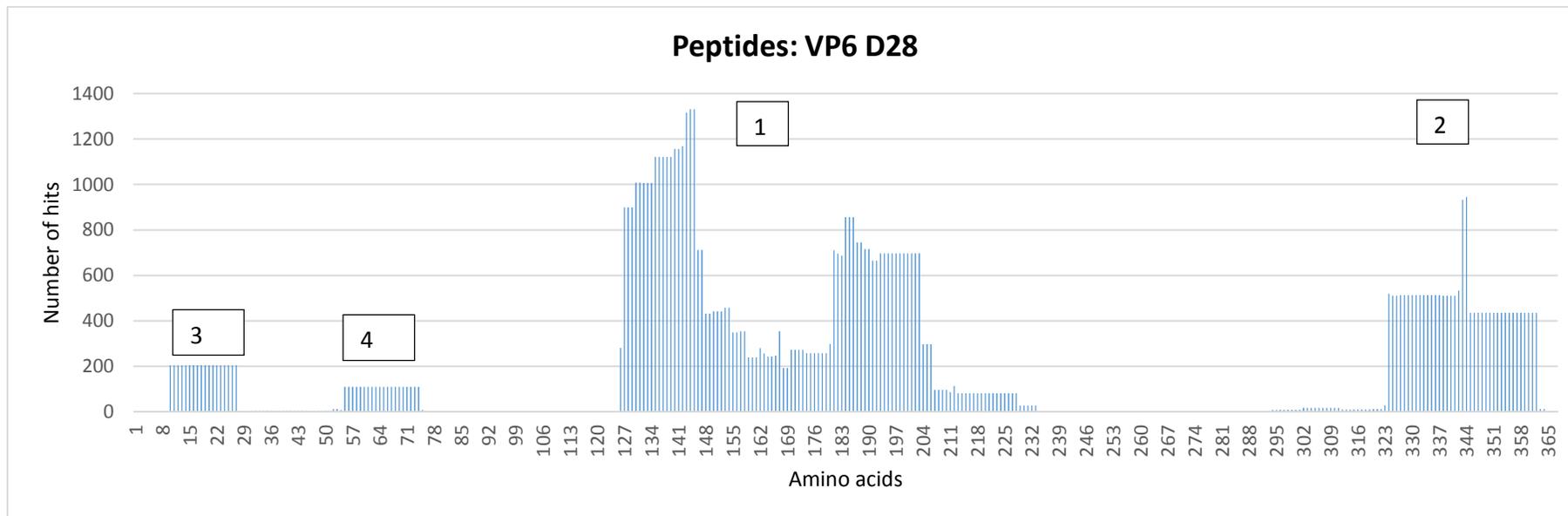
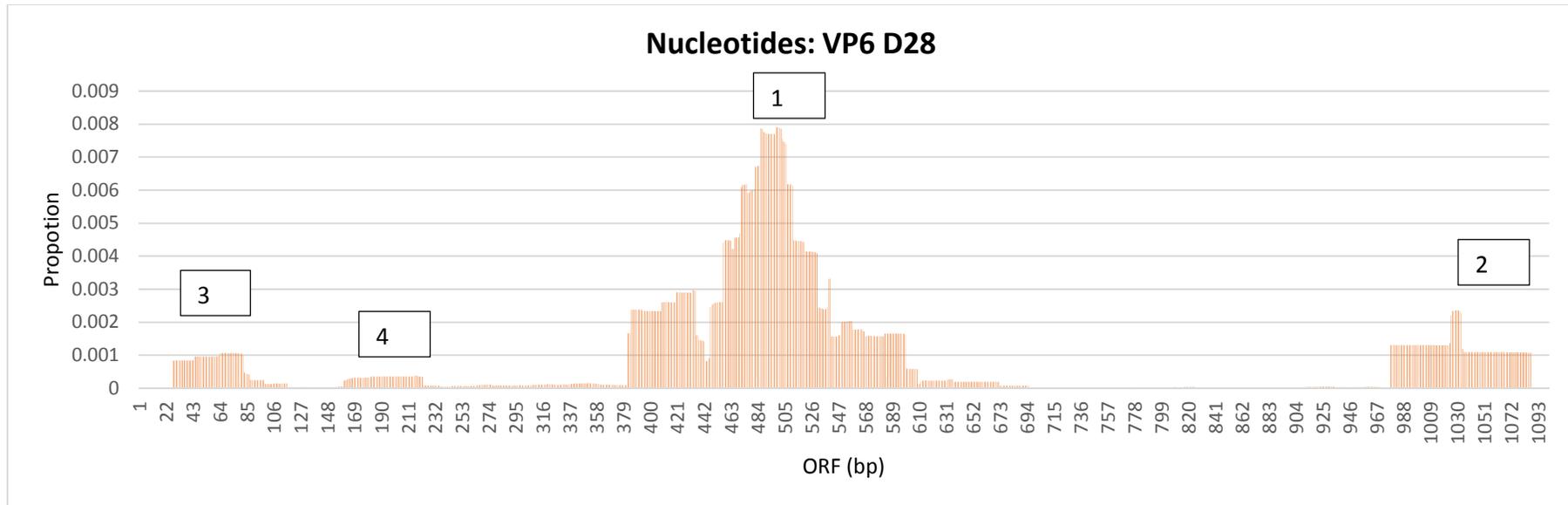


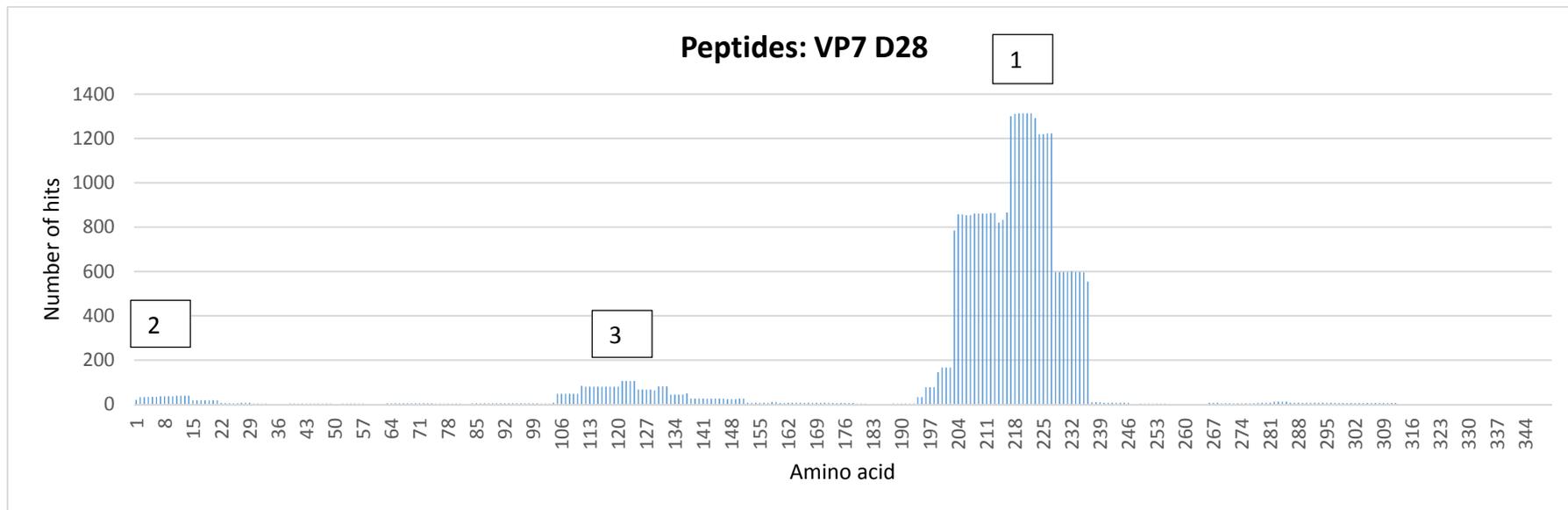
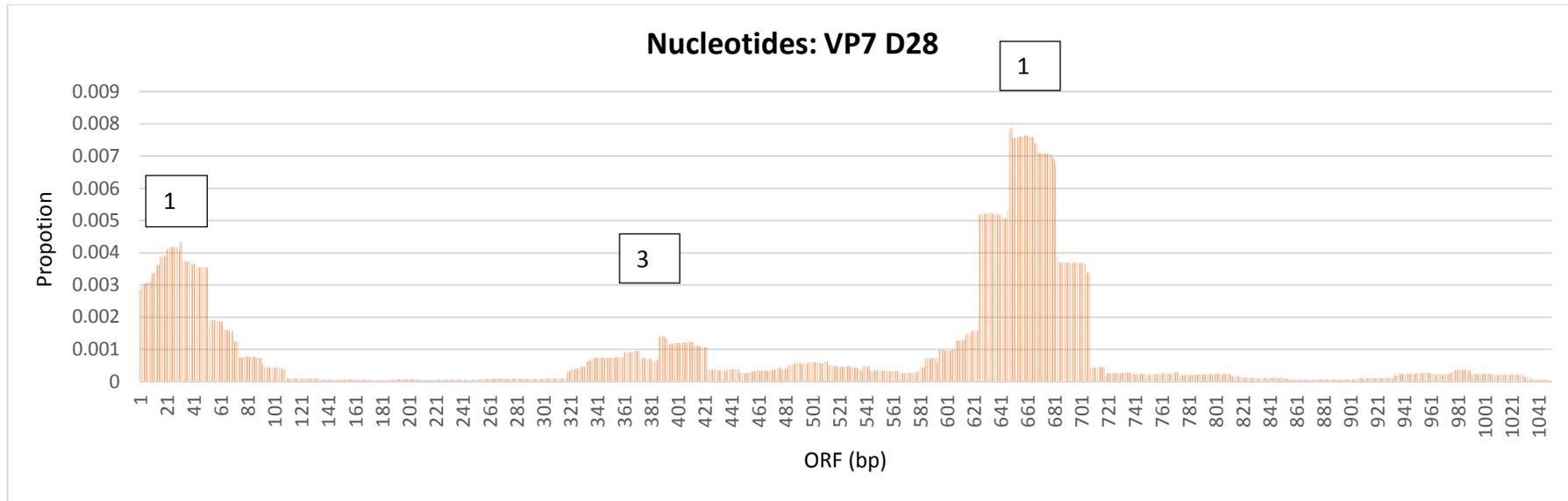


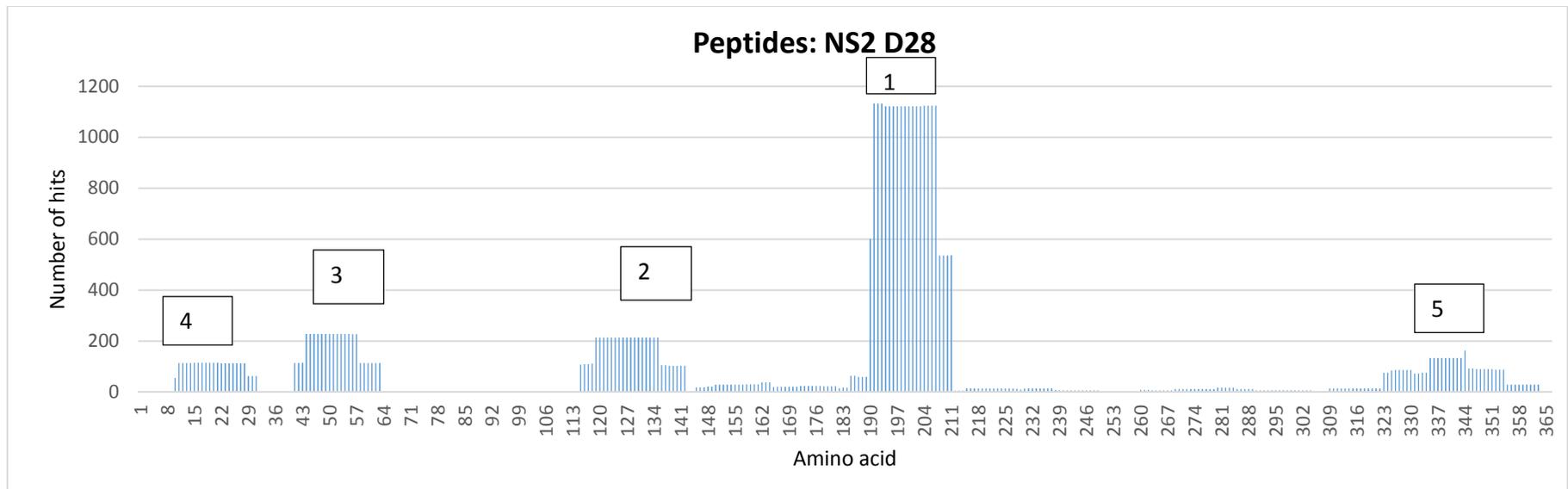
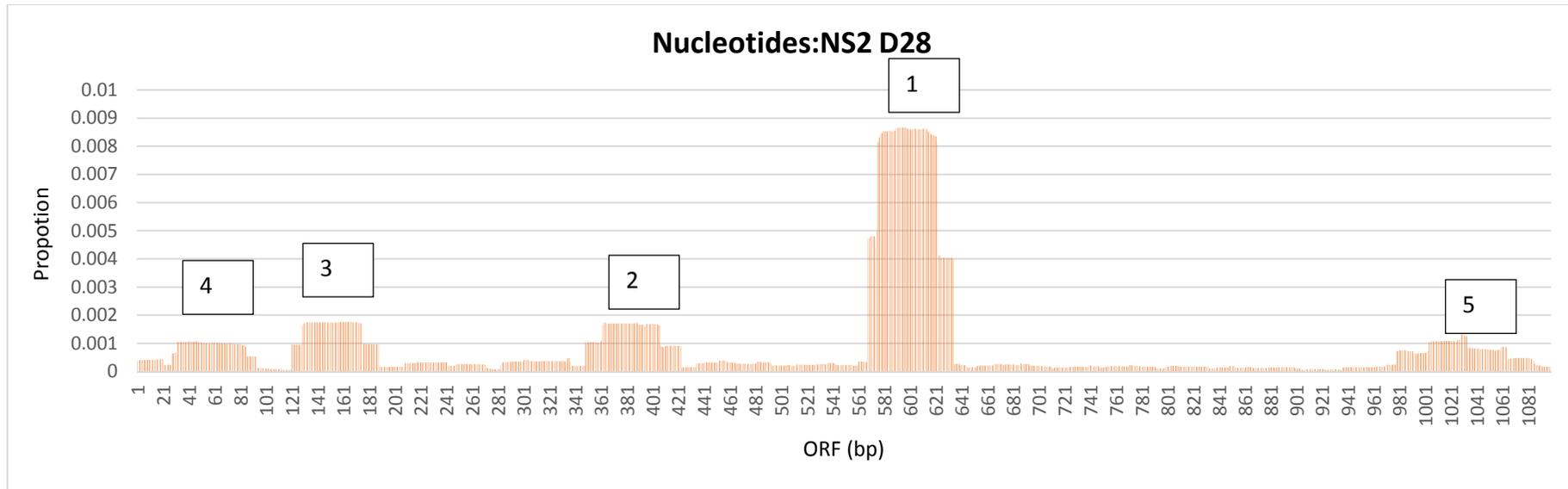












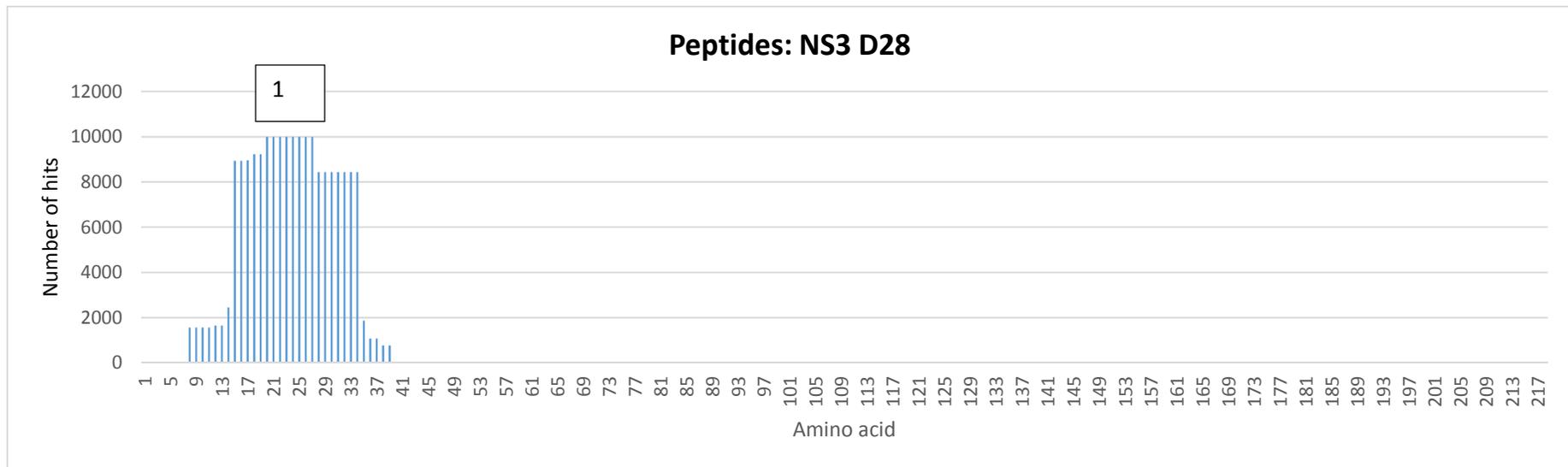
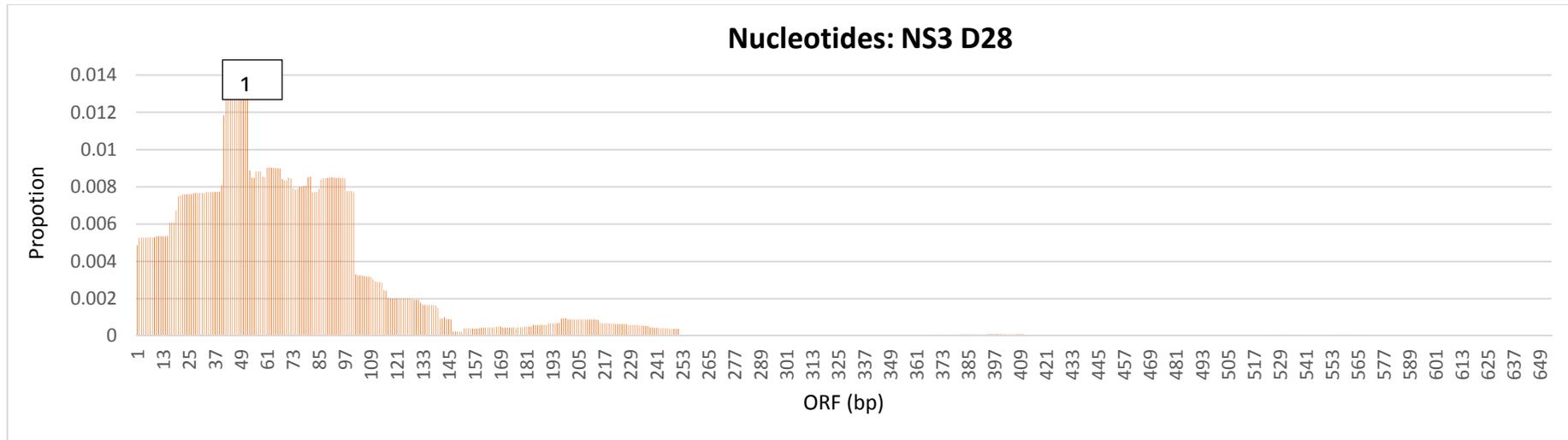


Figure 3.14: Comparison of nucleic acid mapping and deduced amino acid alignments of the selected peptides using horse 2 as an example. The boxed numbers represent the number of identified regions.

The mapped peptides sequences on day 28 horse 2 were further analysed to identify the minimal overlapping amino acids with a potential of being antigenic. These would be the shortest amino acid sequence that was common in all or most of the overlapping peptides on a region. This was done for the dominant mapped region on each of the proteins. Figure 3.15 shows an example of how a potential antigenic region was mapped on the protein that was the most enriched, NS3. Each peptide sequence represent multiple copies of such a clone.

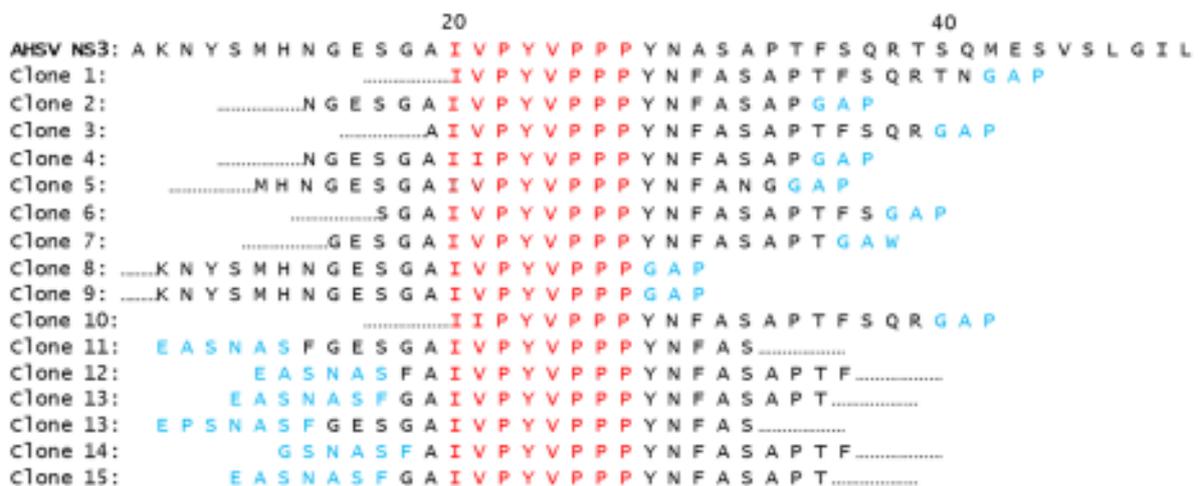


Figure 3.15: An illustration of overlapping peptides selected with horse 2 IgG mapping to the deduced amino acid sequence of NS3 (region 1). Vector sequence is shown in blue and residues shown in red indicates the minimal overlapping region with a potential of being antigenic. “...” indicates incomplete sequence.

Based on the mapping results (Figure 3.13) and confirmation that nucleotide translation to amino acids does not change the mapping pattern (Figure 3.14), regions that were identified by most immunised horse IgGs were selected as potentially antigenic using a genome-targeted AHSV-4 library. The 16 selected regions on AHSV-4 genome and the synthetic peptide tested are listed in Table 3.4. The selected regions were between 18 aa identified on *vp2* to 60 aa on *vp5* and an average of 32 aa. This may suggest the possibility of multiple linear epitopes on the selected regions. Since

a basic linear epitope where all the residues are in contact with those on the paratopes is 6 aa long, the regions identified may suggest that there may have been some level of conformation involved.

Table 3.4: Potentially antigenic regions on AHSV-4 segments

Segment	Peak number	Potential antigenic region position		Size (aa)	Peptides tested*
		Nucleic acid (bp)	Amino acid		
vp1	1	3420-3499	1140-1166	26	ND
vp2	1	60-190	30-63	33	p2-p7
	2	370-450	123-150	27	p14-p19
	3	3110-3163	1036-1054	18	p61-p64
vp3	1	1305-1405	435-468	33	ND
	2	2081-2190	693-730	37	ND
vp4	1	370-482	123-160	37	p14-p20
vp5	3	240-330	80-110	30	ND
	1	600-780	200-260	60	ND
	2	1290-1380	430-460	30	ND
vp6	1	440-530	146-176	30	ND
vp7	2	1-61	1-20	19	p1-p3
	1	621-705	207-235	28	p25-p29
ns3	1	1-150	1-50	49	p1-p5
	2	170-250	56-83	27	p7-p10
ns2	1	570-640	190-213	32	ND

*synthetic peptides tested spanning the selected region on a segment ND=not done
 see appendix for peptide positions

3.5. Peptide ELISA

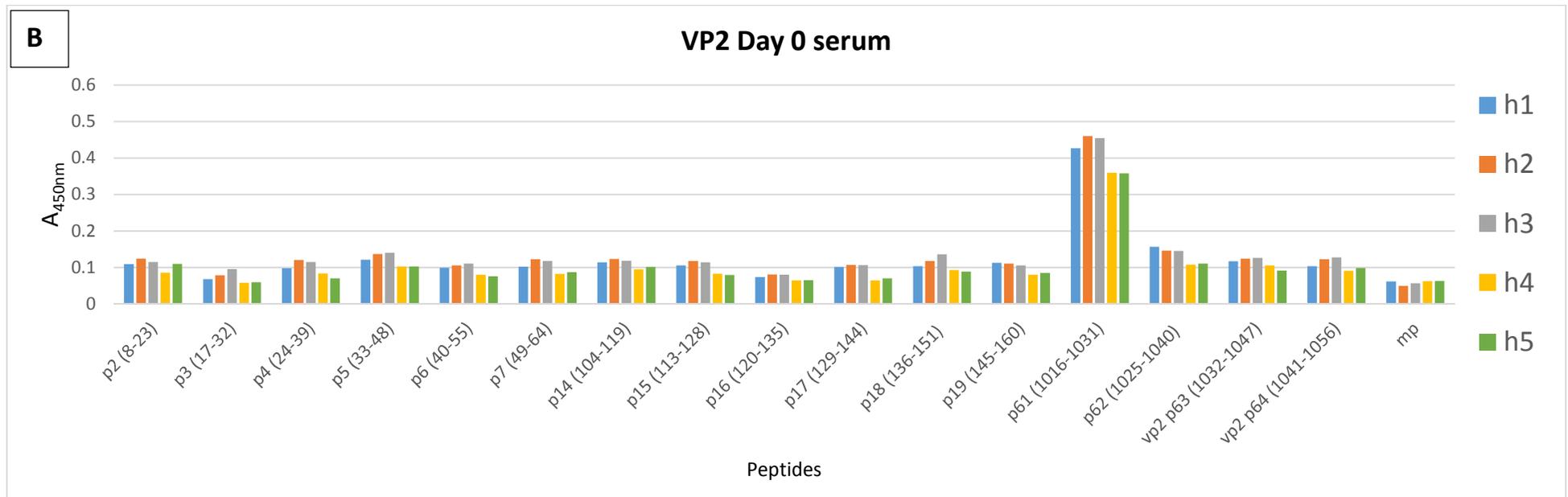
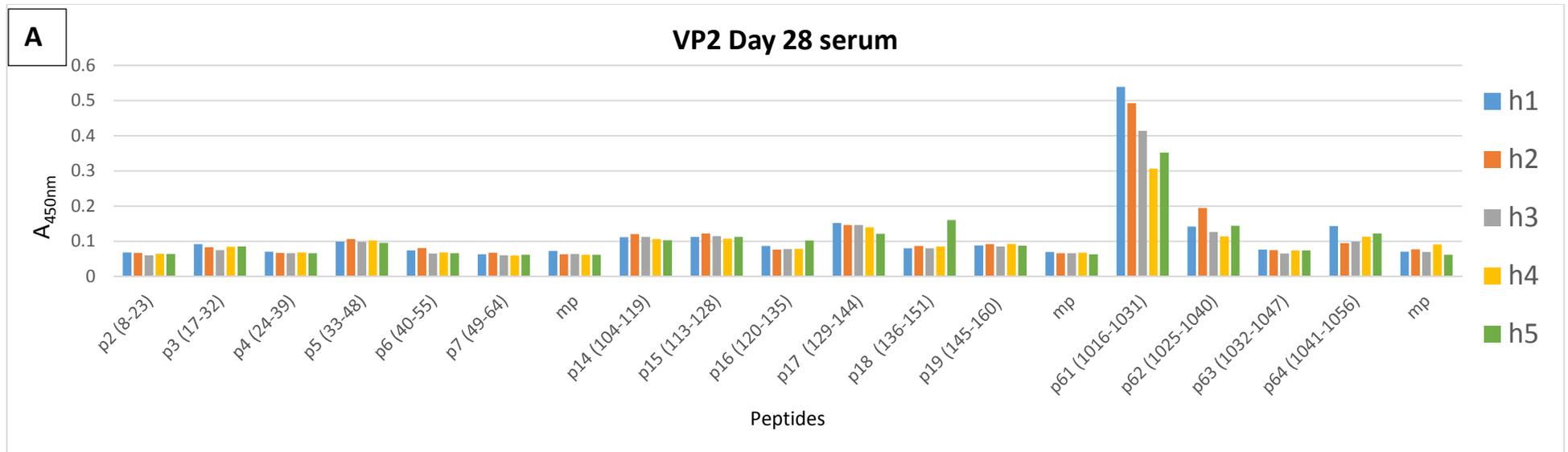
The ability of the selected peptides to be recognised by immunised horse serum was further evaluated with ELISA. To start the process of checking peptide antigenicity, the 16-mer synthetic peptides with 8 overlaps for VP2, VP4, VP7 and NS3 (Table 3.4) were tested against day 28 and day 0 horse antisera since they were already available. Figure 3.16 shows ELISA signals of horse serum reacting with the selected peptides

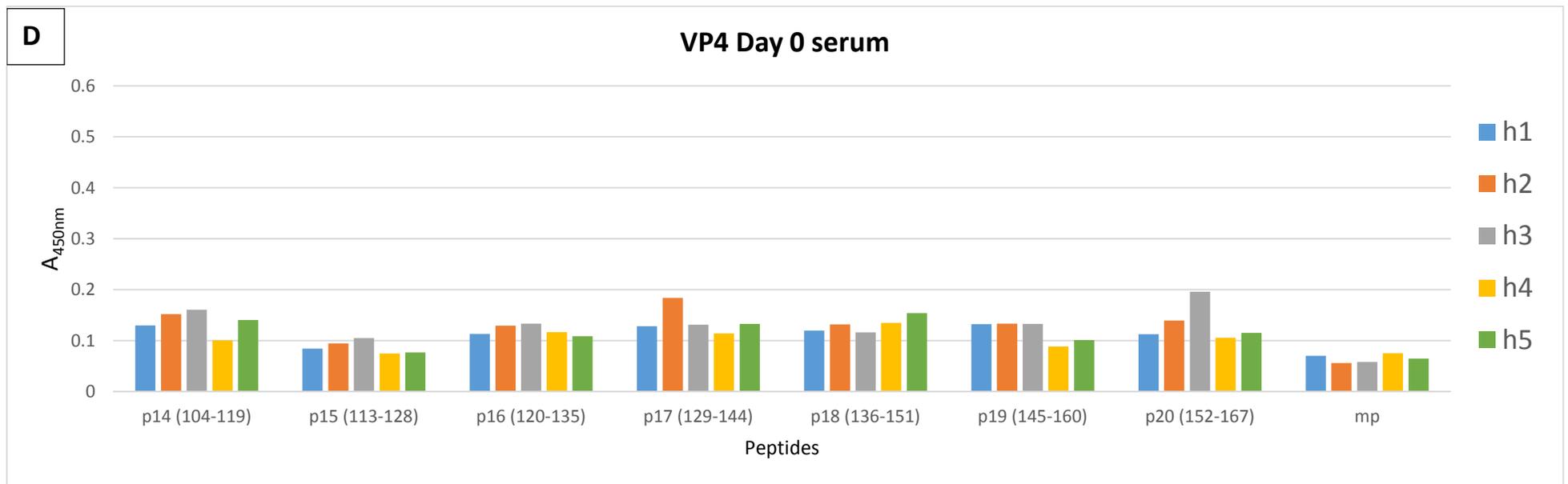
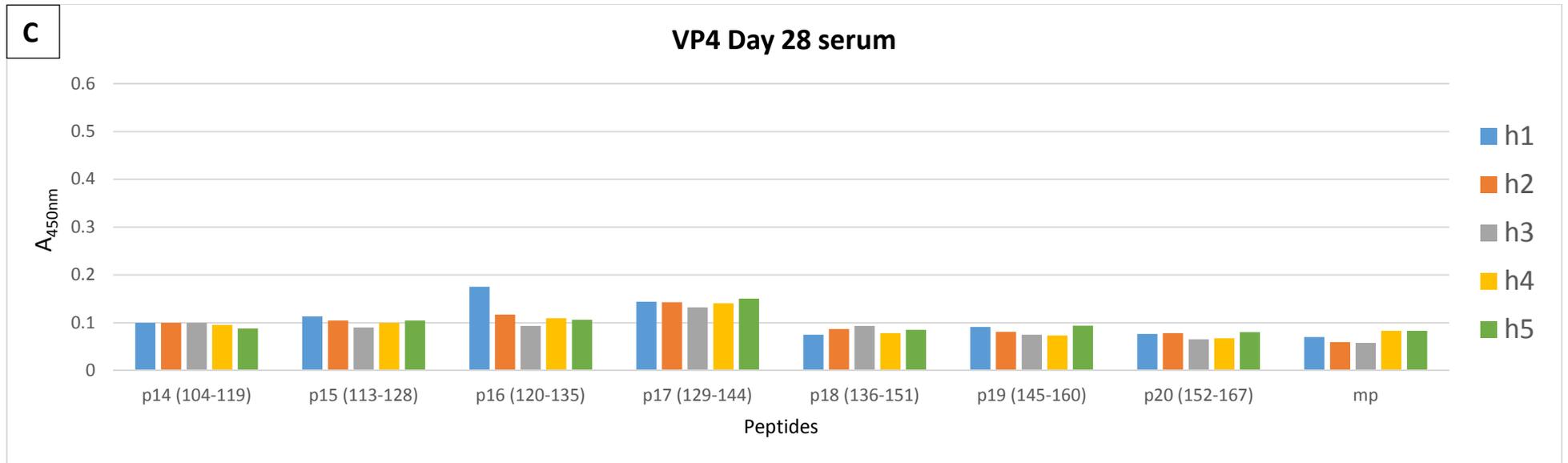
spanning the potentially antigenic regions selected (Table 3.4). A signal was considered positive when it was higher than the background signal. ELISA signals ranged between 0.1 and above 0.5. The signals differed for the different horses.

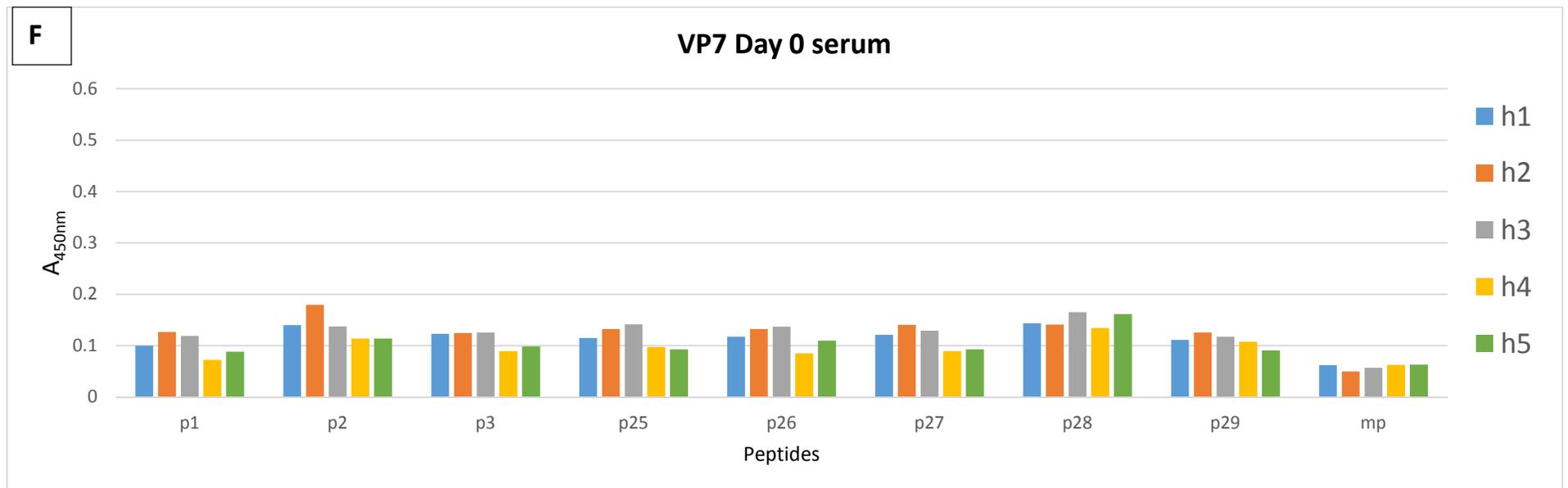
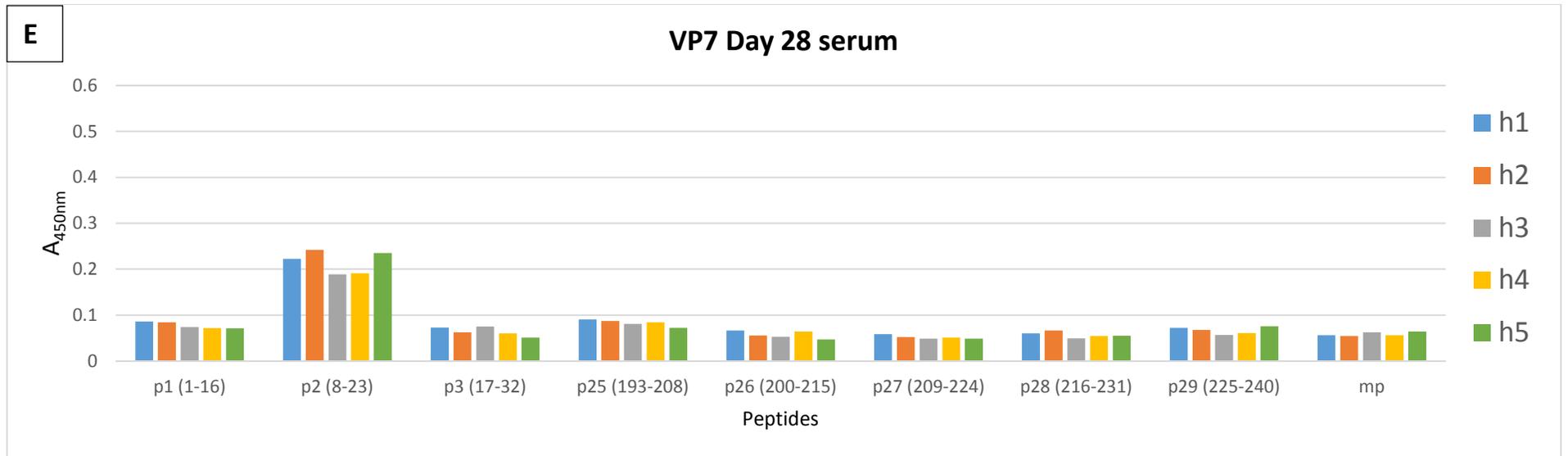
There was a correlation in the binding pattern between all horse sera, where when a peptide was reactive it was with the antisera from all horses. The correlation could confirm that the peptides contains a linear epitope significant to horses. All the peptides had low level signals with an exception of p61 which had high signals for VP2. This peptide at the C-terminal end is in the region marked 3 with the nucleotide mapping results (Figure 3.13B). However, the same region was reactive with day 0 serum. This may indicate that the peptide is a not a targeted peptide (sticky peptide). These results are in contrast to the sequence data results where the dominant region was at the N-terminal end.

A similar pattern was observed between day 28 and day 0 sera for signals with VP4 peptides of the selected region. This may also indicate that the observed potential antigenic region may actually be a non-target sticky peptide. A difference in signal strength between day 28 and day 0 serum ELISA was observed with one peptide, p2, for VP7 and this is a peptide at the N-terminal end. This is also in contrast to the sequence data results where the dominant region was at the centre of the protein (Figure 3.13G). High signals with day 28 serum was observed for p1 to p4 peptides for NS3 in the dominant region marked 1 in nucleotide mapping (Figure 3.13H). The signals were more than three times higher than with day 0 serum, which may indicate that the region is potentially antigenic. The difference in the reactivity observed when using synthetic peptides may be operational. Even though some of the potentially antigenic regions were confirmed by testing in ELISA, this is not the optimal way to use peptides. They were directly immobilised on the surface of ELISA plate wells. A

better way would be to trap them via a tag attached to one end, conjugate them to a bigger protein or capture them on floating beads.







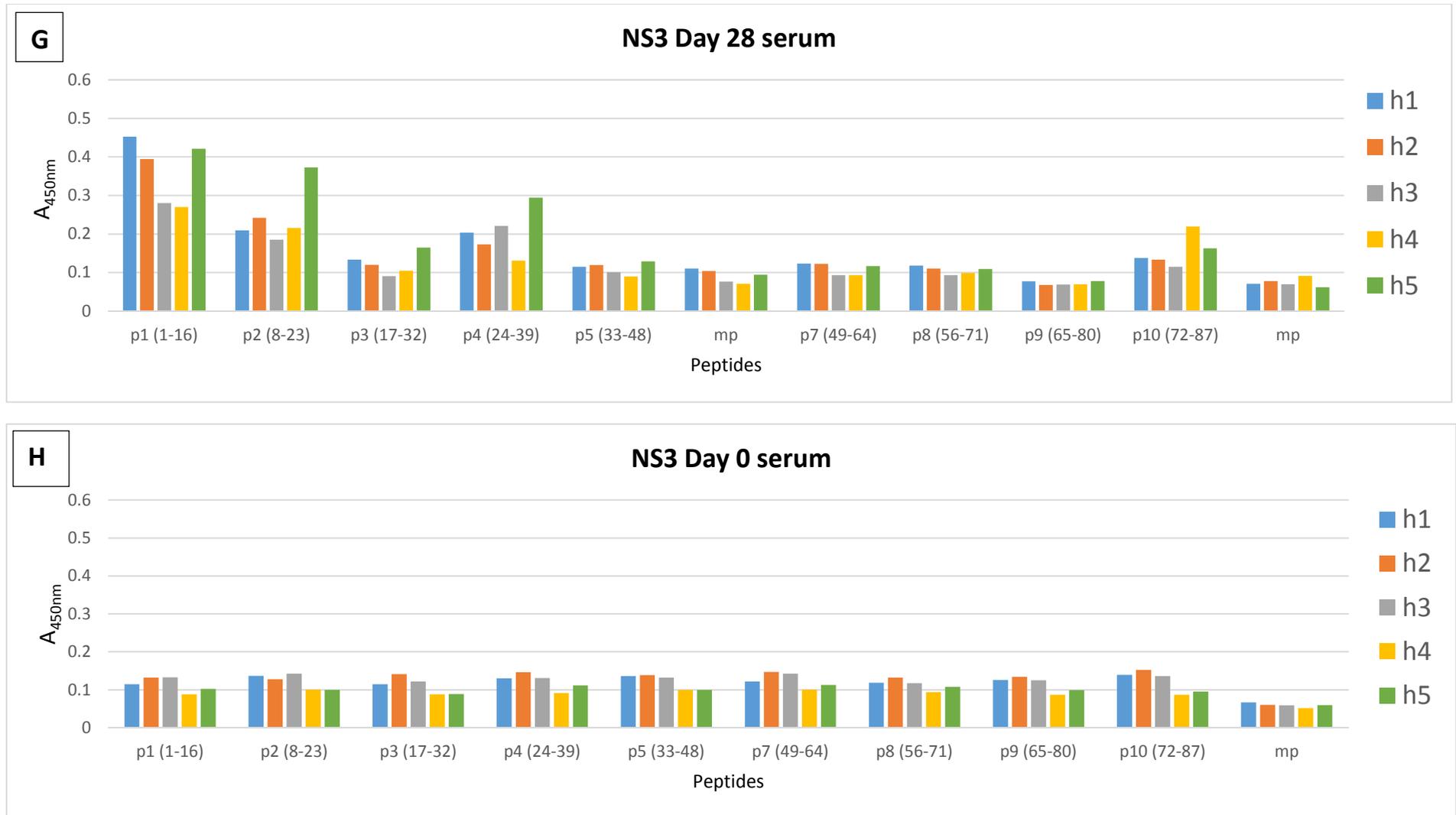


Figure 3.16. ELISA of day 28 and day 0 serum of the five horses reacting with the synthetic 16-mer peptides spanning the selected regions on VP2, VP4, VP7 and NS3. Each plotted value is the average of duplicate reactions. Positions of the peptides are also indicated. Milk powder (MP) acts as the negative control.

CHAPTER 4: DISCUSSION

This study was aimed at identifying B-cell antigenic regions of AHSV-4 that are recognised by polyclonal sera from immunised horses using a genome-targeted phage display library. Pioneered by Smith (1985), phage display allows for a direct link between a displayed fusion peptide and a DNA fragment cloned in a phage genome. The insert DNA fragment expressing the peptide/protein of interest is identified by sequencing the phage genome DNA (Kay, 1994; Ngubane *et al.*, 2013; Rebollo *et al.*, 2014; Christiansen *et al.*, 2015). The direct link makes identification and characterisation of antigenic gene fragments responsible for antibody binding easy. Thus this makes this method convenient as it can be used to screen, identify and characterise genes with unknown function with great resolution. Identifying antigenic regions remains important in vaccine, immunodiagnostic development and antibody production. Coupled with high throughput sequencing, phage display can be used for comprehensive analysis of complex samples. For example, phage display technology and high throughput sequencing were used to comprehensively identify peptides that bind mycobacteria (Ngubane *et al.*, 2013), identify patient specific epitopes motifs in serum (Christiansen *et al.*, 2015) and increased profiling of antigenic regions in vaccinated animals (Domina *et al.*, 2014). To our knowledge, this is the first study to use immunised horse antisera, which is the naturally affected host, to exhaustively study the AHSV humoral immune response. The serum was collected over three different time points during a monovalent AHSV-4 live attenuated vaccine trial from horses with no prior history of vaccination. The horses were vaccinated and a booster was administered 21 days later. Serum was collected three days before first vaccine injection, 28 and 52 days after vaccination.

Gene/genome-targeted phage display libraries have been used in a number of studies to identify B-cell epitopes on viral proteins. It has been used to identify BTV epitopes on VP7 using mouse monoclonal and rabbit polyclonal antisera (du Plessis *et al.* 1994), VP5 using mouse monoclonal (Wang *et al.*, 1995) and NS1 using rabbit antiserum (du Plessis *et al.*, 1995) and VP2 of AHSV-3 using chicken, mouse and one infected horse antiserum (Bentley *et al.*, 2000). The libraries in these studies were constructed by cloning digested DNA fragments to pIII of M13 filamentous phage (Parmley and Smith, 1988). Antigenic regions were identified by sequencing of not more than 20 selected clones. While it was effective, the selection of individual clones approach however, does not offer an in-depth mapping profile of antigenic regions on the protein. Since only a few clones are analysed of the many released after panning, sequences that are enriched but not randomly picked may be missed. Thus, this study improves on all the phage display work mentioned before and merges the approach with next generation sequencing of all the clones released after panning to identify the whole repertoire of potential antigenic regions. The extensive data output from high throughput sequencing enables the detection of phages that would otherwise be missed by the traditional clone picking and sequencing approach. At the same time the combinative approach increased the identification of potentially antigenic regions selected by horse polyclonal serum (Christiansen *et al.*, 2015).

In order to make a genome-targeted phage display library for this study, the AHSV-4 DNA was randomly fragmented with *DNase I* to generate a population of different fragments sizes. These fragments were cloned at the N-terminal of *gene viii* of a non-lytic M13 filamentous phagemid vector, pCVEPI585042, to display on the pVIII protein coat when expressed in *E. coli*. The DNA fragments were dephosphorylated to ensure that each vector contained a single DNA fragment and not concatemers. This makes

screening the library binders easier as the peptide displayed represents single DNA fragments. In preparing the inserts there was a PCR step which has an inherent possibility of introducing errors to the sequences, therefore the library was made to be bigger than required. Other reasons for increasing the library size include frameshifts and DNA fragments cloned out-of-frame which express non-functional peptides that potentially skews the results (Derda *et al.*, 2011). The primary library was 33 times more than the minimum required to represent the whole AHSV-4 genome with 99% probability. This is notwithstanding the notion that only 1 in 18 clones in a library will produce a functional native peptide with 99% probability (Clarke and Carbon, 1976; Jacobsson *et al.*, 2003). The library contained an average DNA insert size of 250 bp, confirmed by PCR. Combo 4 which comprised of *ns3* was not readily cloned as 1 in 10 clones had an insert. The low cloning efficiency may have been caused by toxicity as baculovirus expressed AHSV NS3 was shown to be cytotoxic to both insect cells (*Spodoptera frugiperda*) and bacterial cells (*E. coli*), attributed to two hydrophobic regions on the protein (van Staden *et al.*, 1995; van Staden *et al.*, 1998; Huisman *et al.*, 2004). Nevertheless, these hydrophobic regions were present in the library at a low sequence frequency in this study. This may suggest that cells that contained these hydrophobic peptides had inhibited growth and thus were at a selective growth disadvantage in the library. Nevertheless, the growth inhibitory effect was not a limitation because clones produced for this group were 41x more than theoretically required and covered *ns3* with no gaps as shown by sequencing.

Another protein expected to have low cloning efficiency because of possible toxicity to cells was VP5. Baculovirus expressed AHSV VP5 was cytotoxic in bacteria and insect cells attributed to two amphipathic regions (du Plessis and Nel, 1997; Martinez-Torrecedrada *et al.*, 1999; Wall, 2006; Stassen *et al.*, 2011). However, the

amphipathic region was represented at an average sequence frequency rate in this study, which may indicate that the assumed toxicity was not a limitation. The toxicity of VP5 could not be confirmed here as the segment was pooled into a group of segments with similar sizes i.e. NS1 and VP2 at the beginning of the study. Moreover, sequence analysis of the coverage of each segment in the library showed that all segments were fully covered with no gaps.

The minor structural proteins VP1 and 4 (Combo 3), which are located in the core of the virus particle with VP6 among other proteins, were not readily cloned as 3 in 14 clones had an insert. Difficulty in cloning of African horse sickness virus minor proteins (VP6, VP4) have been previously reported for reasons not completely understood (Grubman and Lewis, 1992; Laviada *et al.*, 1993; Turnbull *et al.*, 1996). The low representation of a segment in the library was not a limitation as the panning process can detect a single clone in a pool of 10^5 clones (Parmley and Smith, 1988). This is because the amplification process ensures that every fragment in the library is significantly represented as each bacterium releases approximately 200 lysogenic phages per hour (Cwirla *et al.*, 1990; Scott, 1992; Jefferies, 1998). However, the larger libraries lower the bias levels which may be otherwise observed with smaller libraries, where over represented clones compete and bind better than the lower represented clones during affinity selection (Derda *et al.*, 2011).

The library was affinity selected for binders using polyclonal IgGs purified from pre-immunised and immunised horse sera. Prior to affinity selection, it was observed that there was low antibody binding to virus in ELISA with pre-immune sera. The signal increased with day 28 sera and declined with day 52 sera. A similar pattern was observed when samples were tested for virus neutralisation where no neutralising antibodies were detected with day 0 sera, some with day 28 sera and very low in day

52 sera. This thus indicated a poor re-vaccination anamnestic response. This was in agreement with observation made with foals in the field that a booster injection with monovalent live-attenuated AHSV-4 vaccine did not increase the immune response (Crafford *et al.*, 2014). Day 0 serum was theoretically therefore expected to have no anti-AHSV antibodies as serum was collected before vaccination. Since ELISA and virus neutralisation results have suggested that there are no anti-AHSV-4 antibodies, the IgGs isolated with sera in day 0 may thus be background (IgGs against an untargeted antigen). The high signals observed with day 28 sera may be because B-cells were already induced by the primary injection, a booster injection led to an increased production of a myriad of antibodies attempting to bind to virus. However, low IgG levels were purified from horse sera in this group. This may indicate that the purification kit was not very effective. Therefore, other methods like Polson method (Polson, 1990) of purification which uses chloroform polyethylene glycol procedure should be considered for future studies. It could also indicate a state of antibody isotype switching as sera was collected seven days after booster injection. The virus has been reported to persist in a horse approximately for nine days on average after infection (CFSPH, 2015). As this was still in the acute phase of infection, it is possible that most of the antibodies produced were IgM and switching to IgG production, hence the low levels of IgG purified (Perkins *et al.*, 2003). However, the low concentrations of purified IgG from day 28 sera was not a limitation as only a small fraction, ~20 µg/ml, was used for panning. By day 52, the antibody concentration decline as observed in ELISA may be in accordance to the inability of booster injection to induce increased production of persistent antibodies as previously observed (Crafford *et al.*, 2014). However, the increased levels of purified day 52 serum IgG from day 28 serum may indicate that although the booster injection did not increase overall antibody

concentration, it improved production of IgG. It may also be assumed that the antibodies observed in day 52 sera may be some of those that have a longer half-life. The immune response to the vaccine was variable among the different horses, also observed in other studies for reasons not fully understood (von Teichman *et al.*, 2010; Crafford *et al.*, 2014; Molini *et al.*, 2015). The IgGs from these sera were purified and used to affinity select the phage library. In principle, biopanning and amplification rounds eliminate most of the non-binders, non-specific binders and enrich the binders in the library (Parmley and Smith, 1988). How much of these undesired phages are removed depends on a number of factors which includes the number of washes between steps, the stringency used and the size of the surface area coated by the ligand.

When an antigen enters a host either by vaccination or infection, B-cells produce and releases an antibody repertoire targeted to the various antigen epitopes (Briles and Davie, 1980). By panning using a diverse pool of polyclonal antibodies, a diverse pool of phages are expected to be selected. One would expect a change in sequence distribution of the selected phages in comparison to the phages of the unpanned library after panning. The change would indicate that the production of specific polyclonal IgGs were induced by an AHSV-4 epitope. There appeared as if the was enrichment of phages selected by day 0 IgGs after four rounds of panning. However, further investigations with ELISA showed that there was no increase in antibody-phage binding signal with day 0 IgGs in all panning rounds, indicating that there was in fact no enrichment. This suggested that the increase in the number of phages observed with day 0 IgGs may have been background from sticky phages binding to the untargeted antigen like plastic, milk powder or other impurities, also collectively referred to as target unrelated peptides (TUPs) (Menendez and Scott, 2005; Vodnik

et al., 2011; Christiansen *et al.*, 2015). This was expected because the sera were collected before vaccination and the horses had no history of vaccination, thus should not have contained anti-AHSV-4 antibodies. However, the pre-immune sera of horse 2 showed some phage enrichment when compared to the other four horses. The possible reasons may be that since these horses are from an AHS endemic region, it is possible that some will have some form of natural immunity that may explain the observed outlier reaction. Also because these are outbred animals, they will not react the same since their immune background may differ. However, the ELISA signal for the unpanned library was similar to that for the fourth round panned phage pool, suggesting that it may have been background reaction i.e. anti-phage antibodies in the sera.

Panning with day 28 IgGs showed a stronger ELISA signal than the unpanned phages, thus signifying phage enrichment, albeit at different levels among the horses. This was expected since day 28 serum also showed a strong ELISA signal indicating presence of high AHSV-4 specific antibodies. This would mean that more IgGs are available to identify, bind and enrich fusion peptides in the library. Day 28 IgG of horses 2 and 5 showed the highest ELISA signals whereas there was not much difference between the three other horses. This is supported by observed variation in vaccination efficacy between serotypes and individual horses which has been attributed to many factors including genetics and immune background (von Teichman *et al.*, 2010; Crafford *et al.*, 2014). The lack of phage enrichment after round three of the panning process indicated that the binding capacity of the immobilised IgGs has reached full capacity, thus further rounds would skew the phage pool towards the most specific binders and loose diversity of the pool.

When the library was panned with day 52 IgGs, one would have expected phage enrichment in relation to antibody binding signals observed with whole serum ELISA. However, this was not the case and low antibody binding to phage signals were observed with ELISA for only horse 1 and 2. The possible reason for the lack of enrichment with the other three horse IgGs could have been due to the varying antibody responses in individual horses alluded to before. Another possibility may be that day 52 IgGs recognise conformationally dependent epitopes that have been destroyed by fragmentation, thus not detected in linear format.

Varying number of panning rounds have been used to identify peptides of interest in phage display. In some studies peptides of interest were identified after a single round of panning (Derda *et al.*, 2011; 'T Hoen *et al.*, 2012; Matochko *et al.*, 2014). This they said, reduced the biasness that may be introduced by iterative rounds of panning which may result in possible loss of weak binding but relevant peptides. Although this approach has potential to be successful, persistence of relevant sequences and reduction of weak binding sequences with iterative selection rounds offer an in-depth and thorough insight of the selection process. Thus, it is not surprising that peptides of interest have also been identified by analysing multiple rounds of panning and sequencing while monitoring persistence of sequences with each round ('T Hoen *et al.*, 2012; Olson *et al.*, 2012; Ngubane *et al.*, 2013; Scott *et al.*, 2014; Christiansen *et al.*, 2015). Since there was no increase of antibodies binding to phage as shown by ELISA after three rounds in this study, this released phage pool was chosen for further characterisation. The sequencing of the panning round where no enrichment is observed after that, along with the control phage pool should give credence to the identified peptides of interest (Christiansen *et al.*, 2015). The DNA inserts of round three panning of each time point were amplified by PCR and sent for high throughput

sequencing using the Illumina platform. The paired sequence data was processed and identity of each sequence was determined by mapping them to the AHSV-4 reference genome. Theoretically, one would expect a change of sequence distribution when compared to the unpanned library. The sequences after panning converged and mapped on defined regions of the segments, thus confirming that peptides of interest have been selected by the purified horse IgGs. The more sequences mapped to a region after sequencing, the more likely it was antigenic.

There generally wasn't much difference in the total sequence output distributions per segment between day 0 and day 52, which was similar to the pattern observed in phage ELISA results. As expected, a change in sequence distribution per segment was observed with day 28 IgGs selected mapping data, which may be indicative of skewing towards potentially antigenic segments. An increased proportion of sequences mapping per segment was observed in all horses for *ns3*, *vp6* and *vp5* while the other segments *vp1*, *vp2*, *vp3*, *vp4*, *vp7*, *ns2*, *ns1* decreased which may indicate the level of potential linear antigenic regions. Since this enrichment pattern was consistent in all horses, it may suggest that *ns3*, *vp6* and *vp5* are involved in live-virus attenuated vaccine immunogenicity. This meant that there were more anti-AHSV antibodies identifying linear regions on NS3, VP6 proteins in day 28 immunised horses' sera. This was unexpected for NS3 because anti-AHSV NS3 antibodies were detected in low concentrations in infected mammalian cells (Laviada *et al.*, 1993; Martinez-Torrecuadrada *et al.*, 1997). However, results may confirm the high concentrations of anti-VP6 and VP5 antibodies detected in vaccinated horse serum in the early stages (Martinez-Torrecuadrada *et al.*, 1997). How the immune system access VP6, which is located within the core of the viral particle, needs further investigations. The distribution pattern observed in this study may suggest that

proteins inside the core are accessible to the host's immune system. This however is possible if it is assumed that the capsid protein layer is permeable and not tightly packed, allowing exposures of proteins in the core to the immune system. Indeed, pores on the core capsid protein layer have been described using cryoelectron microscopy for BTV and Cytopovirus, through which transcribed mRNA, substrates and products for viral transcription complex are transported (Diprose *et al.*, 2001; Yang *et al.*, 2012; Sung and Roy, 2014). However, a similar study using cryoelectron microscopy and structural homology modelling indicated that the five-fold vertices on AHSV-4 VP3 layers are closed. The pore closure is attributed to an extra loop and helix at the N-terminal extending to the region and an additional density at the VP5 layer (Manole *et al.*, 2012). Difference may indicate virus evolution and may suggest that AHSV-4 uses a different way to transport mRNA into the cytoplasm, possibly pores on the capsid protein layers are coordinated by chemical signals (Sung and Roy, 2014). However, further studies are necessary to confirm.

NS3 is a non-structural protein expressed by the smallest segment located inside the core. Since NS3 is not a major outer capsid proteins, thus not one of the first to be exposed to the immune system, then this may suggest that it is somehow exposed during viral replication or viral egress. Antibodies to NS3 have been detected in the serum of AHSV-4 infected horses by SDS-PAGE (Laviada *et al.*, 1993). Anti-AHSV-4 NS3 antibodies were also detected in early and late stages of infection, after horses were vaccinated with live attenuated monovalent AHSV (Martinez-Torrecedrada *et al.*, 1997). It is associated with virulence and virus particle release during virus replication in infected mammalian cells by damaging the host cell membrane (Martin and Meyer, 1998; Stoltz *et al.*, 1996; van Staden *et al.*, 1995). Association with the surface of AHSV infected cells during viral replication were further confirmed where

Sf9 cells infected with baculovirus expressing NS3 resulted in membrane permeability detected by trypan blue stain (van Staden *et al.*, 1995). This is in support of the *ns3* enrichment observed in this study, where serum was collected on seventh day after booster vaccination. This is close to the average incubation of AHSV (~9 days) (CFSPH, 2015), a stage where virus may be preparing to egress the cell. NS3 was shown to be involved in guiding viral egress, thus would be expected to be expressed at high quantities during this time to attach to the cell membrane and consequently be exposed to the immune system when cell lysis to release viral particle. Although the sequences of the *ns3* were represented at a higher frequency to segment size in the primary library, it was the fourth after *vp2*, *vp7* and *ns2*. The high frequency sequence representation of *ns3* was not the reason it was selected as the higher represented segments did not have similar sequence depth. Thus it can be concluded that selection of NS3 peptides was by true affinity. This therefore indicated that antigenic regions on NS3 are more linear than all AHSV proteins as identified by immune horse sera. Studies with inactivated AHSV-4 vaccine did not produce antibodies against NS3 in horses, confirming its role in replication (Laviada *et al.*, 1995). This then allowed for NS3 to be used as a possible marker for a DIVA vaccine (Laviada *et al.*, 1995). Further clarity has been offered when *in vitro* studies with reverse genetics and immunostaining with guinea pig and rabbit monoclonal antibodies showed that only the *ns3* RNA is essential for virus formation and not the NS3/NS3a proteins (van der Water *et al.*, 2015). If this is true, then a potential explanation for the observed NS3 antigenicity dominance may be that it is an immune system by-passing strategy by the virus. The virus uses this protein to divert the immune system's attention from the immunopathogenic hot spots on proteins. This phenomenon has also been described for HIV1 using gp120, termed "deceptive imprinting" (Nara and Garrity, 1998). It

therefore makes sense that a single vaccination using a live BTV vaccine without NS3 protected sheep when challenged with a virulent strain (Feenstra *et al.*, 2014). This could mean that the immune system could not be “fooled” and therefore produced antibodies against the hot spots of the virus to neutralise it. Thus, NS3 seems to be immunogenic but its role in inducing protective or neutralizing antibodies remains unclear.

The distribution pattern of mapping sequences was visualised by means of a bar graph to show the identified regions. These regions form functional peptides in a phage display system when the translated sequences mapped in a similar pattern to nucleotides. The immunodominance of *ns3* day 28 IgG selected sequences was found to be largely attributed to two regions, one with a dominant peak, indicating increase sequence depth of overlapping sequences, and one with a low peak. The peaks with nucleotide sequences were the same as those observed with peptides. This thus confirmed the regions selected produced a functional peptide. This dominant region mapped from 1 to 140 bp at the N-terminal end and was consistent with sera from all horses. The region was still dominant with day 52 IgG which may indicate that NS3 provokes production of long lasting antibodies. This region has never been mapped before as being potentially antigenic. It is an amphipathic helix, which also indicates that it can be potentially antigenic (DeLisi and Berzofsky, 1985; Huismans *et al.*, 2004). This region contains a proline-rich region conserved among serotypes (van Staden *et al.*, 1995). Proline-rich domains on hepatitis E virus and avian influenza virus have been suggested to be necessary for virion release from infected cells (Yamanda *et al.*, 2009; Kenney *et al.*, 2012). A proline-rich region is essential for plasma membrane targeting and viral release in the Ebola and Marburg Virus matrix protein VP40 (Reynard *et al.*, 2011). This may be further proof that NS3 is a transmembrane protein

and the proline-rich domain of AHSV NS3 could be critical in attaching the protein. The shortest peptide sequence that was constant in clones mapping to this region included the residues IVPYVPPP, which is proline rich and support the aforementioned assumptions. An ELISA using synthetic peptides of the selected dominant region to confirm binding to day 28 antibodies showed that the region was reactive at higher levels than with day 0 serum, confirming that the region is a hot spot for linear epitopes. The reasons some peptides showed low or no reactivity may be that they did not adsorb to the wells during coating, or that they attached in the wrong orientation where it attaches to the plastic masking the epitopes. The rest of the NS3 protein does not seem to be antigenic (Figure 3.13F). However, this does not preclude the possibility of there being epitopes in this region when interacting with other neighbouring proteins in its native form thus creating neotopes, or NS3 having conformational epitopes which could not be identified in the phage display system. Therefore phage display and high throughput sequencing has enabled mapping of a potentially antigenic region 139bp long on *ns3* identified by five immunised horses. This region could contain multiple linear epitopes or components of a conformation epitope.

Sequences for *vp6* were the second generally most enriched in all horses with day 28 IgG albeit at different levels for the different horses. VP6 is a largely hydrophobic minor protein located in the core and functions as a helicase that binds and unwinds both RNA and DNA, which means it is critical in virus replication (Roy, 1992; Grimes *et al.*, 1998; Mertens and Diprose, 2004; De Waal and Huismans, 2005). It was the second most enriched consistently in most horses in this study. This is in accordance to the observation that VP6 was one of the strongest inducers of antibodies and a potential early infection marker (Martinez-Torrecedrada *et al.*, 1997). Since it is enclosed within the core, it is not clear how the immune system “reaches into the viral core” and

induce an immune response. One potential way in which VP6 may be exposed to the immune system may also be during virus release by lysing the cell. VP6 was found *in vitro* to be expressed in large concentration in the cytosol during the formation of the viral inclusion bodies in BTV and Ibaraki virus (Stauber *et al.*, 1997; Matsuo *et al.*, 2015). This may be in response to the need of VP6 to bind and unwind RNA and DNA during replication, which is a function similar to NS2 during the formation of VIBs (Modrof *et al.*, 2005; Kar *et al.*, 2007). Since NS2 is found in high concentrations in infected cells VIBs, it is thus understandable that a similar concentration will be expressed for VP6. It is highly likely that not all VP6 are packaged in the viral particle when virus is release by lysing the cell. The VP6 may still be floating in concentrations high enough to induce an immune response. If this is true for AHSV, then it would also explain the high antibody concentrations of NS2, another protein strongly associated with VIBs, being detected in vaccinated horse serum (Martinez-Torrecuadrada *et al.*, 1997). VP6 may also provoke production of antibodies that are persistent and have a long half-life because *vp6* segment still had an increased sequence depth with day 52 IgG compared to the naïve (day 0) IgG. However, a defective virus based candidate vaccine devoid of VP6 gene, with the protein supplied *in-trans*, fully protected sheep against challenge (Matsuo and Roy, 2009; Matsuo *et al.*, 2010; Matsuo *et al.*, 2011). This may suggest that although VP6 provokes production of antibodies, its absence in the virus does not affect viral propagation as infectious particles were produced in BSR cell line (Matsuo *et al.*, 2011). This has been the basis for the development of a disabled infection single cycle vaccine strain (DISC) (Matsuo *et al.*, 2011) which replicates a designed few number of times. However, this may suggest that a vaccine construct that targets VP6 may not be effective in stopping or controlling viral replication. This is similar to what has been reported for NS3. This may thus suggest

that the virus uses the immunodominance of these two proteins to by-pass the immune system, diverting attention away from the immunopathological epitopes.

This immunodominance was found to be caused largely by one region on *vp6* when the day 28 IgG selected sequences of five horses were compared, although five regions were prominent. This region was consistently identified by the sera from five horses, indicating that it is a horse specific potentially antigenic region. This region is in the middle of *vp6* (460-540 bp). The translated sequences mapping to this region showed a similar pattern, and also proved the sequences produced fusion peptides, which confirmed the nucleotide mapping. Recognition of the other regions are shared among the sera from the different horses, which may suggest that recognition is horse dependent. Possible reason for the difference in identification of these potentially antigenic regions may be due to the fact that each horse may have a unique immune response to a similar antigen depending on their genetics and immune background. This does not however exclude the possibility that the multiple regions showing reactivity on VP6 may be linear components of a conformational epitope in its native form, which has been disrupted by fragmentation. All regions on VP6 were still recognised by day 52 sera along with other new regions, which may confirm their antigenicity. The dominant region on AHSV VP6 identified in this study was not one of the six epitopes identified by western blot and ELISA in BTV VP6 using rabbit oligoclonal antibodies raised to synthetic peptides (Hayama and Li, 1994). The possible difference may be experimental design based, where in this case a naturally infected host serum was used and not experimental animal models. This was also observed previously where regions identified using chicken and mouse antibodies against VP2 could not be mapped with horse serum induced by the same protein (Bentley *et al.*, 2000). Sequence analysis between BTV and AHSV has shown a

homology of between 26 – 29% and that antigenic regions identified on BTV were not conserved on AHSV VP6 (Turnbull *et al.*, 1996; de Waal and Huismans, 2005). Thus inference of antigenicity of AHSV VP6 peptides from BTV should be done with great caution, taking the homology factor into consideration. All the regions on *vp6* identified here have never been identified before with immune horse sera. Therefore, phage display and high throughput sequencing has allowed mapping of a previously unknown antigenic region.

The sequences mapping to segment *vp5*, the outer capsid protein, are the third group with increased depth selected with day 28 IgG. During infection, VP5 is associated with permeabilising the cell membrane and release of the viral core from the endosomes into the cytoplasm (Mertens *et al.*, 2005). The ability of VP5 to permeabilise the cell is shown where baculovirus expressed AHSV VP5 was cytotoxic to bacteria and insect cells attributed to two amphipathic regions (du Plessis and Nel, 1997; Martinez-Torrecedrada *et al.*, 1999; Wall, 2006; Stassen *et al.*, 2011). It also assists its neighbouring protein, VP2, to assume functional conformation of cell attachment (Mertens *et al.*, 1987; Roy *et al.*, 1990; Forzan *et al.*, 2004). This is supported by previous studies which showed that treatment of BHK-21 cells with AHSV-4 VP2 or VP5 proteins alone did not induced apoptosis (Vermaak and Theron, 2015). Apoptosis was only observed when the cells were infected with both AHSV outer capsid proteins. Thus, it is under selective pressure from the host yet it must also maintain its structure as it is critical in infection initialisation. Since it is exposed early in infection, it is expected that antibodies against VP5 will be produced during the initial stages of infection. Indeed, antibodies against VP5 were used as early infection markers of AHSV in infected horse sera using western blot (Martinez-Torrecedrada *et al.*, 1997). This is supportive of the VP5 Immunodominance observed in this study. The sera were

collected a week after the booster vaccine, it would be expected that anti-VP5 antibodies would still be present in the serum. This is what was observed with sera from all horses but one, where the sequences slightly declined with horse 1 day 28 IgG. Probably due to immune response differences between horses alluded to before. Further analysis of the sequences on a gene level indicated that three potential antigenic regions were recognised by sera from most horses. The selected nucleotide sequences on the regions identified have been confirmed to form functional peptides when the amino acid per base mapped a similar pattern. Of the three regions, a region at the middle (650 bp - 785 bp) was consistently identified by the sera from all horses but horse 2. This region has never been identified before using horse serum. Another region at the N-terminal half (244 bp -325 bp) was also identified as being immunodominant using mouse monoclonal antibody, rabbit and one infected horse polyclonal sera in western blot and synthetic peptides mapped by PEPSCAN (Martinez-Torrecedrada *et al.*, 1999). The fact that a similar region was identified in this study by sera from more than one horse confirms its antigenicity. Although it was also identified with mouse monoclonal antibody and rabbit polyclonal sera, the results in this study confirm that the region is seen by the horse's immune system during infection. The region at the C-terminal (1280bp -1380 bp) has never been identified before as being antigenic. Thus two new potentially antigenic regions have been identified at the C-terminal half end using this method. The other possibility of the identified epitopes being linear components of one big conformational epitope is also not precluded as previously suggested (Rossitto and MacLahachlan, 1992; Martinez-Torrecedrada *et al.*, 1999). The entire protein is recognised by day 52 IgG, which is in agreement with the suggestion that the entire surface of accessible proteins may be potentially antigenic (van Regenmortel, 1990). Therefore, high throughput sequencing

has allowed for the mapping of new potentially antigenic regions on *vp5* (135 bp; 100 bp and 81 bp) have been identified based on sequence clustering and the number of horses mapping to them. Further experiments to investigate if the identified regions are associated with infection and to confirm antigenicity are thus necessary.

The sequences selected with day 28 IgG mapping to *vp2*, *vp3*, *ns2*, *vp7*, *vp4*, *vp1* although reduced, were still selected to some degree. This indicated that antibodies against these proteins were produced albeit at much lower levels than NS3. The antibodies against the major inner and outer surface structural proteins VP2, VP3 and VP7, with the exception of VP5, were reduced compared to minor protein VP6 and non-structural protein NS3. VP2 is the major outer capsid protein, is the most variable and is responsible for viral attachment to a cell. It is also the most extensively studied antigenic protein, eliciting serotype-specific and neutralising antibodies (Bremer *et al.*, 1990; Iwata *et al.*, 1992; Ranz *et al.*, 1992; Burrage *et al.*, 1993; Martinez-Torrecuadrada *et al.*, 1994; Martinez-Torrecuadrada and Casal, 1995; Williams *et al.*, 1998). This means that this protein also, like VP5, is under host selective disadvantage yet it has to maintain its structure necessary for viral propagation. It is uncoated together with VP5 during the initial stages of infection. It would be expected to induce similar host immune responses as VP5. It was also expected that there will be more anti-VP2 antibodies still present seven days after booster vaccine administration. The sequence distribution still declined for *vp2* with day 28 IgG even when the fragment was represented at a higher frequency than its length in the library. This is thus further confirmation that the higher representation of a fragment sequence in the library did not influence selection. The possible reason for the decline may be that the antigenic regions on VP2 are conformationally dependent and the antibodies produced against it did not recognise the linear peptides (van Regenmortel, 1992; Burrage *et al.*, 1993).

Further confirmation was observed when even the booster shot administered seven days prior to serum collection did not increase VP2 identification of linear peptides, resulting in the low levels of sequences identified by day 28 IgG.

The sequences from day 28 IgG mapping to *vp2* showed that five regions were identified. Although five regions were identified, three were recognised by sera of all horses. The immunodominant region on the N-terminal half (55 - 200 bp) and another (377-471 bp) were identified by all horses. The dominant region at the N-terminal end (55 - 200 bp) and another region at the C-terminal (2539 bp - 2633 bp) were still recognised with day 52 IgG, which may indicate that they are significant regions in the context of horse's immune systems. The rest of the regions are not recognised with day 52 IgG. The N- and C- termini of structural proteins are often surface oriented and more mobile than internal peptides in its native structure (Westhof *et al.*, 1984). It may indicate that these regions are exposed and free from interaction with neighbouring proteins on the viral particle. This is in accordance with the assumption that the antibodies identified the virus in its quaternary structure. This is accordance to observation that some of the immunogenic epitopes AHSV-4 VP2 are located at the tips (Manole *et al.*, 2012). It is therefore possible that the region identified at the N-terminal is a linear component of a conformational epitope. The regions identified in this study adds to the other antigenic regions reported for VP2. Antigenic regions i.e. 600 bp – 1296 bp, which the authors said was dominant using mouse monoclonal antibodies, rabbit and horse polyclonal antibodies and PEPSCAN (Martinez-Torrecedrada *et al.*, 1995; Bentley *et al.*, 2000; Martinez-Torrecedrada *et al.*, 2001), was also identified by sera from horse 1 and 2 in this study although not dominant. The difference may be due to the operational approach, instead of ~20 clones, a repertoire of the whole pool of phages was sequenced to give a comprehensive

binding profile. PEPSCAN only allows analysis of 12-mer peptides and has limited throughput (van Regenmortel, 1986), while phage display can screen and select longer peptides. It is possible that the longer peptides in the study give a more rigid structure that is not possible with 12 aa, which may bind more specifically to reveal new antigenic regions (van Regenmortel, 1986). Synthetic peptides of the binding regions tested in ELISA with day 28 serum and day 0 serum suggested that the identified regions may actually be untargeted peptides. This was because the signals observed for the immunised sera were similar to the control (day 0). However, this may be due to operational factors already mentioned which include peptides not adsorbing to the plate in the correct binding orientation and peptides not coating to the ELISA plate. Results may further confirm that antigenic regions of VP2 are mostly conformationally dependent. The possibility of other regions on the gene being reactive when interacting with neighbouring VP5 is not however excluded.

The proteins VP3 and VP7 are inner core major structural capsid proteins. VP3 forms a scaffold with VP7 between the core and outer capsid. Antibodies against them have also been detected during AHSV infection studies using mouse monoclonal, rabbit and horse sera in western blot and ELISA (Martinez-Torrecuadrada *et al.*, 1997). Antigenicity of VP3 and VP7 remains largely unexplored but the production of antibodies against them suggest that the proteins are exposed to the immune system. How this proteins gets exposed to the immune system remains largely inconclusive, however, VP7, has been shown to sequester and form crystals in infected cells (Basak *et al.*, 1996; Bekker *et al.*, 2014). It is possible that during cell lysis to release virus, this particles are thus exposed to the immune system. Another possibility may be that since co-expressed of VP7 and VP3 forms core-like particles (CLP) resembling the normal viral core in infected insects and normal even in the absence of the genome

(Chuma *et al.*, 1992; Le Blois and Roy 1993; Basak *et al.*, 1996; Maree *et al.*, 1998; Limn *et al.*, 2000), a mutation or incorrect packaging may result in the core-like-particle being release when cell lysis. This seems likely since vaccine strains undergo a lot of mutations to remove virulence. VP3 has been shown to be critical in viral assembly in BTV, recruiting and interacting with RNA genome (Sung and Roy, 2014). A mutation on VP3 may lead to incorrect folding thus incorrect functioning. The low number of sequences selected for VP3 and VP7 may indicate that antigenic regions on these proteins are highly conformational. VP7 is very hydrophobic, mostly insoluble and a serogroup-specific major inner capsid protein. Purified AHSV VP7 from BHK infected cells was able to protect BALB/c mice against lethal challenge and it was suggested that protection may also involve cell mediated responses (Wade-Evans *et al.*, 1997). The protection may, however, be different in its natural host.

The sequences mapping to *vp3* and *vp7* showed that two regions for each segment were identified by the sera from all horses which indicated significance to the horse's immune system. There is one dominant region and one small peak for each segment, which may suggest that they are part of a conformational epitope, which has been suggested for VP7 when it bound strongly in radio-precipitation assay and very weakly by immunoblotting (Laviada *et al.*, 1992; Laviada *et al.*, 1993). However, all the regions on VP7 and VP3 have not yet been mapped with horse sera. The dominant peak of *vp3* is in the middle (1301 bp-1405 bp) and the small peak is at the C-terminal half (2081 bp-2190 bp). On *vp7*, the dominant region is in the middle region (621 bp-701 bp) and the small peak region is at the N-terminal (1 bp-50 bp). These regions were still recognised with day 52 IgG, indicating that antigen induces production of long lasting antibodies. AHSV VP7 consists of top and bottom domains and the dominant region in this study forms part of the bottom domain which make contact with VP3 to

enclose the subcore during infection (Basak *et al.*, 1992; Prasad *et al.*, 1992; Basak *et al.*, 1997; Grimes *et al.*, 1998). One 16-mer peptide on the N-terminal of VP7 was confirmed by ELISA to bind day 28 immunised horse antibodies. All the peptides but one, p2, spanning the dominant region did not show a strong signal. This may have been caused by similar reasons mentioned before. Therefore two potential antigenic regions on *vp3* and *vp7* have been identified using high throughput sequencing.

NS2 is the major component of VIBs and is found in abundance in infected cells (Eaton *et al.*, 1990; Mertens and Diprose, 2004). Antibodies against AHSV and BTV NS2 were one of the early markers of infection when infected sera were tested by western blot (Huisman *et al.*, 1987; Martinez-Torrecedrada *et al.*, 1997). This thus confirms that the proteins in infected cells, which are not packaged in the virus particle, are exposed to the immune systems in concentration high enough to induce an immune response. This may probably occur during cell lysis to release virus as previously explained. However, the low selected NS2 sequences is in contrast to the high antibody levels previously observed in infected cells. This may indicate that antigenic regions on NS2 are conformation dependant and do not recognise linear peptides. Antibodies to NS2 were also reported with AHSV serotype 3, where NS2 and VP7 reacted with hyperimmune guinea-pig sera to all nine serotypes and horse antisera to seven attenuated AHSV (Bremer *et al.*, 1994). This indicates that NS2 is conserved among the AHSV sub-group (Bremer *et al.*, 1990). In another study with BTV, virus-specific cytotoxic T lymphocytes were generated in BALB/c and CBA/ca mouse strains injected with baculovirus expressed NS2 in *Sf9* cell lysate, which conferred protection against infection (Jones *et al.*, 1997) which indicate that it is potentially protective. This is notwithstanding the possibility that results may be different in its natural host.

Analysis of the selected sequences mapping to *ns2* on a gene level showed that five regions were identified by day 28 IgG. Of these regions, one region (573 bp-640 bp) was identified by sera from all horses which may suggest that it is of significance to a horse's immune system. However, this region was identified by only horse 2 day 52 IgG. This may indicate that persistence of this region was horses' dependent, which could be attributed to difference in immune system background. Another region (350 bp -420 bp) is also identified by sera from all horses but with a low peak, which may suggest that it is a part of a dominant conformation epitope in the protein's native form. Two potentially horse specific antigenic regions have been identified on *ns2* by high throughput mapping with day 28 IgG.

Sequences mapping to *vp4* and *vp1* were underrepresented in the library. This was expected as they are minor proteins located in the core. Both proteins are part of the active transcription complex of the viral particle. VP1 functions as an RNA-dependent polymerase (Vreede and Huisman, 1998) and VP4 functions as a transferase during viral replication (Roy *et al.*, 1994). Antigenicity studies on these proteins remain largely unexplored, mostly probably because antibodies against them have not been detected before in infected horses or sheep for BTV. However, detection by antibodies of VP4 and VP1 by phage display indicated that there exists antibodies against them in the sera. The low enrichment is assumed to be due to the low concentration of antibodies against them to bind and enrich peptides. Further analysis of the mapping sequences on these segments at gene level showed that only one region is identified by all horses on *vp1* at the C-terminal half (3421 bp – 3497 bp). The mapping identified by sera from all horses on *vp1* indicate that it is a horse specific antigenic region. This region is not recognised with day 52 IgG which may indicate that the region provokes production of non-persistent antibodies with short half-life. On *vp4*, a region at the N-terminal end

(371 bp - 482 bp) is recognised by sera from all horses which may indicate that it is antigenically meaningful. This region is still identified with horse 4 day 52 IgG, indicating that persistency of this region is horse dependent. One new region (76 bp) has been mapped on *vp1* and one on *vp4* regions (111 bp) with phage display and high throughput sequencing.

The identified regions span between 18 and 60 amino acid residues. Since a basic linear epitope is approximately one to six amino acids long (Novotny, 1991; Rubinstein *et al.*, 2008), there might be multiple epitopes on each region. These regions are big enough to allow the possibility of some form of conformation to occur, which could be one of the reasons to account for the size. Thus the possibility that the identified regions are part of a bigger conformational epitope cannot be ruled out. It is possible that some of the regions not selected could be antigenic when in contact with neighbouring proteins (neotopes), however, this was not elucidated in this study.

Phage display in conjunction with high throughput sequencing methods have shown to be valuable tools to use to get a comprehensive and resolute antigenicity profile of proteins and the specific regions on the proteins simultaneously. It was possible to confirm some of the antigenic regions identified by other methods i.e. by PEPSCAN, thus validating the use of a genome-targeted phage display system with high throughput sequencing. New potential antigenic regions targeted specifically by horses' polyclonal antibodies, the naturally affected host, were also comprehensively mapped with high resolution from a large amount of sequences obtained with high throughput sequencing.

Further studies should focus on conclusively confirming the antigenicity of the regions identified on the selected proteins using better approaches. This could be done by

synthesising peptides conjugated to a bigger protein, or cloning and expressing the overlapping sequences and trapping it with a tag or captured on beads. This will enable and increase peptide accessibility to antibodies and not be involved in adsorbing to the plate. Binding can be confirmed in ELISA and the peptides can also be tested to check if they inhibit neutralising antibodies in VNT. The shortest mapping peptide sequence will be identified *in silico*. If it is observed that the anti-virus antibodies bind the peptide and thereby blocks its neutralising capacity, then that peptide could be a candidate for a vaccine. The selected peptides in these immunodominant regions can further be joined together to form a construct that can be tested for antigenicity and linked to viral pathology or vaccine efficacy. For example, some of the proteins (VP5, VP6, VP7, VP2) were associated with being potentially protective against lethal challenge in some form in the literature. A construct of the selected regions on these proteins could be tested for protective potential in neutralising assays. These B-cell antigenic peptides could further be combined with other epitopes e.g. T-cell (Pretorius *et al.*, 2015), epitopes to form a construct that will target both humoral and cellular immunity. Similar regions could be used to monitor antibody production and vaccine efficiency after vaccination since they are also identified by day 52 data. They can be used to check if the vaccine indeed provokes antibody production. This would be an indication that the vaccine, which is the foundation of protection, provokes antibody production. Such a vaccine would also be DIVA compatible as it can be easily be distinguished from whole virus infection.

Apart from vaccine development, the antigenic regions identified on NS2, VP6, NS3 and VP7 may also assist in development of an AHSV diagnostic test. The peptides of these proteins reacted with the immune sera of all the horses in this study. The antibodies against these proteins are associated with early markers of AHSV infection.

Therefore, the selected regions identified would be used to probe for AHSV antibodies early in infection. A diagnostic tool with such a construct would be able to detect if infection is of AHSV origin (VP7, serogroup) and determine serotype (NS3). Although a construct like this would not be useful during an outbreak as it is dependent on antibody production which could be produced later, it could be useful as a confirmatory tool with ELISA.

Although high throughput sequencing is expensive, it is value for money because it gives a depth of data that allow for informed inferences and deductions. This method can also be used even if the structure or sequence of a reference protein is not known. The disadvantage of this method comes with the laborious task of data handling of translating nucleic acid sequences “*en masse*” in CLC. Thus an improvement on the computer program or use of other method to analyse the high throughput data should suffice. Antigenic regions identified with this method still need to be confirmed in other immunoassays.

This study successfully developed pipeline to analyse the large amount of data that is obtained from high throughput sequencing after selection of phages with antibodies from genome-targeted phage displayed libraries. It was shown that matching the DNA sequences to the target genome gives a true picture, thus the laborious translation is not required. In conclusion, the use of phage display with high throughput system has led to the identification of horse specific potential antigenic regions previously not known in both structural and non-structural proteins of AHSV-4. The results offer more information on the antigenicity of AHSV during infection. Some of the regions on minor and non-structural proteins, which have been previously thought to never leave the core, have been discovered using this approach indicating that they may be exposed to the host’s immune system. High throughput sequencing allowed for comprehensive

determination of antigenic and non-antigenic regions based on the number of reads that mapped to a segment region. Further investigation should be done to ascertain the antigenicity of these antigenic regions and immunogenicity.

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APPENDIX A: BUFFERS AND STOCK SOLUTIONS

10x PBS

NaCl	100 g
KCl	2.5 g
Na ₂ HPO ₄ · 2H ₂ O	8 g
KH ₂ PO ₄	3 g
dH ₂ O	make up to 1 L

10x DNase 1 buffer

100 mM Tris-HCl buffer, pH 7.6,
 25 mM MnCl₂
 1 mM CaCl₂
 0.1 mg/ml BSA

50x TAE

Tris	242 g in 700 ml dH ₂ O
Na ₂ EDTA (pH 8.0)	0.5M in 100 ml
Glacial acetic acid	51.1 ml
dH ₂ O	make up to 1 L

Agarose gel electrophoresis

Add one (1 %) or two (2 %) pellet/s (Bioline) in 1x TEA buffer.
 Dissolve by heating until everything is mixed. Add DNA stain (SYBR Safe) and pour on container with comb. Run gel in 1x TEA buffer.

Crystal violet gel

Prepare as for agarose gel. Instead of SYBR Safe, add crystal violet to a final concentration of 10 µg/ml. Mix well pour on container with comp.
 Run gel in 1x TEA containing 10 µg/ml crystal violet.

DNase 1 stopping solution

70% glycerol
 75 mM EDTA
 0.3% bromophenol blue

PEG/NaCl

PEG 6000	200 g
NaCl	146.1 g
dH ₂ O	make to 1L
Autoclave	

1M Glycine-HCl pH 2.2

Glycine 7.51 g into 800 ml
 Mix well and adjust pH with concentrated HCl
 dH₂O Make up to 1 L

1M Tris-HCl pH 9.0

Tris base 121.14 g into 800 ml dH₂O
 Mix well and adjust pH with concentrated HCl
 Make up to 1 L with dH₂O.

0.1M Carbonate buffer pH 9.6.

Na₂CO₃ 1.59g

NaHCO₃ 2.93g

Mix well in 900 ml of dH₂O, adjust pH and fill up to 1L.

APPENDIX B: MEDIA

Antibiotics

Antibiotic	Stock solution	Working solution
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	25 mg/ml	25 µg/ml

2x TY broth

Tryptone 16 g

Yeast extract 10 g

NaCl 5 g

dH₂O make to 900 ml

Mix well and autoclave

Prior to use, add ampicillin and glucose to a working concentration of 100 µg/ml and 2% or kanamycin to the final concentration of 25 µg/ml.

LB broth

Tryptone 10 g

Yeast extract 5 g

NaCl 5 g

dH₂O make up to 1 L

Mix and autoclave. Prior to use, add ampicillin and glucose to a final concentration of 100 µg/ml and 2%.

LB Agar plates

Tryptone 10 g

Yeast extract 5 g

NaCl 5 g

Agar bacteriological 15 g

dH₂O make up to 1 L

Mix well and autoclave. Cool and add ampicillin to a final concentration of 100 µg/ml and glucose to a final concentration of 2%.

TYE Agar plates

Tryptone 10 g

Yeast extract 5 g

NaCl 8 g

dH₂O make to 1 L

Mix well and autoclave. Add ampicillin to a final concentration of 100 µg/ml and glucose to a final concentration

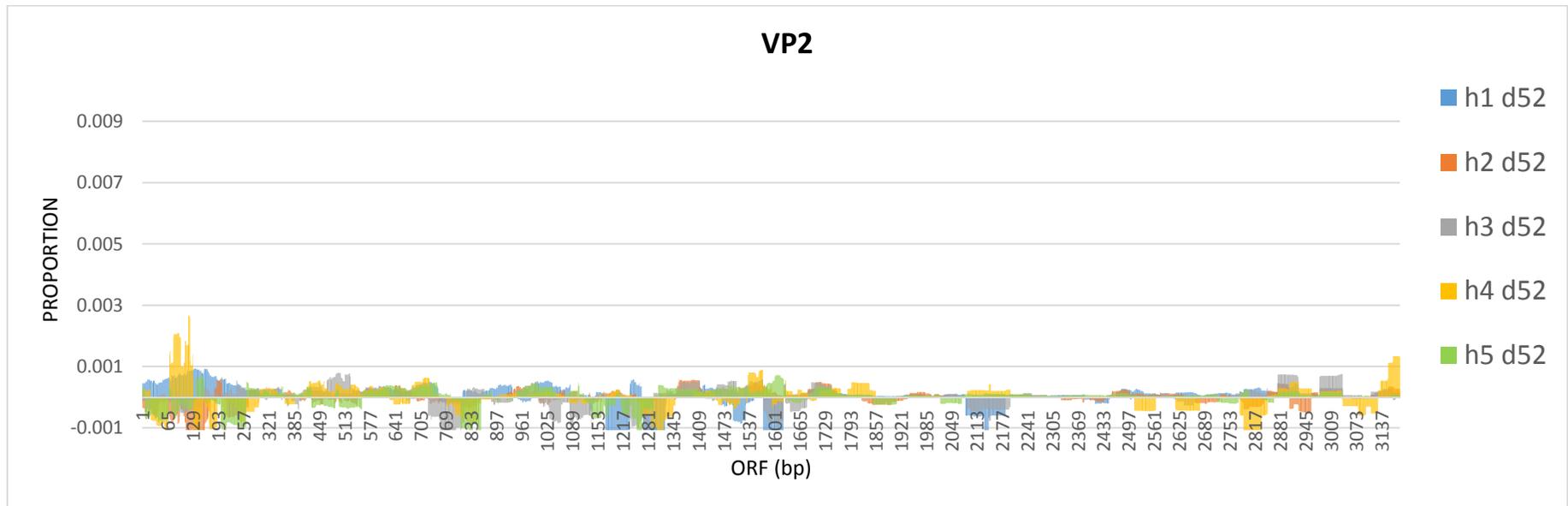
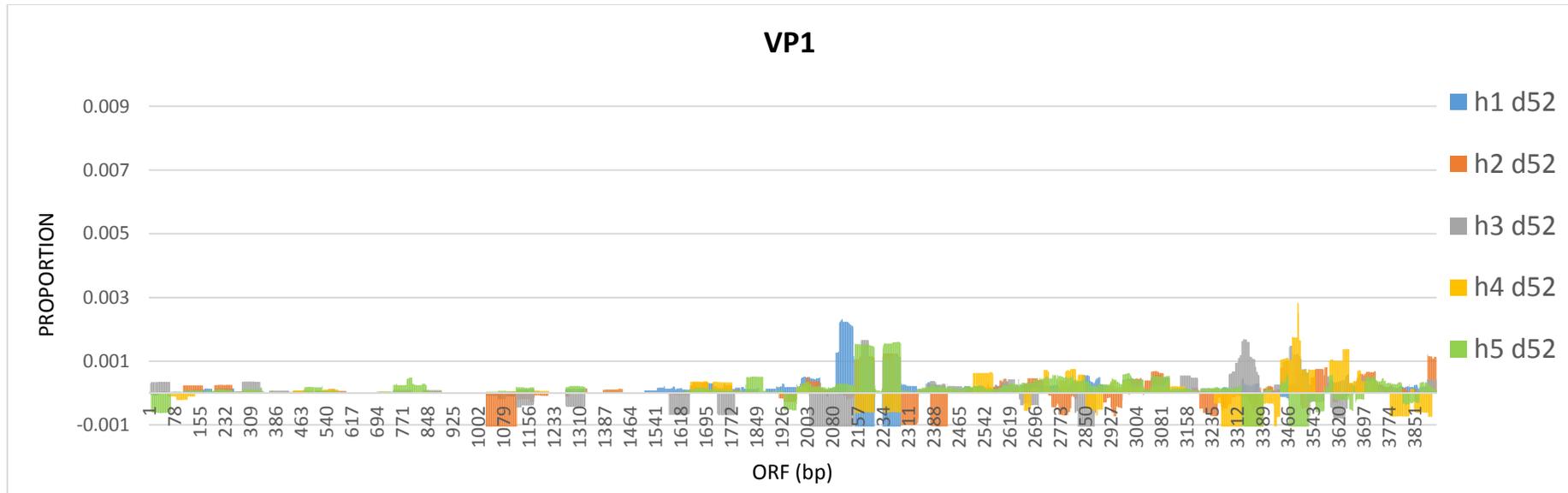
APPENDIX C: SUPPLEMENTARY TABLES AND FIGURES

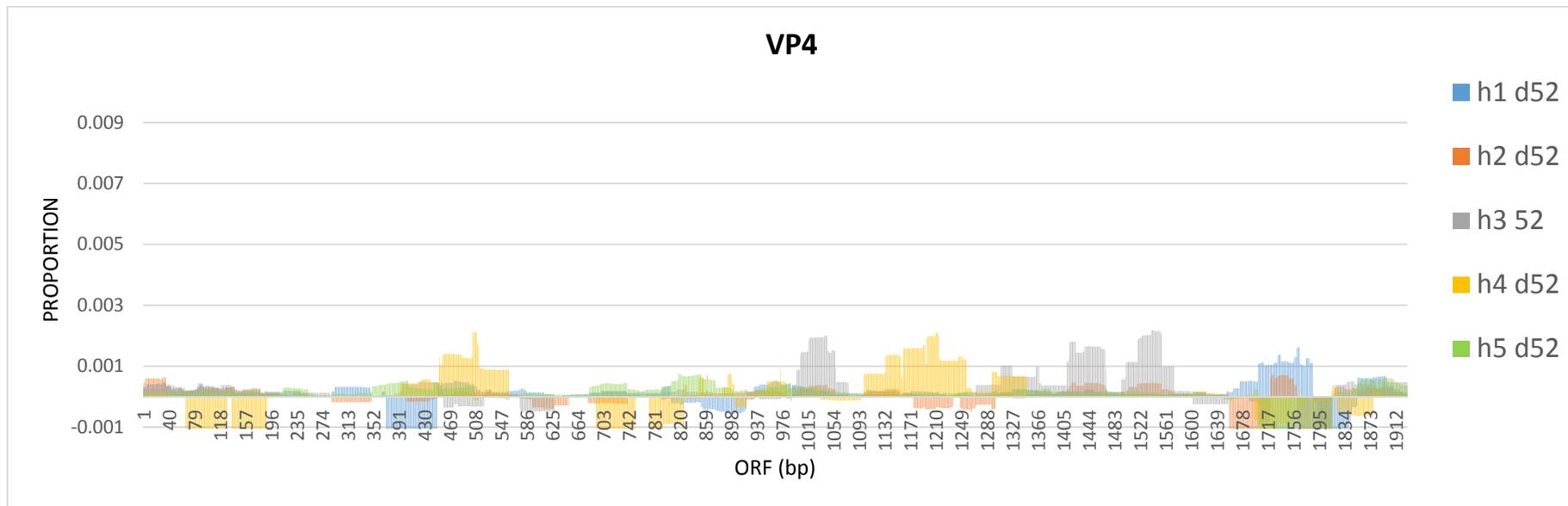
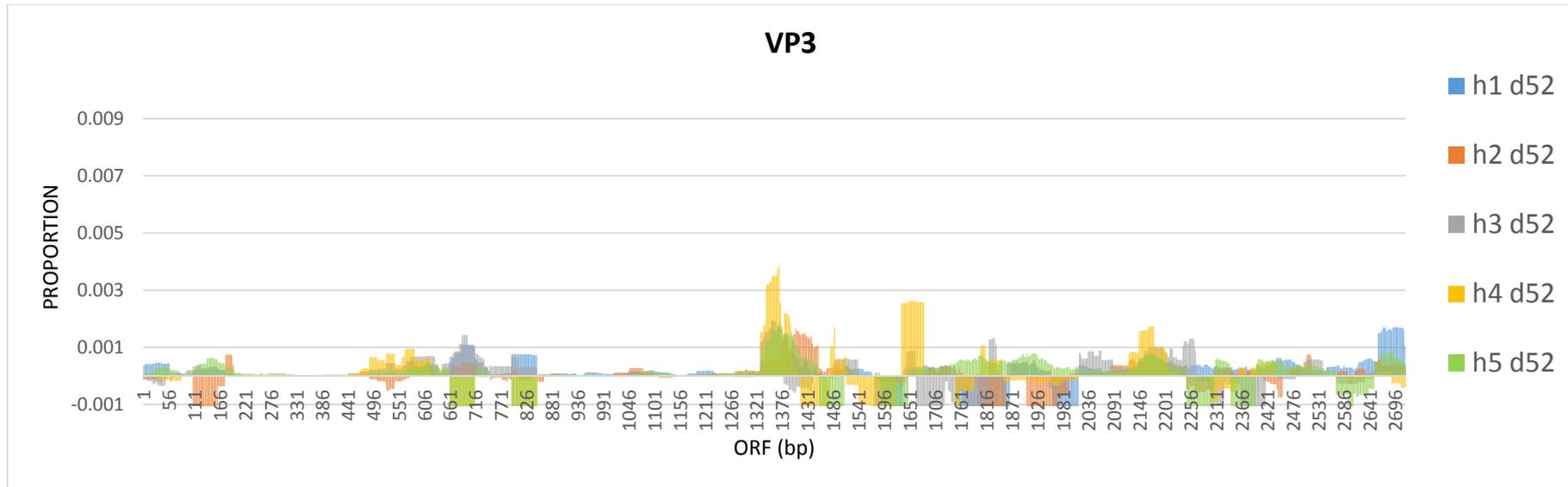
Table AC1: phage numbers during panning process. Number of phages subjected to panning (input), the number of phages released after washing (output). Percentage recovery is calculated as (phage output/phage input) x 100.

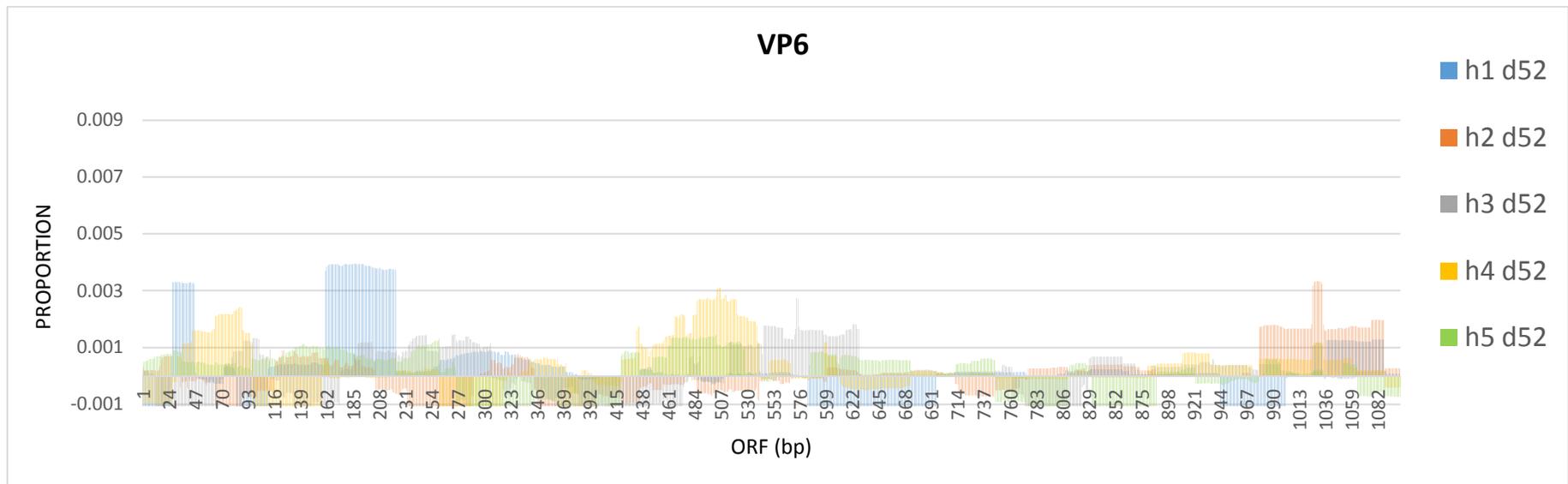
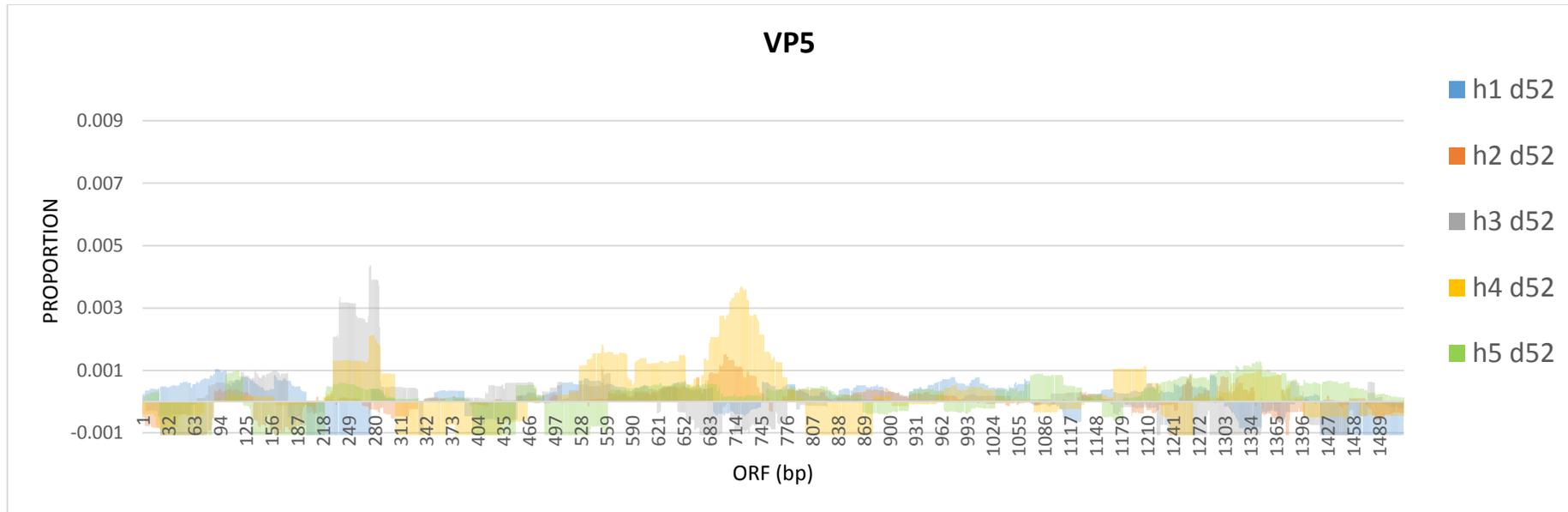
Library		Round1			Round 2			Round 3			Round 4		
		input (TU)	output (TU)	% recovery	input (TU)	output (TU)	% recovery	input (TU)	output (TU)	% recovery	Input (TU)	Output (TU)	% recovery
D0	H1	5.63X10 ⁵	2x10 ³	0.36	2x10 ³	4.5x10 ⁴	2250	4.5x10 ⁴	1.0x10 ⁶	2667	1.0x10 ⁶	1.3x10 ⁶	130
	H2		6x10 ⁴	10.66	6x10 ⁴	7.5x10 ⁴	125	7.5x10 ⁴	2.0x10 ⁶	2133	2.0x10 ⁶	2.3x10 ⁶	115
	H3		3x10 ³	0.53	3x10 ³	3.0x10 ⁴	1000	3.0x10 ⁴	3.0x10 ⁶	9333	3.0x10 ⁶	5.4x10 ⁶	180
	H4		1x10 ³	0.18	1x10 ³	8.5x10 ⁴	8500	8.5x10 ⁴	1.0x10 ⁶	1529	1.0x10 ⁶	2.9x10 ⁶	290
	H5		6x10 ⁴	10.66	6x10 ⁴	1.1x10 ⁵	183.3	1.1x10 ⁵	2.0x10 ⁶	1364	2.0x10 ⁶	2.4x10 ⁶	120
D28	H1	5.63X10 ⁵	3.8x10 ⁴	6.75	3.8x10 ⁴	7.7x10 ⁵	2026	7.7x10 ⁵	1.0x10 ⁶	156	1.0x10 ⁶	1.4x10 ⁶	140
	H2		6.0x10 ⁶	1066	6.0x10 ⁶	8.6x10 ⁶	143	8.6x10 ⁶	1.0x10 ⁷	140	1.0x10 ⁷	8.6x10 ⁶	86
	H3		8.1x10 ⁵	144	8.1x10 ⁵	2.7x10 ⁶	333	2.7x10 ⁶	8.0x10 ⁶	307	8.0x10 ⁶	8.1x10 ⁶	101
	H4		9.0x10 ⁵	160	9.0x10 ⁵	9.3x10 ⁶	103	9.3x10 ⁶	7.4x10 ⁵	80	7.4x10 ⁵	9.5x10 ⁵	128
	H5		1.9x10 ⁵	34	1.9x10 ⁵	2.2x10 ⁷	11579	2.2x10 ⁷	6.0x10 ⁶	25	6.0x10 ⁶	6.3x10 ⁶	105
D52	H1	5.63X10 ⁵	7.0x10 ⁶	1243	7.0x10 ⁶	9.5x10 ⁷	1357	9.5x10 ⁷	8.0x10 ⁷	86	8.0x10 ⁷	8.1x10 ⁷	101
	H2		4.1x10 ⁷	7282	4.1x10 ⁷	2.5x10 ⁷	61	2.5x10 ⁷	5.0x10 ⁷	212	5.0x10 ⁷	4.6x10 ⁷	92
	H3		1.4x10 ⁶	249	1.4x10 ⁶	2.0x10 ⁶	143	2.0x10 ⁶	3.0x10 ⁷	1600	3.0x10 ⁷	2.6x10 ⁷	86
	H4		3.6x10 ⁶	639	3.6x10 ⁶	1.7x10 ⁷	472	1.7x10 ⁷	2.0x10 ⁷	112	2.0x10 ⁷	2.2x10 ⁷	110
	H5		6.0x10 ⁵	107	6.0x10 ⁵	3.0x10 ⁶	500	3.0x10 ⁶	6.0x10 ⁷	1933	6.0x10 ⁷	4.5x10 ⁷	75

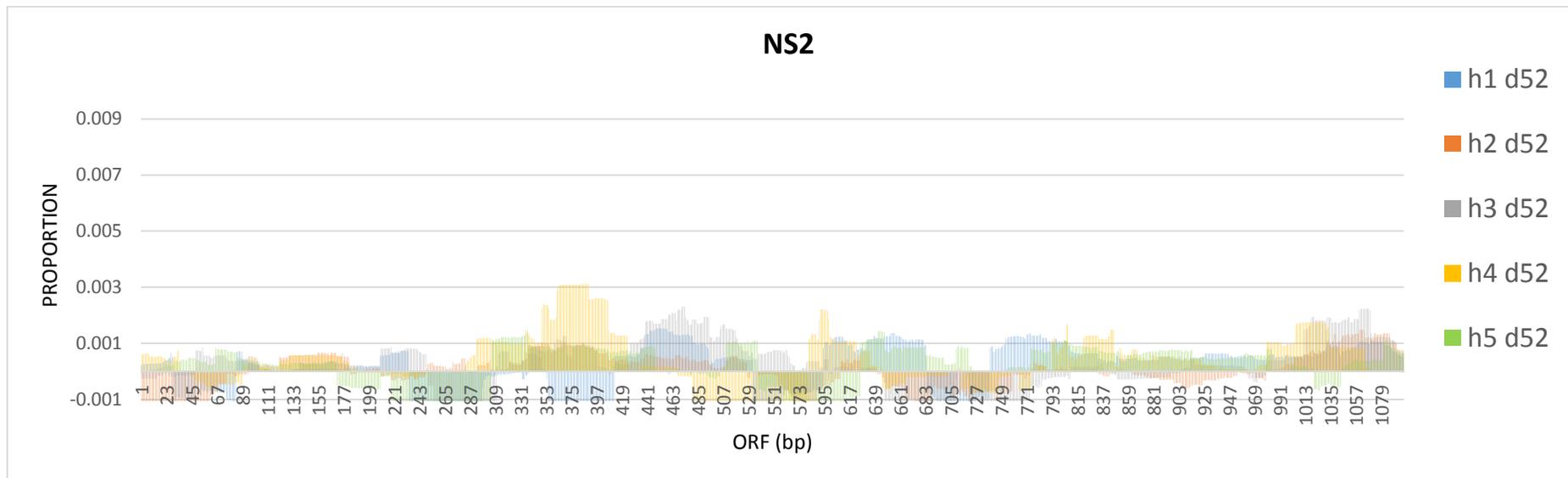
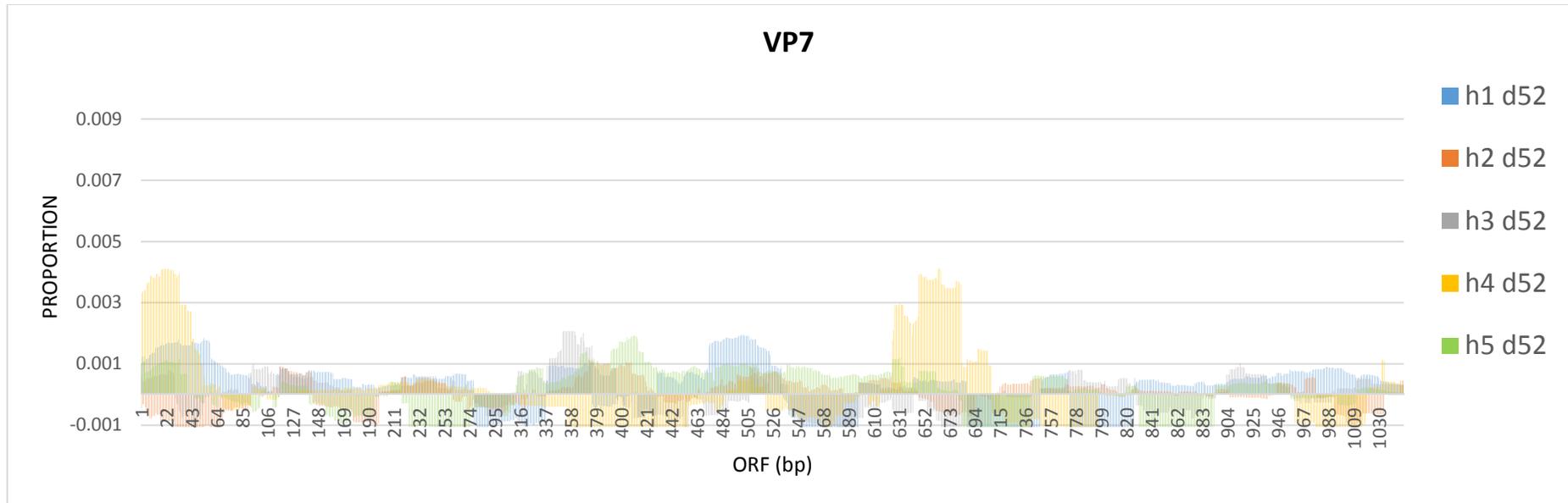
*H = horse

*D = day









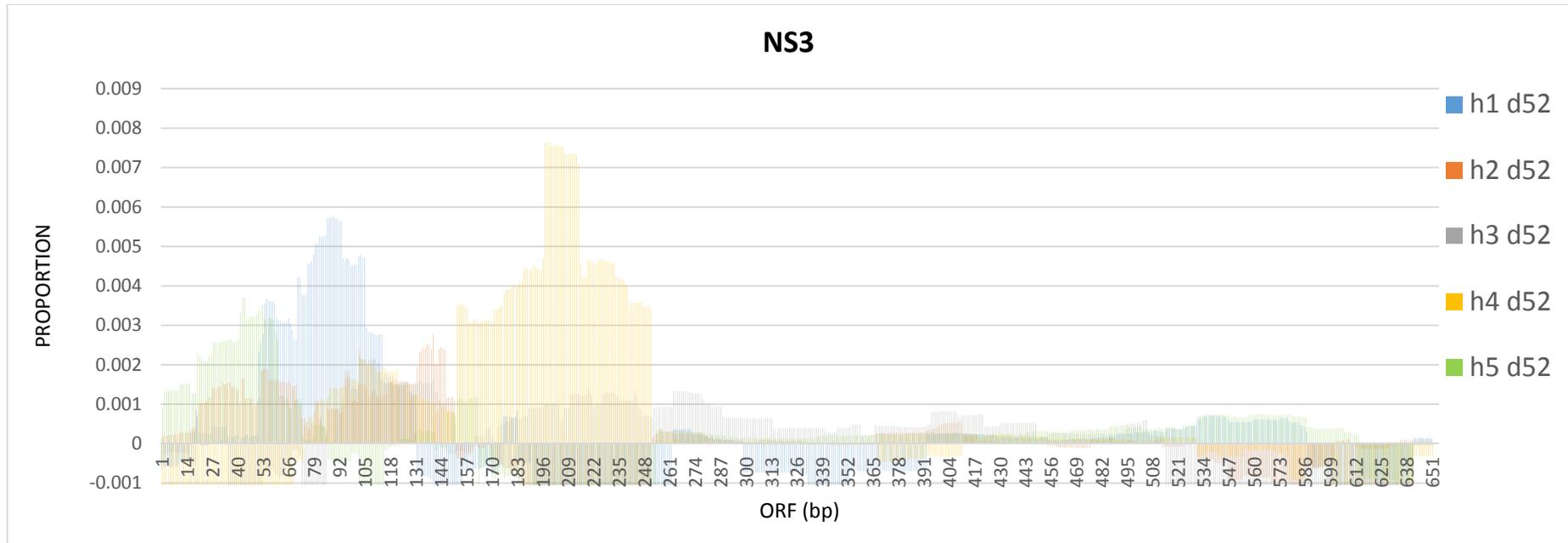


Figure AC2: Day 52 sequence data converted to matches per nucleotide position as a proportion of the total nucleotide matches per gene segment. Data of all five horses combined on one graph. Numbers represent the identified potentially antigenic regions. Data was normalised by subtraction of day 0 matches (baseline data).

Table AC2: Synthetic 16-mer peptides with 8 amino acid overlaps for AHSV-4 proteins VP2, VP4, VP7 and NS3 and a presentation illustrating the 16-mer peptides with 8 overlaps

VP2

DNA bp		Amino acids		Peptide	Sequence
start	end	start	end		
24	69	8	23	VP2-1_P2	MTNEKFDPSLEKTICD
49	96	17	32	VP2-1_P3	SLEKTICDVIVTKKGR
72	117	24	39	VP2-1_P4	VIVTKKGRVKHKEVDG
97	144	33	48	VP2-1_P5	VKHKEVDGVCGYEWDE
120	165	40	55	VP2-1_P6	VCGYEWDETNRHFGLC
145	192	49	64	VP2-1_P7	TNHRFGLCEVEHDMSI
312	357	104	119	VP2-1_P14	DRDDNEMRKILIQPYA
337	384	113	128	VP2-1_P15	KILIQPYAGEMYFSPE
360	405	120	135	VP2-1_P16	GEMYFSPECYPSVFLR
385	432	129	144	VP2-1_P17	CYPSVFLRREARSQKL
408	453	136	151	VP2-1_P18	REARSQKLDIRINYIG
433	480	145	160	VP2-1_P19	DIRINYIGKRVEFYEE
3048	3093	1016	1031	VP2-2_P61	TLKIYNRGSMDTLILI
3073	3120	1025	1040	VP2-2_P62	SMDTLILISSGVYTFG
3096	3141	1032	1047	VP2-2_P63	SSGVYTFGNKFLLSKL
3121	3168	1041	1056	VP2-2_P64	NKFLLSKLLAKTE

VP2

MTNEKFDPSLEKTICD
 SLEKTICDVIVTKKGR
 VIVTKKGRVKHKEVDG
 VKHKEVDGVCGYEWDE
 VCGYEWDETNRHFGLC
 TNHRFGLCEVEHDMS

DRDDNEMRKILIQPYA
 KILIQPYAGEMYFSPE
 GEMYFSPECYPSVFLR
 CYPSVFLRREARSQKL
 REARSQKLDIRINYIG
 DIRINYIGKRVEFYEE

TLKIYNRGSMDTLILI
 SMDTLILISSGVYTFG
 SSGVYTFGNKFLLSKL
 NKFLLSKLLAKTE

VP4

DNA bp		Amino acid		Peptide	Sequence
start	end	start	end		
312	357	104	119	VP4_P14	IGRRRVTLRKSFGNML
337	384	113	128	VP4_P15	RKSFGNMLRAYAFQHV
360	405	120	135	VP4_P16	RAYAFQHVTVLHGSEA
385	432	129	144	VP4_P17	TVLHGSEAETLSYADP
408	453	136	151	VP4_P18	ETLSYADPKRHVVKGQ
433	480	145	160	VP4_P19	KRHVVKGQPKAAPMYD
456	501	152	167	VP4_P20	PKAAPMYDHPDRWWRD

VP4

IGRRRVTLRKSFGNML
 RKSFGNMLRAYAFQHV
 RAYAFQHVTVLHGSEA
 TVLHGSEAETLSYADP
 ETLSYADPKRHVVKGQ
 KRHVVKGQPKAAPMYD
 PKAAPMYDHPDRWWRD

VP7

DNA bp		Amino acid		Peptide	Sequence
start	end	start	end		
1	48	1	16	VP7_P1	MDAIAARALSVVRACV
24	69	8	23	VP7_P2	LSVVRACVTVTDARVS
49	96	17	32	VP7_P3	TVTDARVSLDPGVMET
577	624	193	208	VP7_P25	IFCDPQGASLESAPGT
600	645	200	215	VP7_P26	SLESAPGTFVTVDGVN
625	672	209	224	VP7_P27	FVTVDGVNVAAGDVVA
648	693	216	231	VP7_P28	VAAGDVVAWNTIAPVN
673	720	225	240	VP7_P29	WNTIAPVNVGNPGARR

VP7

MDAIAARALSVVRACV
 LSVVRACVTVTDARVS
 TVTDARVSLDPGVMET

IFCDPQGASLESAPGT
 SLESAPGTFVTVDGVN
 FVTVDGVNVAAGDVVA
 VAAGDVVAWNTIAPVN
 WNTIAPVNVGNPGARR

NS3

DNA bp		Amino		Peptide	Sequence
start	end	start	end		
1	48	1	16	NS3_P1	MNLASIAKNYSMHNGE
24	69	8	23	NS3_P2	NYSMHNGESGAIVPYV
49	96	17	32	NS3_P3	SGAIVPYVPPPYNFAS
72	117	24	39	NS3_P4	PPPYNFASAPTFSQRT
97	144	33	48	NS3_P5	APTFSQRTSQMESVSL
145	192	49	64	NS3_P7	GILNQAMSSTTGASGA
168	213	56	71	NS3_P8	STTGASGALKDEKAAF
193	240	65	80	NS3_P9	LKDEKAAFGAMEALR
216	261	72	87	NS3_P10	GAMEALRDPEPIRQI

NS3

MNLASIAKNYSMHNGE
 NYSMHNGESGAIVPYV
 SGAIVPYVPPPYNFAS
 PPPYNFASAPTFSQRT
 APTFSQRTSQMESVSL

GILNQAMSSTTGASGA
 STTGASGALKDEKAAF
 LKDEKAAFGAMEALR
 GAMEALRDPEPIRQI

Animal Ethics approval certificate



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	Identifying B-cell epitopes of African horse sickness virus serotype 4 recognised by antisera of immune horses
PROJECT NUMBER	V082-13
RESEARCHER/PRINCIPAL INVESTIGATOR	EM Mathebula

STUDENT NUMBER (where applicable)	134 157 44
DISSERTATION/THESIS SUBMITTED FOR	MSc

Use of serum samples previously obtained

ANIMAL SPECIES	n/a	
NUMBER OF ANIMALS	n/a	
Approval period to use animals for research/testing purposes	January 2013 – December 2014	
SUPERVISOR	Dr. J Fehresen	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	25 November 2013
CHAIRMAN: UP Animal Ethics Committee	Signature	

Protocol approval

Ref: V082/13

22 January 2014



University of Pretoria

Faculty of Veterinary Science
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Onderstepoort
0110

Tel: +27 12 529 8000
Fax: +27 12 529 8300

Dr J Fehrsen
Department Veterinary Tropical Diseases
(fehrsenj@arc.agric.za)

Dear Dr Fehrsen

**PROTOCOL V082/13: IDENTIFYING B-CELL EPITOPES OF AFRICAN HORSE SICKNESS VIRUS
RECOGNIZED BY ANTISERA OF IMMUNE HORSES – *EM Mathebula***

I am pleased to inform you that the abovementioned protocol was approved by the Research Committee.

Kindly take note of the attached document.

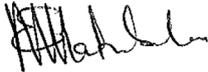
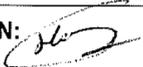
Kind regards

A handwritten signature in black ink, appearing to read 'Niesje Tromp'.

NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Prof JAW Coetzer, Deputy Dean: Research (koos.coetzer@up.ac.za)
EM Mathebula, Researcher (mathebulaem@arc.agric.za)
Prof D Abernethy, HOD (darrell.abernethy@up.ac.za)
Prof M Oosthuizen, Departmental Research Coordinator (marinda.oosthuizen@up.ac.za)
Ms M Human, Student Administration (magda.human@up.ac.za)

Title approval

UNIVERSITY OF PRETORIA	
FACULTY OF VETERINARY SCIENCE	
APPLICATION FOR APPROVAL OF TITLE OF DISSERTATION OR THESIS	
PLEASE NOTE:	
This form must be completed in TYPING .	
Please send the application form to: HEAD: ACADEMIC ADMINISTRATION .	
Name of candidate:	Evans Mantiri Mathebula
Student number:	13415744
Degree:	rMSc (VetSci)
Course code:	VWE801
Department:	Veterinary Tropical Diseases
Name of supervisor/Leader:	Dr J Fehrsen
Name of co-supervisor(s)/co-leader(s)	Dr A Pretorius
Protocol approved : Yes <input checked="" type="checkbox"/> No Date submitted/approved: 22/01/2014 Please attach a summary/abstract.	
Title of dissertation/thesis: Identifying B-cell epitopes of African horse sickness virus serotype 4 recognised by antisera of immunised horses.	
SIGNATURES OF:	
CANDIDATE: 	Date of submission: 09/09/2015
SIGNATURE OF SUPERVISOR/PROMOTER: 	DATE: 9/9/2015
APPROVED BY HEAD OF DEPARTMENT: 	DATE: 10/9/2015
APPROVED BY POST GRAD COM CHAIRMAN: 	DATE: 29/09/15