Identification of T cell and linear B cell epitopes on African horse sickness virus serotype 4 proteins VP1-1, VP2, VP4, VP7 and NS3

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Highlights

- AHSV4 VP1, -2, -4, -7 and NS3 contain regions with CD8+ T cell epitopes.
- Overlapping peptides spanning these AHSV4 proteins were synthesized.
- Recall immune assays identified T cell and linear B cell epitopes.
- Conserved T cell and linear B cell epitopes will be used in NGV.

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Abstract

The viral proteins VP1-1, VP2, VP4, VP7 and NS3, of African horse sickness virus serotype 4 (AHSV4), have previously been identified to contain CD8+ T cell epitopes. In this study, overlapping peptides spanning the entire sequences of these AHSV4 proteins were synthesized and used to map epitopes. Peripheral blood mononuclear cells (PBMC) isolated from five horses immunized with an attenuated AHSV4 were stimulated *in vitro* with the synthesized peptides. Various memory immune assays were used to identify the individual peptides that contain CD8+ T cell epitopes, CD4+ T cell epitopes and linear B cell epitopes. The newly discovered individual peptides of AHSV4 proteins VP1-1, VP4, VP7 and/or NS3 that contain CD8+ T cell, CD4+ T cell or linear B cell epitopes could contribute to the design and development of new generation AHS peptide-based vaccines and therapeutics.

Keywords:

Overlapping synthetic peptides, T cell epitopes, linear B cell epitopes, new generation AHS peptide-based vaccines, conserved epitopes

[1]

[1] **Abbreviations:** AHS, African horse sickness; AHSV1, African horse sickness virus serotype 1; APCs, antigen presenting cells; BCRs, B cell receptors; DIVA, differentiate infected from vaccinated animals; FATT-CTL, fluorescent antigen-transfected target cells-cytotoxic T lymphocytes; GC, germinal centre; IFN-γ, interferon gamma; LPA, lymphocyte proliferation assay; PBMC, peripheral blood mononuclear cells; TCM, central memory T cells; TCRs, T cell receptors; TEM, effector memory T cells; Th1, T helper type 1; TRM, tissue-resident memory T cells; TSCM, stem cell memory T cells

1. Introduction

African horse sickness (AHS) is an insect-transmitted, infectious but noncontagious disease of equids that can cause up to 95% mortality in susceptible horses. African horse sickness virus (AHSV), an orbivirus of the family *Reoviridae* causes this economically important disease. The AHSV dsRNA genome encodes seven structural proteins (VP1, VP2, VP3, VP4, VP5, VP6 and VP7) and five non-structural proteins (NS1, NS2, NS3, NS3A and NS4) (Mellor and Hamblin, 2004; Carpenter et al., 2017; Dennis et al., 2019). To date, nine antigenically distinct AHSV serotypes, AHSV serotype 1 (AHSV1) to 9 (AHSV9) have been identified based primarily on the antigenic variation of AHSV protein VP2 (Mellor and Hamblin, 2004). In South Africa, the disease is controlled by preventative vaccination of susceptible horses in endemic areas with the commercially available polyvalent AHSV live attenuated vaccine (Mellor and Hamblin, 2004; Carpenter et al., 2017; Dennis et al., 2019). The attenuated vaccines currently available are not compatible with DIVA (differentiate infected from vaccinated animals) serological assays. Additionally, various bio-safety concerns relating to the possible reversion of the vaccine viruses to virulent phenotype through genetic drift or recombination and reassortment, have contributed to numerous studies focusing on the development of safer new generation AHS vaccines. The protective role of serotype-specific neutralizing antibodies against AHSV VP2 is well established (Dennis et al., 2019). This forms the basis of novel vaccine formulations that almost exclusively induce the humoral immune response in order to specifically induce serotype-specific neutralizing antibodies against AHSV protein VP2 (Calvo-Pinilla et al., 2018; Dennis et al., 2019). However, despite significant time and monitory input resulting in multiple experimental trials, none of the new generation AHS vaccines has been commercialized to date (Dennis et al., 2019). The development of new generation AHS vaccines is complex, partly due to the variability of VP2, the most variable protein between the different AHSV serotypes (Van Niekerk et al., 2001; Dennis et al., 2019) as well as the lack of knowledge pertaining to the cellular immune responses induced by attenuated and virulent AHSV strains. Recently, the contribution of the cellular immune response to protective immunity have received additional attention (Calvo-Pinilla et al., 2018), yet in-depth investigations into the role of the cellular immune response against this disease are still lacking.

In order to investigate the cellular immune response induced against AHSV, an optimised assay was previously developed to measure the antigen-specific effector CD8+ T cell (CTL) killing, or lysis, of viral protein expressing target cells, using fluorescent antigen-transfected target cells-cytotoxic T lymphocytes (FATT-CTL). The presence of CD8+ T cell epitopes contained within the structural (VP1, VP2, VP4 and VP7) and non-structural (NS3) AHSV4 proteins, was identified by utilizing the antigen-specific memory CTL-induced cell lysis from the peripheral blood mononuclear cells (PBMC) of horses vaccinated with attenuated AHSV4 (Faber et al., 2016). The CTLs do not kill their target cells by directly causing lysis to the cell membranes, but induce apoptosis in target cells and the apoptotic cells progress to secondary necrosis (cell lysis) *in vitro* (Jerome et al., 2003). In this study, overlapping peptides consisting of 16-mer amino acids (aa) were synthesized, to span the entire sequences of AHSV4 proteins VP2, VP4, VP7, NS3 and part of VP1 (1-628 aa of VP1-1), with 8 aa overlap between the peptides. The PBMC were isolated from five horses immunized with attenuated AHSV4 and stimulated *in vitro* with the synthesized peptides. Various recall (also known as memory) immune assays were used to identify the individual peptides that contain CD8+ T cell epitopes, CD4+ T cell epitopes and linear B cell epitopes. The position of the individual peptides identified to contain T cell or linear B cell epitopes was determined within the AHSV4 proteins as well as their sequence homology were determined by analysing all the available AHSV sequences.

The identification of individual peptides that contain CD8+ T cell, CD4+ T cell or linear B cell epitopes that are conserved between the nine different AHSV serotypes may contribute to the design and development of new generation AHS peptide-based vaccines (e.g. multi-epitope or synthetic peptide-based vaccines) or therapeutics. Synthetic peptide-based vaccines have been suggested to mitigate the disadvantages associated with conventional whole organism vaccines (Skwarczynski and Toth, 2016).

2. Materials and methods

2.1. Immunization of horses

Five AHSV naïve 30 month-old horses were subcutaneously immunized twice with the attenuated AHSV4 vaccine strain, using 5×10^4 viable virus in 2 ml DMEM medium (Lonza) prepared at the

Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) (Faber et al., 2016; Pretorius et al., 2016). The first immunization was administered on day 0 followed by the second immunization on day 21. All animal vaccination protocols were approved by the animal ethics committees at the ARC-OVR, Onderstepoort Biological Products (OBP), Ltd and the Department of Agriculture, Land Reform and Rural Development (DALRRD) of South Africa, under Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984).

2.2. Collection of blood and isolation of horse peripheral blood mononuclear cells (PBMC)

This study performed recall (memory) immune assays using PBMC prepared from blood collected 5 months (peptide pools) and 6 months (individual peptides) after the second immunization on day 21. Blood (50 ml per horse) was collected in BD Vacutainer®- EDTA tubes (Becton, Dickinson) and PBMC was isolated on a density gradient medium (Histopaque®-1077; Sigma-Aldrich®) as described previously (Pretorius et al., 2012). The PBMC was washed three times and the live cells were counted using GIBCO® trypan blue stain (Invitrogen) and resuspended at 4 x 10^6 cells/ml in cRPMI (GIBCO® RPMI+GlutaMAX™-I (Invitrogen) supplemented with 55 mM 2-mercaptoethanol and 1% (v/v) of 100x GIBCO® Pen Strep (Invitrogen) and 10% heat inactivated horse serum (Invitrogen).

2.3. Synthetic peptides

In this study, a total of 359 individual peptides were designed to transverse the complete VP2, VP4, VP7, NS3 and part of VP1 (1-628 aa of VP1-1) proteins of AHSV4. The 16-mer peptides overlapping by 8 aa were synthesized by GenScript (Piscataway, NJ, USA). The 359 peptides comprised of 80 for VP4 (VP4_P1-P80 peptides), 27 for NS3 (NS3_P1-P27 peptides), 43 for VP7 (VP7_P1-P43 peptides), 78 for VP1-1 (VP1-1_P1-P78 peptides) and 131 to subdivide VP2 (1-543 aa of VP2: VP2-1_P1-P67 peptides and 544-1060 aa of VP2: VP2-2_P1-P64 peptides). The majority of the peptides were > 98% pure, with a few exceptions that were > 90%, > 80% or crude and their quantity were between 1-4 mg. The peptides were dissolved in 1ml PBS (Sigma) containing 20% v/v DMSO (Sigma) and diluted to 200 µg/ml in cRPMI (GIBCO® RPMI+GlutaMAX™-I (Invitrogen) supplemented with 55 mM 2-mercaptoethanol and 1% (v/v) of 100x GIBCO® Pen Strep (Invitrogen) and 10% heat inactivated horse serum (Invitrogen). The individual peptides (20 µg/ml) were grouped into pools, and 10 µg/ml of

each pool were used in the recall immune assays.

2.3.1. Peptide pools and individual peptides

In the first experiment, the 359 individual peptides were arranged in a matrix of 18 vertical pools (vp1-18) and 20 horizontal pools (hp19-38), where each peptide is present in two pools (Supplementary Fig. S1). Each peptide was allocated to one vertical and one horizontal pool. The individual peptides with positive results were identified at the vertical/horizontal intersection (Supplementary Fig. S1). However, the pooling method is ambiguous because a positive result could have been obtained, either when all the individual peptides in the vertical/horizontal intersections or if only some of them were positive (Supplementary Fig. S2). Because of this ambiguity, it is necessary to repeat all the recall immune assays again with the individual peptides. In the second experiment, individual peptides identified from the pools in the first experiment were tested either individually or in 2-3 peptides mixtures using the recall immune assays. All the combinations of 1-3 peptides will subsequently be referred to as individual peptides.

2.4. Recall immune assays

Recall immune assays were performed using PBMC obtained from five horses immunized with attenuated AHSV4. The PBMC were stimulated *in vitro* with peptide pools in the first experiment and with the individual peptides in the second. Only the results that were significant in at least four out of the five horses were included in this study. Unstimulated PBMC were used as negative controls and concanavalin A (ConA), the live viable virulent AHSV4 field isolate and recombinant AHSV4 proteins, VP1-1, VP2-2, VP4, VP7 and NS3 from our previous study (Faber et al., 2016) were used in the first experiment as positive controls in all assays. Additional controls used are included in the applicable sections below.

2.4.1. Fluorescent antigen-transfected target cells-cytotoxic T lymphocytes (FATT-CTL) assay

A published FATT-CTL assay that quantifies the CTL-mediated elimination of antigen-GFP expressing cells by flow cytometry and uses PBMC as both the target and effector cells (van Baalen et al., 2008) was adapted for use with horse PBMC and modified for recall immunity (Faber et al., 2016). Triplicate

wells of effector cells at 5-50 x 10^5 cells/ml were stimulated with 10 µg/ml peptide pools, individual peptides and the recombinant AHSV4 proteins per well or with the live viable virulent AHSV4 field isolate (5 x 10^3 virus/ml) in medium in a 48 well plate. Unstimulated controls of effector cells in medium were included. Target cells were electroporated with either empty pIRES-hrGFP II vector (TpGFPempty) for negative control or with the pIRES-hrGFP II vectors containing the AHSV4 gene (TpGFP_AHSV4gene). Aliquots containing 2.5 x 10^6 cells were washed with PBS and resuspended in 250 µl electroporation buffer (BioRad). Each aliquot of cells was electroporated with 10 µg of TpGFPempty or TpGFP_AHSV4gene at 250V and at 1000µF; ∞ Ω. Target cells were seeded in triplicate in a 48 well plate at 5 x 10^5 cells per well. These target and effector cells were incubated for 96 h at 37 °C in a humidified 5% CO_2 incubator (United Scientific), and the effector cells were loosened and mixed at a effector : target cell ratio of 1 : 1. Target and effector cells only controls were also included. The percentage lysis was determined by acquiring the number of GFP positive cells using a cytomics FC 500 flow cytometer® with Kaluza 2.1 software (Beckman Coulter). The percentage lysis was calculated similar to Faber et al. (2016) with some modifications. To reduce the number of calculations, the number of GFP positive cells were measured and converted to events/second, since concentration cannot be measured on the FC500 flow cytometer. Each peptide was evaluated to its own controls (empty vector control, naïve VP/NS expression control, naïve VP/NS protein, immune VP/NS expression control and immune VP/NS protein). Samples of the effector cells were used for phenotypic analyses in the first and second experiments, whereas the optimized FATT-CTL assay was used only in the second.

2.4.2. Phenotypic analyses of the effector cells using flow cytometry

2.4.2.1. Cell surface staining

Samples of the effector cells stimulated with peptide pools in the first experiment and with individual peptides in the second, as described in section 2.4.1., were collected and stained to determine the cell phenotype using flow cytometry (Pretorius et al., 2012). Briefly, triplicate wells of PBMC were pooled before 2 x 10^5 cells/well were stained for CD4, CD8, and CD45RO (which is a marker of memory T cells; individual peptides only) as well as B cell markers in duplicate using a single-staining method.

The relevant primary monoclonal antibody (1:70 dilution) was added individually to each well. The antibodies used were mouse anti-equine CD4 (cell line HB61D, IgG1, WSU), mouse anti-equine CD8 (cell line HT14A, IgG1, WSU), mouse anti-equine CD45RO (cell line GC44A, IgG3, WSU) and mouse anti-equine B-cell marker (cell line E18A, IgG2a, WSU). This was followed by staining with the appropriate secondary antibody: goat anti-mouse IgG1-PE (1:40 dilution, Serotec) or goat anti-mouse IgG2a-FITC (1:10 dilution, AbD Serotec) or IgG3-FITC (1:10 dilution, AbD Serotec). Flow cytometry data acquisition and analyses were performed on a cytomics FC 500 flow cytometer® with Kaluza 2.1 software (Beckman Coulter). Data was obtained by gating a low side scatter, CD2 positive T cell population.

2.4.2.2. Intracellular interferon gamma (IFN-γ) staining

Intracellular IFN-γ staining was done using the BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences) with BD GolgiStop™ protein transport inhibitor containing monensin, according to the instructions of the manufacturer. Samples of the effector cells stimulated with individual peptides as described in section 2.4.1., were collected and stained. Briefly, 4 µl BD GolgiStop™ was added for every 6 ml of cell culture and incubated for 4 h prior to harvesting. Triplicate wells of PBMC were pooled before 2 x 10^5 cells/well were surface stained for either CD8 (cell line HT14A, IgG1, WSU) or CD4 (cell line HB61D, IgG1, WSU) in duplicate using a single-staining method followed by staining with the appropriate secondary antibody (goat anti-mouse IgG1-PE diluted 1:40, Serotec). Cells were resuspend in 100 µl Cytofix/Cytoperm™ solution for 20 min at 4°C. Cells were washed twice in 1X Perm/Wash™ solution, pelleted and the supernatant removed. Fixed/permeabilized cells were resuspend in 50 µl of Perm/Wash™ solution containing fluorochrome-conjugated anti-IFNγ/FITC (Alexa fluor®488, Serotec) and incubated at 4°C for 30 min in the dark. Cells were washed twice with 1X Perm/Wash™ solution and resuspended in staining buffer prior to flow cytometric analysis. Flow cytometry data acquisition and analyses were performed on a cytomics FC 500 flow cytometer® with Kaluza 2.1 software (Beckman Coulter). Data was obtained by gating a low side scatter, CD2 positive T cell population.

2.4.3. Lymphocyte proliferation assay (LPA)

Proliferation assays were carried out using PBMC stimulated with peptide pools in the first experiment and with individual peptides in the second, in triplicate wells of half-area flat bottom 96 well plates (Costar) at 37°C in a humidified atmosphere containing 5% CO_2 for six days as described by Van Kleef et al. (2000). Briefly, the plates were incubated for 6 days at 37°C in a humidified atmosphere containing 5% CO_2. The cells were labelled with 1 µCi [methyl-H3] Thymidine (Amersham) on day 5. The cells were harvested 18 h later using a Micromate™196 cell harvester (Packard) according to the instructions of the manufacturer. Ultima Gold™F (PerkinElmer) was added to the dry membranes and the cells were counted using a MicroBeta®TriLux (EG&G®WALLAC). The results were presented as a stimulation index (SI) ± standard deviation (SD), where SI is the mean counts per minute (cpm) of cells stimulated with antigens divided by cpm of unstimulated cells. The SI index of the antigen tested was compared to the SI of cells stimulated with an unrelated negative control. A SI ≥ 2 was considered to be an indication of antigen-specific proliferation.

2.4.4. IFN-γ ELISPOT assay

The ELISPOT assay was done using the Bovine/Ovine/Equine IFN-γ ELISPOT^{PLUS} kit (Mabtech) according to the instructions of the manufacturer. Briefly, ELISPOT plates were coated with 100 µl/well bIFNγ-I coating antibody (7.5 µg/ml) in sterile PBS (Sigma) and incubated overnight at 4°C. One hour prior to addition of PBMC and antigen, blocking medium RPMI-1640 with 10% horse serum (HS) was added to the plates and incubated at room temperature. Plates were seeded with $4x10^6$/ml PBMC, 50 µl cells per well. In the first experiment the peptide pools and in the second experiment the individual peptides were added to the PBMC in triplicate wells. The plates were incubated for 20 h at 37°C in a humidified atmosphere containing 5% CO_2. Plates were incubated at room temperature for 2 h with 100 µl/well, 0.25 µg/ml detection antibody (PAN-biotin) in PBS-0.5% HS and 1 h with 100 µl/well Streptavidin-ALP (1:1000) in PBS-0.5% HS. Substrate solution (BCIP-NBT-plus) was added to the plates (100 µl/well) and the colour development was stopped by washing the plates with tap water. The plates were left to dry at room temperature in darkness. The images from the developed ELISPOT plates were quantified using an automated ELISPOT reader (Zeiss KS ELISpot Compact 4.5). The value of

spots/well was calculated by multiplying the value of unstimulated PBMC in triplicate wells and the value of the peptide stimulated PBMC in triplicate wells. The results were positive if the spots/well values were greater than two times (> 2) the value of unstimulated PBMC in triplicate wells.

2.5. Bioinformatics analysis and identification of predicted epitopes and sequence homology of the selected AHSV4 peptides

2.5.1. T cell and Linear B cell epitope prediction tools

Immune Epitope Database (IEDB) (https://www.iedb.org/) was used to predict T cell and linear B cell epitopes. This was performed to investigate a possible correlation between experimental analysis of overlapping synthetic peptides spanning an entire protein with employing epitope prediction tools. Representative design of the overlapping synthetic peptides of NS3 and the data obtained from the epitope prediction tools for NS3 are shown in Supplementary B.

2.5.2. Amino acid sequence alignments

Individual alignments were generated using between 238 and 519 the amino acid sequences of AHSVs VP1, VP2, VP4, VP7 and NS3. These protein sequences represented all nine AHSV serotypes and were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). The sequences of the positively identified peptides were included in the alignments in order to determine the sequence conservation of each peptide. All alignments and sequence comparison analyses were performed using the CLC Genomics Workbench v9.0 (http://www.clcbio.com/products/clc-genomics-workbench/).

2.7. Statistical analysis

The significance of differences between immunological assay results was determined by means of the Student's *t*-test. *P*-values ≤ 0.05 were regarded as significant.

3. Results

During the first round of assays, 38 pools of peptides were evaluated based on phenotypic analyses, IFN-γ ELISPOT assay and the LPA assay. Peptide pools with results significant in four of the five horses, were selected and divided into CD8 pools (Supplementary Table S1), CD4 pools (Supplementary Table

S2) and B cell pools (Supplementary Table S3). Certain of the peptide pools (e.g. pool 8) overlapped between the divided pools (Supplementary Table S1, S2 and S3). The individual peptides from the CD8 pools, CD4 pools and B cell pools were respectively sorted into the CD8 group, CD4 group and B cell group (Supplementary Table S4) for the second experiment.

3.1. FATT-CTL assay

Antigen-specific CTL-induced cell lysis (apoptotic cells that progressed to a secondary necrotic state) was detected in PBMC. Significant cell lysis were induced in target cells by effector cells stimulated with recombinant AHSV4 proteins, VP1-1, VP2, VP4, VP7 and NS3 as well as the selected individual peptides from the CD8 group. The results indicated that several of the individual peptides [VP1-1 (n=7), VP2-1 (n=8), VP2-2 (n=9), VP4 (n=5), VP7 (n=3) and NS3 (n=5)] contain CD8+ T cell (CTL) epitopes (Table 1). Phenotypic analyses of the effector cells demonstrated that CD8+CD45RO+ T cells eliminated antigen presenting cells (APCs) via apoptosis upon antigen-specific recognition of peptide/MHC class I complexes, these individual peptides (n=22) are indicated in red in Table 1.

3.2. Phenotypic analyses of the effector cells using flow cytometry

3.2.1. Cell surface staining

Cell surface staining with CD8, CD4 or B cell surface markers of the samples of effector cells stimulated with the peptide pools showed a significant increase of CD8+ T cells (Supplementary Table S1), CD4+ T cells (Supplementary Table S2) and B cells (Supplementary Table S3). Similarly, the individual peptides from the CD8, CD4 and B cell groups showed an increase of CD8+CD45RO+ T cells (Fig. 1A), CD4+CD45RO+ T cells (Fig. 1B) and B cells (Fig. 1B). A significant increase of CD8+CD45RO+ T cells was detected following the stimulation with several individual peptides of VP1-1, VP2-1 and VP2-2, but only one or two individual peptides from VP4, VP7 and NS3 (Fig. 1A). An increase in the number of peptides representing NS3 and VP7 produced similar results for CD4+CD45RO+ T cells (Fig. 1B). The significant increase of B cells after stimulation with individual peptides of VP2-1 (n=1), VP2-2 (n=1) and NS3 (n=3) (Fig. 1B) was expected, since both VP2 (outer capsid protein of virus) and NS3 (later, extracellular domain during virus replication) are exposed to the extracellular environment.

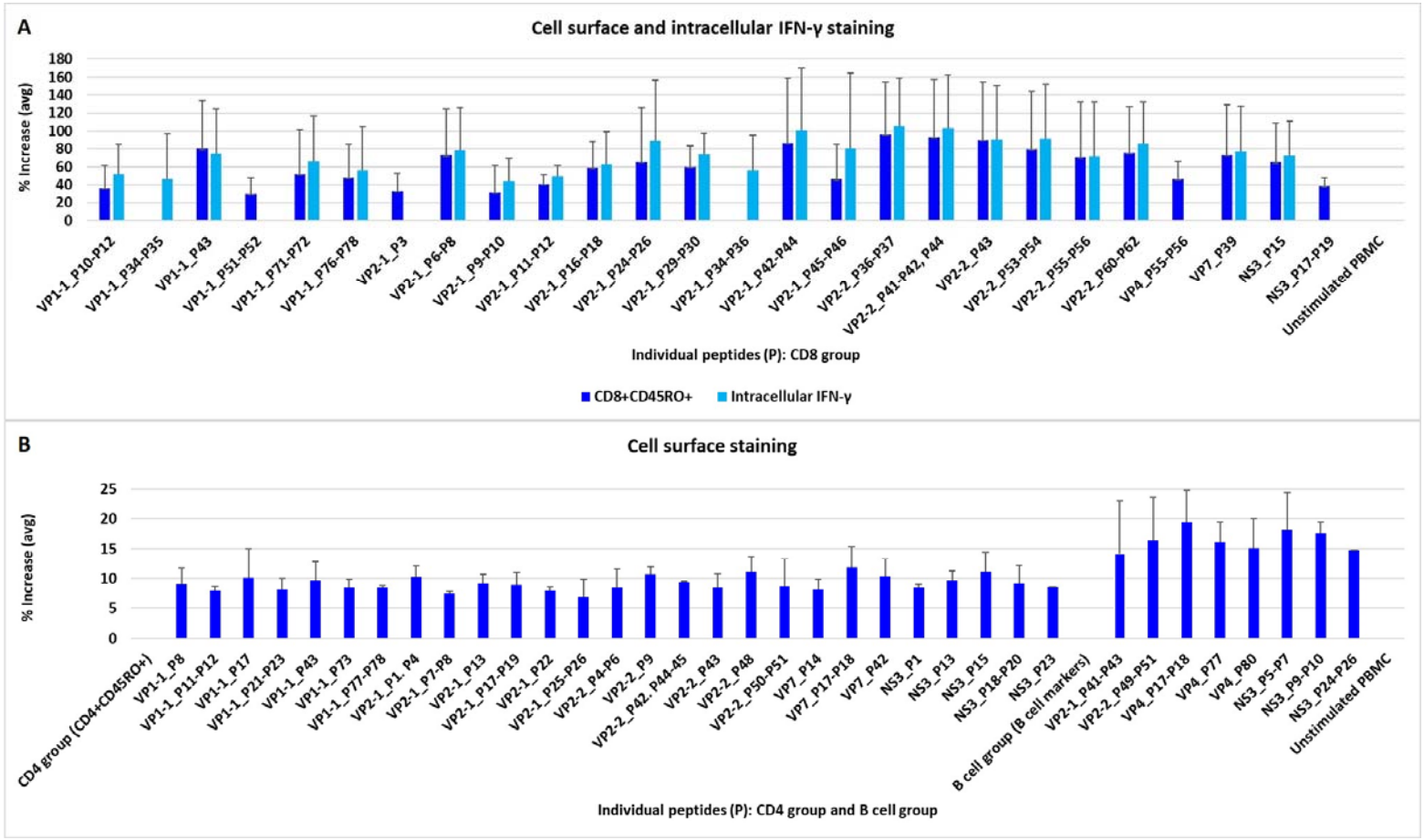

Fig. 1. (A) Cell surface and intracellular IFN-γ-staining: Significant (*P*-values ≤ 0.05) % increase for CD8+CD45RO+ T cells and % increase for intracellular IFN-γ producing CD8+CD45RO+ T cells stimulated with individual peptides from the CD8 group. **(B)** Cell surface staining: Significant (*P*-values ≤ 0.05) % increase for CD4+CD45RO+ T cells and B cells stimulated with individual peptides from the CD4 group and B cell group, respectively. The average (avg) results and standard deviation (SD), indicated with the error bars, of the individual peptides from the CD4, CD8 and B cell groups with significant results in PBMC from at least four out of the five horses compared to unstimulated PBMC are shown in the figure. Unstimulated PBMC was subtracted from the avg results.

Table 1. FATT-CTL assay. Significant (*P*-values ≤ 0.05) % lysis induced in target cells electroporated with pGFP_AHSV4gene (VP1, VP2, VP4, VP7 or NS3) by effector cells stimulated with recombinant AHSV4 proteins (VP1-1, VP2-1, VP2-2, VP4, VP7 or NS3) and the individual peptides from the CD8 group. The average (avg) results and standard deviation (SD) of the recombinant AHSV4 proteins and the individual peptides that induced significant cell lysis in PBMC from at least four out of the five horses compared to the controls are shown. The naïve PBMC (before vaccination) controls (light blue) and the immune PBMC (attenuated AHSV4 immunized horse PBMC used in this study) controls, which include the recombinant AHSV4 proteins (dark blue) are shown in the table.

FATT-CTL (CD8 group)					
Individual peptides (P)	**% Lysis (avg)**	**SD**	**Individual peptides (P)**	**% Lysis (avg)**	**SD**
VP1-1_P7	32.0	17.6	VP7_P40-P41	21.9	9.2
VP1-1_P10-P12	27.7	6.1	NS3_P11-P12	24.0	9.8
VP1-1_P13-P14	20.7	11.0	NS3_P15	39.1	18.1
VP1-1_P43	41.2	13.9	NS3_P17-P19	35.3	18.3
VP1-1_P48-P50	31.2	6.2	NS3_P22	39.3	7.6
VP1-1_P51-P52	29.1	7.5	NS3_P25-P27	33.9	15.8
VP1-1_P71-P72	22.2	2.0	**Negative control:**		
VP2-1_P3	31.1	18.5	Empty vector control	0.7	1.4
VP2-1_P6-P8	29.6	19.9	**Naïve PBMC:**		
VP2-1_P11-P12	32.1	10.0	VP1 Expression control	0.4	0.7
VP2-1_P16-P18	23.1	21.7	VP1 protein	0.0	0.0
VP2-1_P24-P26	38.9	10.5	VP2 Expression control	0.4	0.7
VP2-1_P29-P30	40.5	15.3	VP2 protein	0.0	0.0
VP2-1_P42-P44	40.5	21.1	VP4 Expression control	0.4	0.7
VP2-1_P45-P46	29.7	21.0	VP4 protein	0.0	0.0
VP2-2_P11-P12	36.2	18.9	VP7 Expression control	0.4	0.7
VP2-2_P36-P37	33.0	25.0	VP7 protein	0.0	0.0
VP2-2_P41-P42, P44	41.2	20.5	NS3 Expression control	0.5	1.1
VP2-2_P43	18.2	10.8	NS3 protein	0.0	0.0
VP2-2_P47	33.3	20.9	**Positive control:**		
VP2-2_P50-P52	26.7	32.1	**Immune PBMC:**		
VP2-2_P53-P54	31.5	15.7	VP1 Expression control	1.8	3.1
VP2-2_P55-P56	36.6	21.6	VP1 Protein	26.6	12.1
VP2-2_P60-P62	40.9	20.5	VP2 Expression control	2.9	5.8
VP4_P55-P56	14.9	11.1	VP2 Protein	37.0	17.4
VP4_P60-P62	15.6	9.1	VP4 Expression control	0.4	0.7
VP4_P65	18.9	15.7	VP4 Protein	29.4	5.4
VP4_P68-P70	23.0	13.5	VP7 Expression control	3.5	2.4
VP4_P71-72	24.3	9.3	VP7 Protein	34.0	1.3
VP7_P1-P2	29.2	8.3	NS3 Expression control	4.5	5.1
VP7_P39	34.0	11.5	NS3 Protein	35.9	16.7

In contrast, a significant increase of B cells after stimulation with individual peptides of VP4 (n=3) was surprising because VP4, a capping enzyme located within the viral core (Dennis et al., 2019), is not exposed to the extracellular environment.

3.2.2. Intracellular IFN-γ staining

Intracellular IFN-γ staining analyses revealed that samples of the effector cells stimulated with individual peptides from the CD8 group produced IFN-γ. Based on both intracellular IFN-γ and cell surface staining (CD8 and CD45RO), the results showed that CD8+CD45RO+ T cells produced IFN-γ after stimulation with individual peptides of VP1-1 (n=4), VP2-1 (n=8), VP2-2 (n=6), VP7 (n=1) and NS3 (n=1) (Fig. 1A)

3.3. LPA

Significant lymphocyte proliferation was detected in PBMC after stimulation with multiple peptide pools (Supplementary Table S1, S2 and S3) and several of the individual peptides from the CD8, CD4 and B cell groups (Table 2). The LPA assay cannot discriminate between the lymphocyte subsets. However, based on cell surface staining results (CD8, CD4, CD45RO or B cell surface markers) it is most likely that CD8+CD45RO+ T cells, CD4+CD45RO+ T cells and B cells are contributing to the observed proliferation after stimulation with the individual peptides from their respective groups and those positive for both assays are shown in red in Table 2.

3.4. IFN-γ ELISPOT assay

Significant secretion of IFN-γ from PBMC was observed following stimulation with numerous peptide pools from the CD8 and CD4 peptide pools (Supplementary Table S1 and S2), as well as several of the individual peptides from the CD4 group (Fig. 2). Based on cell surface staining (CD4 and CD45RO) and the IFN-γ ELISPOT assay, the results demonstrated that CD4+CD45RO+ T cells secreted IFN-γ after stimulation with individual peptides (dark green columns) from the CD4 group (Fig. 2).

3.5. Locations of CD8+ T cell, CD4+ T cell and linear B cell epitopes on AHSV4 proteins

The positions of the individual peptides that contain CD8+ T cell, CD4+ T cell and/or linear B cell epitopes are graphically presented on VP1-1, VP2, VP4, VP7 and NS3 of AHSV4 (Fig. 3).

Table 2. LPA assay. Significant (*P*-values ≤ 0.05) positive (SI values ≥ 2) results were considered to be an indication of peptide specific proliferation. The average (avg) results and standard deviation (SD) of the individual peptides from the CD4, CD8 and B cell groups that induced significant lymphocyte proliferation in PBMC from at least four out of the five horses compared to unstimulated PBMC are shown. Unstimulated PBMC was subtracted from the avg results.

LPA					
CD8 group			**CD4 group**		
Individual peptides (P)	**SI (avg)**	**SD**	**Individual peptides (P)**	**SI (avg)**	**SD**
VP1-1_P2-P4	3.4	1.2	VP1-1_P3	17.1	27.4
VP1-1_P10-P12	3.6	1.8	VP1-1_P11-P12	10.0	13.3
VP1-1_P45	5.2	4.1	VP1-1_P17	3.8	2.3
VP1-1_P48-P50	3.3	1.1	VP1-1_P21-P23	4.8	2.9
VP1-1_P71-P72	2.9	1.0	VP1-1_P26	16.9	23.7
VP1-1_P76-P78	4.4	1.7	VP1-1_P29-P30	13.4	12.6
VP2-1_P16-P18	2.8	0.6	VP1-1_P77-P78	8.9	12.4
VP2-1_P42-P44	6.6	3.4	VP2-1_P1, P4	5.6	4.9
VP2-2_P36-P37	10.8	4.8	VP2-1_P7-P8	12.6	17.0
VP2-2_P41-P42, P44	6.3	2.9	VP2-1_P13	8.5	7.1
VP2-2_P50-P52	4.0	1.6	VP2-1_P17-P19	16.3	17.0
VP2-2_P60-P62	7.6	5.3	VP2-1_P22	10.2	5.7
VP4_P55-P56	3.4	1.0	VP2-1_P25-P26	23.1	33.8
VP4_P60-P62	2.6	1.1	VP2-1_P67	9.0	7.1
VP4_P65	2.4	0.7	VP2-2_P4-P6	13.0	10.5
VP7_P1-P2	5.5	2.0	VP2-2_P9	7.2	5.5
VP7_P39	4.3	2.1	VP2-2_P11	3.2	1.8
VP7_P40-P41	3.2	0.6	VP2-2_P12	13.5	21.1
NS3_P25-P27	2.7	1.4	VP2-2_P14-P15	8.0	8.2
Unstimulated PBMC	0.0	0.0	VP2-2_P38	4.0	4.1
B cell group			VP2-2_P42. P44-45	11.7	12,5
Individual peptides (P)	**SI (avg)**	**SD**	VP2-2_P43	4.8	5.3
VP2-1_P36-P37	6.8	5.5	VP2-2_P48	10.4	10.0
VP2-2_P10, P13-P14	9.5	7.5	VP2-2_P50-P51	11.1	8.2
VP4_P5	8.8	11.1	VP2-2_P57	14.5	23.3
VP4_P13-P15	7.5	5.2	VP2-2_P61-P63	13.7	17.5
VP7_P1-P2	9.7	7.2	VP4_P75	9.5	9.6
VP7_P15-P17	9.2	7.8	VP4_P78-P80	17.6	20.7
VP7_P28-P29	8.2	10.5	VP7_P5	7.5	6.9
VP7_P39	9.9	5.9	VP7_P9-P11	12.9	13.2
NS3_P9-P10	6.5	4.2	VP7_P17-P18	10.8	4.0
NS3_P16	7.2	6.6	VP7_P42	10.7	9.8
NS3_P24-P26	9.8	10.5	NS3_P1	8.8	10.4

Unstimulated PBMC	0.0	0.0	NS3_P4	9.4	11.3
			NS3_P7-P8	7.8	8.8
			NS3_P26	9.1	8.2
			Unstimulated PBMC	0.0	0.0

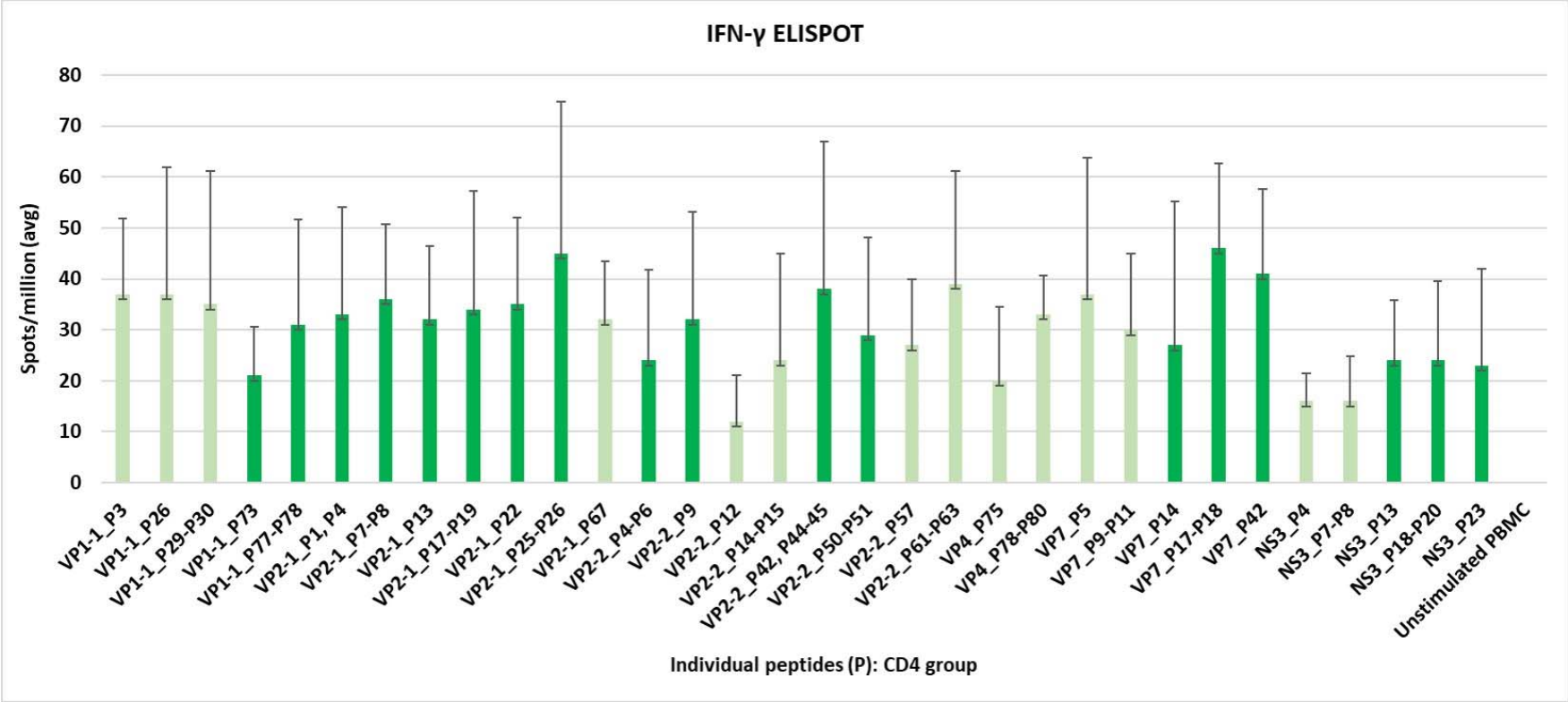

Fig. 2. IFN-γ ELISPOT assay. Significant (*P*-values ≤ 0.05) positive (> 2 × value of unstimulated PBMC) results were considered to be an indication of IFN-γ secretion from PBMC stimulated with individual peptides from the CD4 group (light green). IFN-γ secretion from CD4+CD45RO+ T cells stimulated with individual peptides from the CD4 group are shown in dark green. The average (avg) results and standard deviation (SD), indicated with the error bars, of the individual peptides from the CD4 group with significant results in PBMC from at least four out of the five horses compared to unstimulated PBMC are shown in the figure. Unstimulated PBMC was subtracted from the avg results.

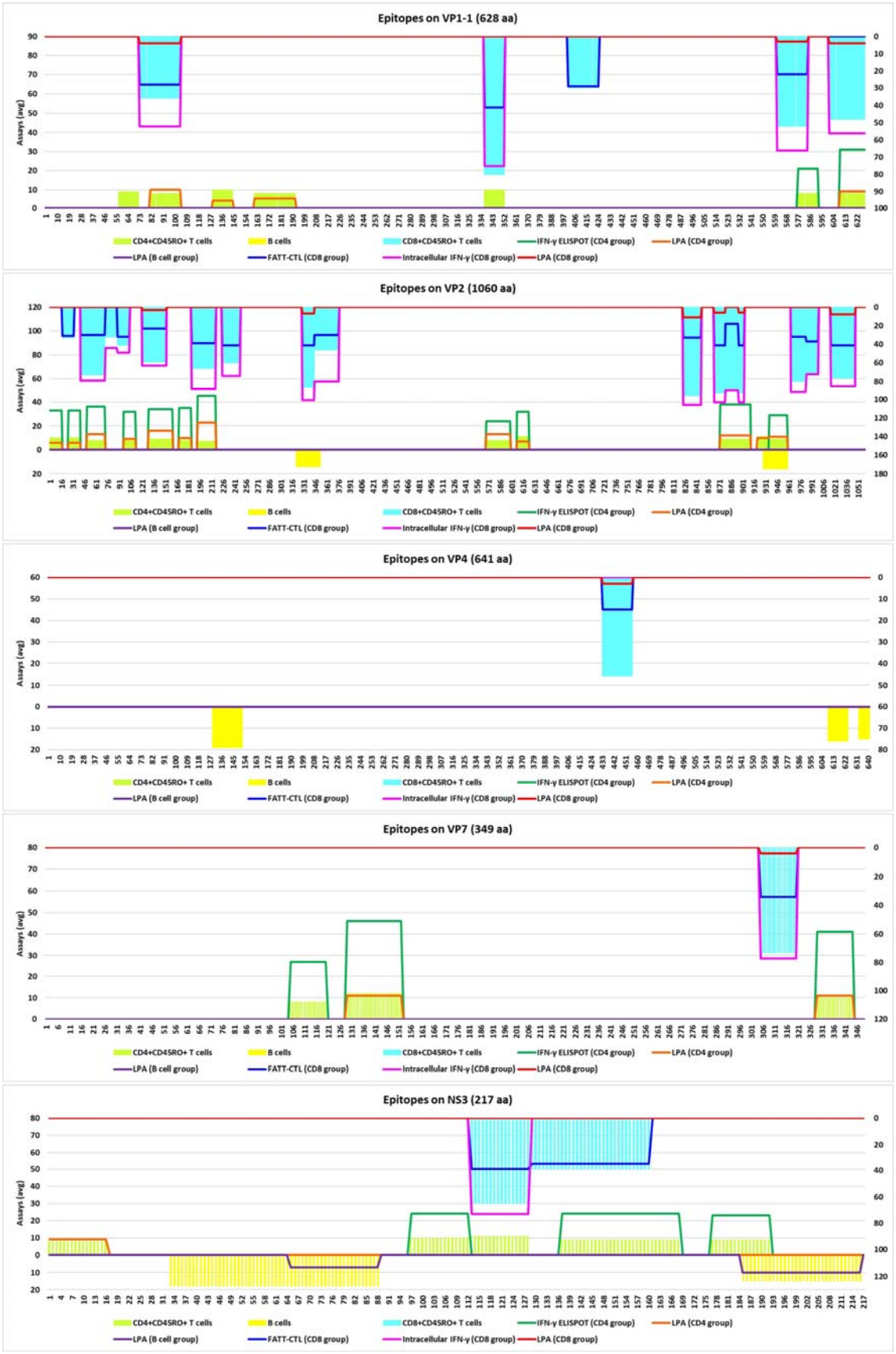
Epitopes on VP1-1 (628 aa)
Epitopes on VP2 (1060 aa)
Epitopes on VP4 (641 aa)
Epitopes on VP7 (349 aa)
Epitopes on NS3 (217 aa)
Assays (avg)
CD4+CD45RO+ T cells
B cells
CD8+CD45RO+ T cells
IFN-γ ELISPOT (CD4 group)
LPA (CD4 group)
LPA (B cell group)
FATT-CTL (CD8 group)
Intracellular IFN-γ (CD8 group)
LPA (CD8 group)

Fig. 3. The locations of the individual peptides that contain CD8+ T cell, CD4+ T cell and/or linear B cell epitopes on AHSV4 proteins, VP1-1, VP2, VP4, VP7 and NS3 sequences as determined by the significant cell surface staining results. The CD8+ T cell epitope locations are plotted on the secondary axis, CD4+ T cell epitopes on the primary axis (above 0) and the linear B cell epitopes on the primary axis (below 0). The FATT-CTL assay, intracellular IFN-γ staining, LPA and/or IFN-γ ELISPOT assay results of the individual peptides that were identified by cell surface staining to contain CD8+ T cell, CD4+ T cell and/or linear B cell epitopes are also shown in the figure.

3.5.1. T cell and Linear B cell epitope prediction tools

The significant horse [Eqca-16*00101 (ELA-A2) and Eqca-1*00101(ELA-A3)] and human HLA allele reference set (HLA-A* and HLA-B*) MHC class I binding predictions covered the complete sequence of the AHSV4 NS3 protein, for the most part (Fig. 4 and Supplementary B). Individual peptides NS3_P15 and NS3_P17-P19 were experimentally shown in this study to contain CD8+ T cell epitopes (Fig. 4). Of the top 10 best predicted epitopes, three overlapped with NS3_P17-P19 for both ELA-A2 and ELA-A3, whereas one prediction overlapped with NS3_P17-P19 for human HLA. None of the horse nor human top 10 best predicted epitopes overlapped with NS3_P15.

The human HLA reference set (HLA-DRB*, HLA-DQA*, HLA-DQB*, HLA-DPA* and HLA-DPB*) MHC class II binding predictions were predominantly in the 82-128 aa regions of the NS3 protein sequence (Supplementary Fig. S3 and Supplementary B). It was demonstrated in this study that individual peptides NS3_P1, NS3_P13, NS3_P15, NS3_P18-P20 and NS3_P23 (Supplementary Fig. S3) contain CD4+ T cell epitopes. Six (lengths: 11 to 13 aa) and ten (lengths: 14 to 16 aa) of the top 10 best predicted epitopes from the HLA reference set overlapped with NS3_P13. Several of the significant human HLA reference set predicted epitopes overlapped with NS3_P15 and a few with NS3_P18-P20. In contrast, none of the significant human HLA reference set predicted epitopes overlapped with NS3_P1 nor NS3_P23.

It was predicted that AHSV4 protein NS3 contains seven linear B cell epitopes (between 5 to 22 aa in length) (Supplementary Fig. S4 and Supplementary B). NS3_P5-P7, NS3_P9-P10 and NS3_P24-P26 were shown to contain linear B cell epitopes in this study (Supplementary Fig. S4). Four of the predicted linear B cell epitopes had some overlapping regions with NS3_P5-P7 and NS3_P24-P26.

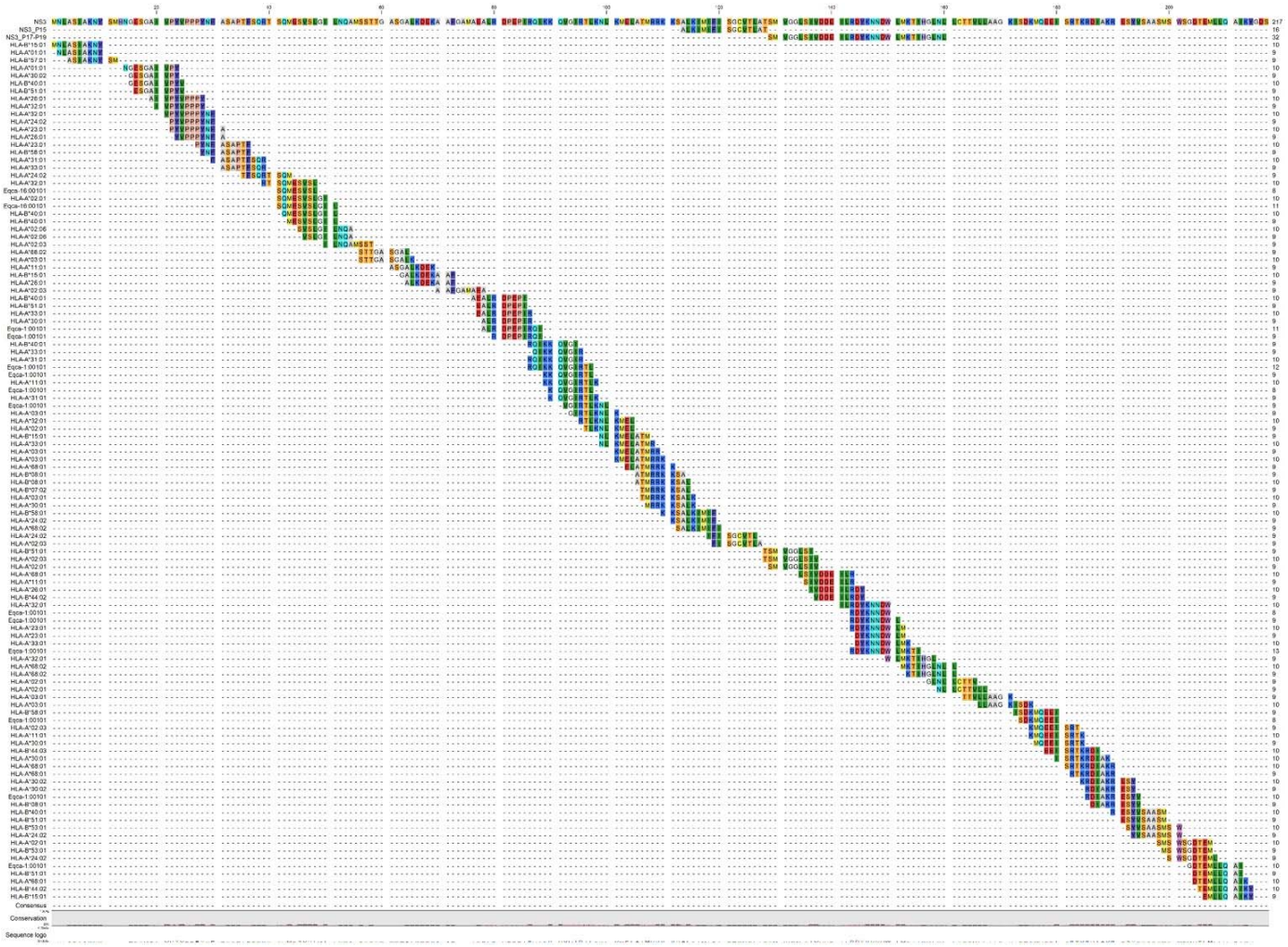

Fig. 4. CD8+ T cell epitopes. Individual peptides NS3_P15 and NS3_P17-P19 from this study and all the significant predicted horse [Eqca-16*00101 (ELA-A2) and Eqca-1*00101(ELA-A3)] and human (HLA-A* and HLA-B*) epitopes are mapped to the AHSV4 protein NS3 sequence.

3.6. Amino acid sequence alignments

Based on the aforementioned data, 62 peptides were identified as potential epitopes for future diagnostics, vaccine or therapeutic targets. In order to evaluate the level of sequence conservation, the amino acid sequence of each peptide was compared to all the available sequences for a specific viral protein. The total number of available sequences differ between 238 and 519 (Supplementary Table S5). The percentage sequence identity of each amino acid was determined by using protein specific alignments and this was used to calculate the average percentage sequence identity of each peptide (Supplementary Table S5). The enzyme proteins VP1 and VP4 as well as the core protein VP7 had the highest percentage sequence identity ranging between 92.7 and 99%. In contrast, the variable proteins VP2 and NS3 had sequence conservation between 45.2 and 96.7% (Supplementary Table S5).

4. Discussion

In this study, recall immune assays were used to identify individual AHSV4 peptides that contain T cell or linear B cell epitopes. PBMC isolated from five horses immunized with attenuated AHSV4 (Faber et al., 2016) were stimulated *in vitro* with peptide pools in the first round of experiments and with the individual peptides in the second. Peptides 16-mer in length that overlapped by 8-mers, were synthesized in order to identify the locations of T and B cell epitopes on AHSV4 proteins VP1-1, VP2, VP4, VP7 and NS3. MHC class I molecules bind short peptide fragments (8-10 aa) and antigen-specific T cell receptors (TCRs) of CD8+ T cells recognize these peptide/MHC class I complexes displayed on the cell surfaces of APCs or all nucleated cells (Blum et al., 2013; Rock et al., 2016). However, MHC class II molecules bind longer peptide fragments (13-25 aa) for CD4+ T cell presentation (Sanchez-Trincado et al., 2017) and although some might be longer, the majority of linear B cell epitopes are typically in the range of 5-22 aa (Singh et al., 2013). Thus, the 16-mer peptides used in this study were sufficient in length for the identification of the locations of most CD8+ T cell epitopes, but only the adjacent CD4+ T cell and linear B cell epitopes ≤ 16 aa in length on these AHSV4 proteins.

Immune assays employed in this study as well as the expression of CD45RO, which is a marker of memory T cells (Courville and Lawrence, 2021), showed that predominantly memory T cells were

responsible for the observed effector responses. Specifically, CD8+CD45RO+ T cells eliminated APCs, and both CD8+CD45RO+ and CD4+CD45RO+ T cells secreted IFN-γ and proliferated after stimulation with specific individual peptides of VP1-1, VP2, VP4, VP7 and NS3. Both memory CD8+ T cells (CTLs) and CD4+ T cells are heterogeneous and consist of multiple subsets that vary in their localizations, homing characteristics, expression of surface markers and functions (Pennock et al., 2013). The major memory T cell subsets include effector memory T cells (TEMs), central memory T cells (TCMs), tissue-resident memory T cells (TRMs) and the recently described stem cell memory T cells (TSCMs) (Martin and Badovinac, 2018; Eagar and Miller, 2019). Since TEMs recirculate between blood and peripheral tissues (Pennock et al., 2013; Eagar and Miller, 2019) it is more likely that the majority of CD8+CD45RO+ T cells and CD4+CD45RO+ T cells were TEMs. TEMs have lower proliferative potential but display rapid effector functions. After antigen-specific re-exposure, effector memory CD8+ T cells and effector memory CD4+ T cells quickly produce effector cytokines (e.g. IFN-γ) (Pennock et al., 2013; Martin and Badovinac, 2018; Eagar and Miller, 2019). The main function of effector memory CD8+ T cells is to eliminate target cells (e.g. virus-infected cells) via their cytotoxic activities (Martin and Badovinac, 2018).

This study mainly focussed on effector memory CD4+ T cells that secreted the T helper type 1 (Th1) characterizing cytokine IFN-γ. However, the lack of IFN-γ secretion after stimulation with some of the individual peptides of VP1-1, VP2 and NS3 indicated that different memory Th cell subsets were also present. Future experiments are required to identify these Th phenotypes (e.g. Th2, Th17, Tregs, and Tfh cells) and additional epitopes that are recognized by them.

Comparative experiments with mouse models have demonstrated a correlation between the synthesized overlapping peptide and predicted epitope methods, despite the fact that the sets of epitopes identified by the two approaches were not entirely overlapping and certain peptides were only identified by a single method (Sidney et al., 2020). In this study, there was some similarities between the experimentally verified individual synthesized overlapping peptides of AHSV4 protein NS3 and the significant predicted T cell epitopes, while others were not predicted at all (e.g. CD4+ T cell epitopes on NS3_P1 and NS3_P23). However, it should be taken into consideration that CD4+ T cells and CD8+ T cells are

MHC restricted, where every TCR is specific for both a distinct peptide antigen as well as a particular allelic form of a MHC molecule. A TCR only recognizes and binds to a specific peptide/MHC combination. Furthermore, MHC class I and MHC class II molecules are highly polymorphic. There are hundreds of different alleles of the MHC genes due to MHC polymorphism in all large outbred populations, as a whole, but the individuals in a population will express different combinations of MHC molecules (Blum et al., 2013; Rock et al., 2016; Sidney et al., 2020). Likewise, ELA-DRB exon 2 typing demonstrated polymorphism between the five horses, where ELA-DRB of two horses varied from the other three (Faber et al., 2016).

Therefore, the specific immunodominant epitopes and/or subdominant epitopes selected for presentation from the various AHSV proteins will be dependent on the collection of MHC molecules expressed by individual horses. For the development of new generation AHS peptide-based vaccines, it remains to be investigated if this collection of MHC molecules expressed by these five horses (Faber et al., 2016) are part of the collections of larger horse populations. Additionally, it needs to be determined if the individual peptides that contain CD8+ T cell and CD4+ T cell epitopes identified in this study will also be selected as immunodominant or subdominant epitopes for presentation by different collections of MHC molecules and recognized by TCRs. T cell epitope prediction tools may assist with the identification and selection CD8+ T cell and CD4+ T cell epitopes. However, similarly to synthesized overlapping peptides, these predicted T cell epitopes must also be evaluated experimentally, in smaller and in much larger outbred populations.

During AHSV infections, antigen-specific neutralizing antibodies are predominantly detected against VP2 (Dennis et al., 2019), and to a lesser degree against VP5 and VP7 (Martínez-Torrecuadrada et al., 1996). As such, VP2 is the major vaccine candidate in most AHS studies, which has proven to be problematic because VP2 is the most variable protein between the different AHSV serotypes (Van Niekerk et al., 2001; Dennis et al., 2019). Similarly, in this study, the variability of VP2 was observed with all of the individual peptides that contain linear B cell epitopes. Additionally, this study showed that AHSV4 protein VP2 contains numerous CD8+ T cell and CD4+ T cell epitopes. Sequence diversity

was observed in VP2 for all of the individual peptides that contain CD8+ T cell and CD4+ T cell epitopes. The peptides with a high percentage sequence identity could be considered, but peptides of VP2 would probably be excluded from new generation AHS peptide-based vaccine formulations based on this criteria. NS3 is the second most variable protein after VP2 (Van Niekerk et al., 2001; Dennis et al., 2019). However, NS3 contains a number of highly conserved structural features (Van Niekerk et al., 2001). The individual peptides that contain CD8+ T cell and CD4+ T cell epitopes of AHSV4 protein NS3 share 69% and 96% sequence identity respectively between the different AHSV serotypes. This indicated that the peptides are located on the conserved regions and should be evaluated as potential new generation AHS peptide-based vaccine candidates. The individual peptides that contain CD8+ T cell and CD4+ T cell epitopes of AHSV4 proteins VP1-1, VP4 (CD8 only) and VP7 are conserved between the different AHSV serotypes. Depending on their selection by the wider varieties of collections of MHC molecules from multiple horses, the individual peptides of VP1-1, VP4 (CD8 only) and VP7 could possibly be effective new generation AHS peptide-based vaccine candidates to induce CD4+ T cell and CD8+ T cell responses.

Cell surface staining with B cell surface markers of the samples of effector cells showed a significant increase for B cells after stimulation with individual peptides of AHSV4 proteins VP2, VP4 and NS3, and proliferation after stimulation with individual peptides of NS3. The results indicated that the individual peptides of AHSV4 proteins VP2, VP4 and NS3 contain linear B cell epitopes. VP5 was not included in this experiment; a future study is required to determine if this protein contains linear B cell epitopes. No linear B cell epitopes ≤ 16 aa in length was detected on the individual peptides of VP7, indicating that the linear epitopes are likely longer or that only conformational epitopes on this protein are recognized. Nonetheless, it is advisable to do an in-depth investigation with VP7 that includes synthesizing longer overlapping peptides and using linear B cell epitope prediction tools because VP7 has surface exposure and is highly conserved within the different AHSV serotypes (Dennis et al., 2019). Neutralizing antibodies bind conformational epitopes on VP2 (Martínez-Torrecuadrada et al., 1996; Calvo-Pinilla et al., 2018), whereas non-neutralizing antibodies bind linear B cell epitopes on VP2 (Martínez-Torrecuadrada et al., 1996). It has been suggested that non-neutralizing antibodies are

induced against NS3 (Mathebula et al., 2017). Future studies should investigate if it is possible that non-neutralizing antibodies bind linear B cell epitopes on the individual peptides of VP2 and NS3 identified in this study. The non-neutralizing antibodies have a wide range of important effector functions (Hoffman et al., 2016; Lu et al., 2018), and it is conceivable that both neutralizing antibodies and non-neutralizing antibodies contribute to protective immunity against AHSV. Thus, it is advisable that linear B cell epitopes that induce the production of neutralizing antibodies and non-neutralizing antibodies should be included in new generation AHS vaccine formulations.

Memory B cells responding to the individual peptides that contain linear B cell epitopes of VP4 was unexpected since this protein is not exposed to the extracellular environment. Basic Local Alignment Search Tool (BLAST) analyses revealed protein sequence similarities to AHSV protein VP4 only, indicating that VP4-specific B cell responses occurred. It has been speculated that depending on various factors, any of the AHSV proteins might be exposed to the extracellular environment during the loss of plasma membrane integrity of AHSV-infected cells due to an inflammatory cell death, secondary necrosis or cell damage. In turn, recall immunity will be observed if memory was generated against those particular AHSV proteins in the primary immune response (Faber et al., 2022). The significant memory B cell increase after stimulation with individual peptides of VP4 in this study showed that the loss of plasma membrane integrity hypothesis warrants investigation in the future. In turn, additional linear B cell epitopes on VP4 as well as the other AHSV proteins that are not extracellularly exposed should be identified and their potential as vaccine candidates must be evaluated.

This study showed that AHSV4 proteins VP2, VP4 and NS3 contain linear B cell epitopes. The variability of VP2 (Van Niekerk et al., 2001; Dennis et al., 2019) was observed with all of the individual peptides, which should be excluded from additional testing. In contrast, the individual peptides that contain linear B cell epitopes of VP4 are conserved between the different AHSV serotypes. However, in-depth investigations are required to determine if VP4 is a viable vaccine candidate. Likewise, the individual peptides that contain linear B cell epitopes of NS3 are conserved between the different AHSV serotypes and may be evaluated as potential new generation AHS peptide-based vaccine candidates,

which include uncovering if neutralizing and/or non-neutralizing antibodies are produced. In contrast to the predicted T cell epitopes, no predicted linear B cell epitopes completely overlapped with any of the experimentally verified individual peptides of AHSV4 protein NS3. This possibly could be due to the much wider range in different lengths of B cell epitopes. Emphasizing the fact that predicted linear B cell epitopes must be synthesized and tested to investigate if they are recognized by B cells.

It should be taken into consideration that fractions of the germinal centre (GC)-memory B cells, mostly IgG memory B cells, directly differentiate into plasmablasts and rapidly secrete high-affinity antibodies upon antigen re-encounter (Seifert et al., 2015; Hoffman et al., 2016; Cyster and Allen, 2019). This occurs independent of Th cell help (Seifert et al., 2015). Since the memory immune response was investigated in this study, it appeared that individual peptides NS3_P5-P7 and NS3_P9-P10 activated memory B cells without Th cell help. In contrast, overlapping aa sequences on NS3_P23 (CD4+ T cell epitopes) and NS3_P24-P26 (linear B cell epitopes) showed that these memory B cells likely presented peptide/MHC class II complexes to CD4+ T cells and were thus activated with Th cell help. Crucially, the GC response is dependent on Th cell help during both primary and memory immune responses. The extrafollicular memory B cells enter GCs upon antigen re-exposure (Hoffman et al., 2016), and the GC-memory B cells re-enter the GC to produce further diversified secondary memory B cells and long-lived plasma cells (Hoffman et al., 2016; Cyster and Allen, 2019). It is therefore essential that linear B cell epitopes must also contain CD4+ T cell epitopes. Unfortunately, in many instances, different individual peptides of NS3 from the CD4 and B cell groups were used in this study. A future experiment is required for a more comprehensive characterization of linear B cell/CD4+ T cell epitopes.

In conclusion, this study identified several conserved individual peptides of AHSV4 proteins VP1-1, VP4, VP7 and/or NS3 that contain CD8+ T cell, CD4+ T cell or linear B cell epitopes, which may contribute to the design and development of new generation AHS peptide-based vaccines. Future experiments will include the use PBMC isolated from a larger horse population with a broader MHC diversity, determined by comprehensive MHC typing, to further evaluate these individual peptides. As well as the identification of the specific peptide/MHC combinations and additional linear B cell epitopes that

preferably also contain CD4+ T cell epitopes.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Appendix B. Supplementary data

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