Supplementary figures and tables:

Vertical pools

Horizontal pools	vp1	vp2	vp3	vp4	vp5	vp6	vp7	vp8	vp9	vp10	vp11	vp12	vp13	vp14	vp15	vp16	vp17	vp18
hp19	1	2	3	4	5	**6**	7	8	9	10	11	12	13	14	15	16	17	18
hp20	19	20	21	22	23	**24**	25	26	27	28	29	30	31	32	33	34	35	36
hp21	37	38	39	40	41	**42**	43	44	45	46	47	48	49	50	51	52	53	54
hp22	55	56	57	58	59	**60**	61	62	63	64	65	66	67	68	69	70	71	72
hp23	73	74	75	76	77	**78**	79	80	81	82	83	84	85	86	87	88	89	90
hp24	91	92	93	94	95	**96**	97	98	99	100	101	102	103	104	105	106	107	108
hp25	109	110	111	112	113	**114**	115	116	117	118	119	120	121	122	123	124	125	126
hp26	**127**	**128**	**129**	**130**	**131**	**132**	**133**	**134**	**135**	**136**	**137**	**138**	**139**	**140**	**141**	**142**	**143**	**144**
hp27	145	146	147	148	149	**150**	151	152	153	154	155	156	157	158	159	160	161	162
hp28	163	164	165	166	167	**168**	169	170	171	172	173	174	175	176	177	178	179	180
hp29	181	182	183	184	185	**186**	187	188	189	190	191	192	193	194	195	196	197	198
hp30	199	200	201	202	203	**204**	205	206	207	208	209	210	211	212	213	214	215	216
hp31	217	218	219	220	221	**222**	223	224	225	226	227	228	229	230	231	232	233	234
hp32	235	236	237	238	239	**240**	241	242	243	244	245	246	247	248	249	250	251	252
hp33	253	254	255	256	257	**258**	259	260	261	262	263	264	265	266	267	268	269	270
hp34	271	272	273	274	275	**276**	277	278	279	280	281	282	283	284	285	286	287	288
hp35	289	290	291	292	293	**294**	295	296	297	298	299	300	301	302	303	304	305	306
hp36	307	308	309	310	311	**312**	313	314	315	316	317	318	319	320	321	322	323	324
hp37	325	326	327	328	329	**330**	331	332	333	334	335	336	337	338	339	340	341	342
hp38	343	344	345	346	347	**348**	349	350	351	352	353	354	355	356	357	358	359	

Fig. S1. The panel of 359 individual peptides were arranged in a matrix of 18 vertical pools (vp1-18) and 20 horizontal pools (hp19-38). Each peptide occurs once in the vertical pool and once in the horizontal pool. Individual positive peptides are identified at the vertical/horizontal intersection. As an example, if pools vp6 and hp26 (green highlighted rows and columns) are positive, then the positive individual peptide 132 (red) would be at the vertical/horizontal intersection.

Vertical pools

Horizontal pools	vp1	vp2	vp3	vp4	vp5	vp6	vp7	vp8	vp9	vp10
hp19	1	2	3	4	5	6	7	8	9	10
hp20	19	**20**	**21**	**22**	**23**	**24**	**25**	26	27	28
hp21	37	**38**	**39**	**40**	**41**	**42**	**43**	44	45	46
hp22	55	**56**	**57**	**58**	**59**	**60**	**61**	62	63	64
hp23	73	**74**	**75**	**76**	**77**	**78**	**79**	80	81	82
hp24	91	**92**	**93**	**94**	**95**	**96**	**97**	98	99	100
hp25	109	**110**	**111**	**112**	**113**	**114**	**115**	116	117	118
hp26	127	128	129	130	131	132	133	134	135	136
hp27	145	146	147	148	149	150	151	152	153	154
hp28	163	164	165	166	167	168	169	170	171	172

Fig. S2. A hypothetical example of positive results obtained by pools vp2-7 and hp20-25 (light green highlighted rows and columns). It could either be that all the individual peptides in the vertical/horizontal intersections (dark green highlighted square) are positive, but it would give the same results if only peptides 20, 39, 58, 77, 96 and 115 (red) are positive. This ambiguity demonstrates the necessity to repeat the assays again with the identified individual peptides to confirm the results.

Table S1. The peptide pools with significant (*P*-values ≤ 0.05) results for phenotypic analyses (CD8, % increase in CD8+ T cells), IFN-γ ELISPOT assay (> 2 × value of unstimulated PBMC) and the LPA assay (SI values ≥ 2) in PBMC from at least four out of the five horses compared to unstimulated PBMC were selected and divided into CD8 pools. The average (avg) positive results and standard deviation (SD) that were significant in 4/5 horses are shown. Unstimulated PBMC was subtracted from the avg results.

CD8 Pools[a]	Phenotype: CD8+ T cells		IFN-γ ELISPOT		LPA	
	% Increase (avg)	SD	Spots/million (avg)	SD	SI (avg)	SD
Pool 1	18.0	13.6	43.0	66.9	3.7	1.9
Pool 2	23.2	12.5	58.0	93.6	4.7	4.1
Pool 6	14.6	7.2	32.0	57.4	4.0	3.9
Pool 7	18.6	12.0	47.0	76.8	6.2	4.1
Pool 8	25.5	24.4	62.0	103.8	5.7	2.8
Pool 11	11.8	9.7	23.0	59.4	7.8	5.2
Pool 14	16.1	13.3	20.0	64.1	2.2	1.2
Pool 15	17.0	11.1	44.0	86.6	3.4	1.3
Pool 16	13.5	9.5	20.0	64.4	5.0	3.0
Pool 17	20.7	14.5	19.0	57.4	3.6	1.5
Pool 18	16.8	11.2	24.0	64.9	3.1	1.6
Pool 22	17.2	9.9	28.0	57.9	4.1	3.5
Pool 24	16.9	8.0	42.0	64.7	4.8	2.7
Pool 27	10.6	7.1	75.0	101.6	5.9	3.1
Pool 29	17.2	6.0	48.0	112.5	3.0	1.8
Pool 31	17.9	15.3	31.0	73.4	3.6	2.9
Pool 32	20.5	11.8	56.0	91.7	5.1	3.4
Pool 33	21.8	14.5	36.0	83.1	4.9	0.9
Pool 38	29.4	36.1	51.0	78.0	5.0	3.2
Unstimulated PBMC	0.0	0.0	0.0	0.0	0.0	0.0
AHSV4	5.1	8.9	0.0	0.0	2.1	0.2
ConA	36.0	51.2	>1000		157.1	73.6
VP1-1	22.8	11.8	34.0	61.3	4.5	3.6
VP2-1	22.3	10.8	98.0	101.5	9.6	7.6
VP2-2	19.5	2.9	90.0	99.2	7.5	6.6
VP4	21.6	10.3	88.0	99.4	4.3	2.3
VP7	22.0	7.3	102.0	96.8	6.7	5.4
NS3	16.6	6.7	83.0	99.8	5.1	5.7

[a]Unstimulated PBMC was used as a negative control and concanavalin A (ConA), which is a T cell mitogen, was used as a positive control. AHSV4 and recombinant AHSV4 proteins VP1-1, VP2-1, VP2-2, VP4, VP7 and NS3 were also included as additional positive controls for antigen-specific memory T cell responses.

Table S2. The peptide pools with significant (*P*-values ≤ 0.05) results for phenotypic analyses (CD4, % increase in CD4+ T cells), IFN-γ ELISPOT assay (> 2 × value of unstimulated PBMC) and the LPA assay (SI values ≥ 2) in PBMC from at least four out of the five horses compared to unstimulated PBMC were selected and divided into CD4 pools. The average (avg) positive results and standard deviation (SD) that were significant in 4/5 horses are shown. Unstimulated PBMC was subtracted from the avg results.

CD4 Pools[a]	Phenotype: CD4+ T cells		IFN-γ ELISPOT		LPA	
	% Increase (avg)	SD	Spots/million (avg)	SD	SI (avg)	SD
Pool 3	5.0	2.0	45.0	73.4	4.9	1.9
Pool 7	8.7	5.3	47.0	76.8	6.2	4.1
Pool 8	5.7	2.1	62.0	103.8	5.7	2.8
Pool 9	4.2	1.4	25.0	57.3	5.5	1.8
Pool 12	7.8	4.2	30.0	65.8	5.1	2.5
Pool 15	10.1	1.4	44.0	86.6	3.4	1.3
Pool 16	5.5	5.1	20.0	64.4	5.0	3.0
Pool 23	6.8	4.6	31.0	65.2	3.4	1.5
Pool 24	4.1	4.1	42.0	64.7	4.8	2.7
Pool 25	5.2	2.6	34.0	65.2	4.3	1.3
Pool 27	5.5	3.3	75.0	101.6	5.9	3.1
Pool 28	5.2	2.3	41.0	75.2	4.4	2.2
Pool 31	6.0	4.7	31.0	73.4	3.6	2.9
Pool 32	5.2	4.5	56.0	91.7	5.1	3.4
Pool 35	5.1	3.8	25.0	55.2	7.9	4.8
Pool 37	3.9	2.5	33.0	65.0	4.5	3.0
Pool 38	5.9	4.7	51.0	78.0	5.0	3.2
Unstimulated PBMC	0.0	0.0	0.0	0.0	0.0	0.0
AHSV4	7.6	3.5	0.0	0.0	2.1	0.2
ConA	3.3	5.1	>1000		157.1	73.6
VP1-1	8.8	2.9	34.0	61.3	4.5	3.6
VP2-1	4.0	4.2	98.0	101.5	9.6	7.6
VP2-2	6.0	3.2	90.0	99.2	7.5	6.6
VP4	3.8	2.4	88.0	99.4	4.3	2.3
VP7	4.3	2.1	102.0	96.8	6.7	5.4
NS3	4.3	3.6	83.0	99.8	5.1	5.7

[a]Unstimulated PBMC was used as a negative control and concanavalin A (ConA), which is a T cell mitogen, was used as a positive control. AHSV4 and recombinant AHSV4 proteins VP1-1, VP2-1, VP2-2, VP4, VP7 and NS3 were also included as additional positive controls for antigen-specific memory T cell responses.

Table S3. The peptide pools with significant (*P*-values ≤ 0.05) results for phenotypic analyses (B cell markers, % increase in B cells) and the LPA assay (SI values ≥ 2) in PBMC from at least four out of the five horses compared to unstimulated PBMC were selected and divided into B cell pools. The average (avg) positive results and standard deviation (SD) that were significant in 4/5 horses are shown. Unstimulated PBMC was subtracted from the avg results.

B cell Pools[a]	Phenotype: B cells		LPA	
	% Increase (avg)	SD	SI (avg)	SD
Pool 5	15.2	17.9	4.3	1.3
Pool 8	9.7	3.9	5.7	2.8
Pool 9	11.6	7.2	5.5	1.8
Pool 13	9.1	7.2	2.3	0.4
Pool 14	4.7	4.9	2.2	1.2
Pool 15	4.3	5.2	3.4	1.3
Pool 17	8.2	9.0	3.6	1.5
Pool 18	17.3	6.9	3.1	1.6
Pool 19	7.8	8.9	4.2	3.0
Pool 23	7.0	2.8	3.4	1.5
Pool 24	10.0	10.2	4.8	2.7
Pool 25	11.4	10.6	4.3	1.3
Pool 26	10.2	7.3	6.6	3.3
Pool 33	12.8	9.9	4.9	0.9
Pool 34	14.1	12.6	5.5	1.9
Pool 35	12.4	9.3	7.9	4.8
Pool 36	17.4	11.5	4.6	2.7
Pool 37	14.6	9.7	4.5	3.0
Unstimulated PBMC	0.0	0.0	0.0	0.0
AHSV4	13.6	10.6	2.1	0.2
ConA	15.2	9.4	157.1	73.6
VP1-1	3.7	5.1	4.5	3.6
VP2-1	17.2	18.8	9.6	7.6
VP2-2	8.4	4.7	7.5	6.6
VP4	12.7	10.4	4.3	2.3
VP7	8.3	8.2	6.7	5.4
NS3	11.7	12.2	5.1	5.7

[a]Unstimulated PBMC was used as a negative control and concanavalin A (ConA), which is a T cell mitogen, was used as a positive control. AHSV4 and recombinant AHSV4 proteins VP1-1, VP2-1, VP2-2, VP4, VP7 and NS3 were also included as additional positive controls. However, our team recently demonstrated that the *Escherichia coli* (*E. coli*) contamination products present in the recombinant AHSV4 proteins have a considerable effect on the humoral immune response. Specifically, lipopolysaccharide (LPS) that is a B cell mitogen activated potent innate-like B cell and antibody-mediated responses. Based on their structures and/or extracellular exposure, it is conceivable that only soluble recombinant AHSV4 proteins NS3 and potentially VP2-2 could have been recognized by memory follicular (FO) B cells (Faber et al., 2022). Therefore, in addition to AHSV4, it is likely that only NS3 and possibly VP2-2 functioned as positive controls for antigen-specific memory B cell responses.

Reference:

Faber E, Tshilwane SI, Van Kleef M, Pretorius A. The impact of Escherichia coli contamination products present in recombinant African horse sickness virus serotype 4 proteins on the innate and humoral immune responses. Mol Immunol. 2022;152:1-13. doi: 10.1016/j.molimm.2022.09.013.

Table S4. The individual peptides from the CD8 pools were sorted into the CD8 group, individual peptides from the CD4 pools were sorted into the CD4 group and individual peptides from the B cell pools were sorted into the B cell group.

Individual peptides (P)		
CD8 group[a]	**CD4 group**	**B cell group**
VP1-1_P2-P4	VP1-1_P3	VP1-1_P43
VP1-1_P7	VP1-1_P8	VP2-1_P33
VP1-1_P10-P12	VP1-1_P11-P12	VP2-1_P36-P37
VP1-1_P13-P14	VP1-1_P17	VP2-1_P41-P43
VP1-1_P34-P35	VP1-1_P21-P23	VP2-1_P45-P46
VP1-1_P39-P41	VP1-1_P26	VP2-1_P51
VP1-1_P43	VP1-1_P29-P30	VP2-1_P54-P55
VP1-1_P45	VP1-1_P43	VP2-1_P59-P61
VP1-1_P48-P50	VP1-1_P73	VP2-1_P63-P64
VP1-1_P51-P52	VP1-1_P77-P78	VP2-2_P2
VP1-1_P71-P72	VP2-1_P1, P4	VP2-2_P5-P6
VP1-1_P76-P78	VP2-1_P7-P8	VP2-2_P11
VP2-1_P3	VP2-1_P13	VP2-2_P12
VP2-1_P6-P8	VP2-1_P17-P19	VP2-2_P10, P13-P14
VP2-1_P9-P10	VP2-1_P22	VP2-2_P16-P17
VP2-1_P11-P12	VP2-1_P25-P26	VP2-2_P22
VP2-1_P16-P18	VP2-1_P67	VP2-2_P25-P26
VP2-1_P21	VP2-2_P4-P6	VP2-2_P30-P32
VP2-1_P24-P26	VP2-2_P9	VP2-2_P34-P35
VP2-1_P27-P28	VP2-2_P11	VP2-2_P40
VP2-1_P29-P30	VP2-2_P12	VP2-2_P43
VP2-1_P34-P36	VP2-2_P14-P15	VP2-2_P44-P45
VP2-1_P39	VP2-2_P38	VP2-2_P49-P51
VP2-1_P42-P44	VP2-2_P42, P44-45	VP2-2_P53-P54
VP2-1_P45-P46	VP2-2_P43	VP4_P5
VP2-2_P11-P12	VP2-2_P48	VP4_P8-P9
VP2-2_P36-P37	VP2-2_P50-P51	VP4_P13-P15
VP2-2_P41-P42, P44	VP2-2_P57	VP4_P17-P18
VP2-2_P43	VP2-2_P61-P63	VP4_P77
VP2-2_P47	VP4_P75	VP4_P80
VP2-2_P50-P52	VP4_P78-P80	VP7_P1-P2
VP2-2_P53-P54	VP7_P5	VP7_P7
VP2-2_P55-P56	VP7_P9-P11	VP7_P10-P11
VP2-2_P60-P62	VP7_P14	VP7_P15-P17
VP4_P55-P56	VP7_P17-P18	VP7_P19-P20
VP4_P60-P62	VP7_P39	VP7_P25
VP4_P65	VP7_P42	VP7_P28-P29
VP4_P68-P70	NS3_P1	VP7_P33-P35
VP4_P71-72	NS3_P4	VP7_P37-P38
VP7_P1-P2	NS3_P7-P8	VP7_P39
VP7_P39	NS3_P13	NS3_P1
VP7_P40-P41	NS3_P15	NS3_P5-P7
NS3_P11-P12	NS3_P18-P20	NS3_P9-P10
NS3_P15	NS3_P23	NS3_P15
NS3_P17-P19	NS3_P26	NS3_P16
NS3_P22		NS3_P19-P20
NS3_P25-P27		NS3_P24-P26

[a]Cell surface staining and intracellular IFN-γ staining assays were not done with VP4_P71-72. The fluorescent antigen-transfected target cells-cytotoxic T lymphocytes (FATT-CTL) assay was not done with the following individual peptides: VP1-1_P2-P4, VP1-1_P34-P35, VP1-1_P39-P41, VP1-1_P45, VP1-1_P76-P78, VP2-1_P9-P10, VP2-1_P21, VP2-1_P27-P28, VP2-1_P34-P36 and VP2-1_P39 from the CD8 group.

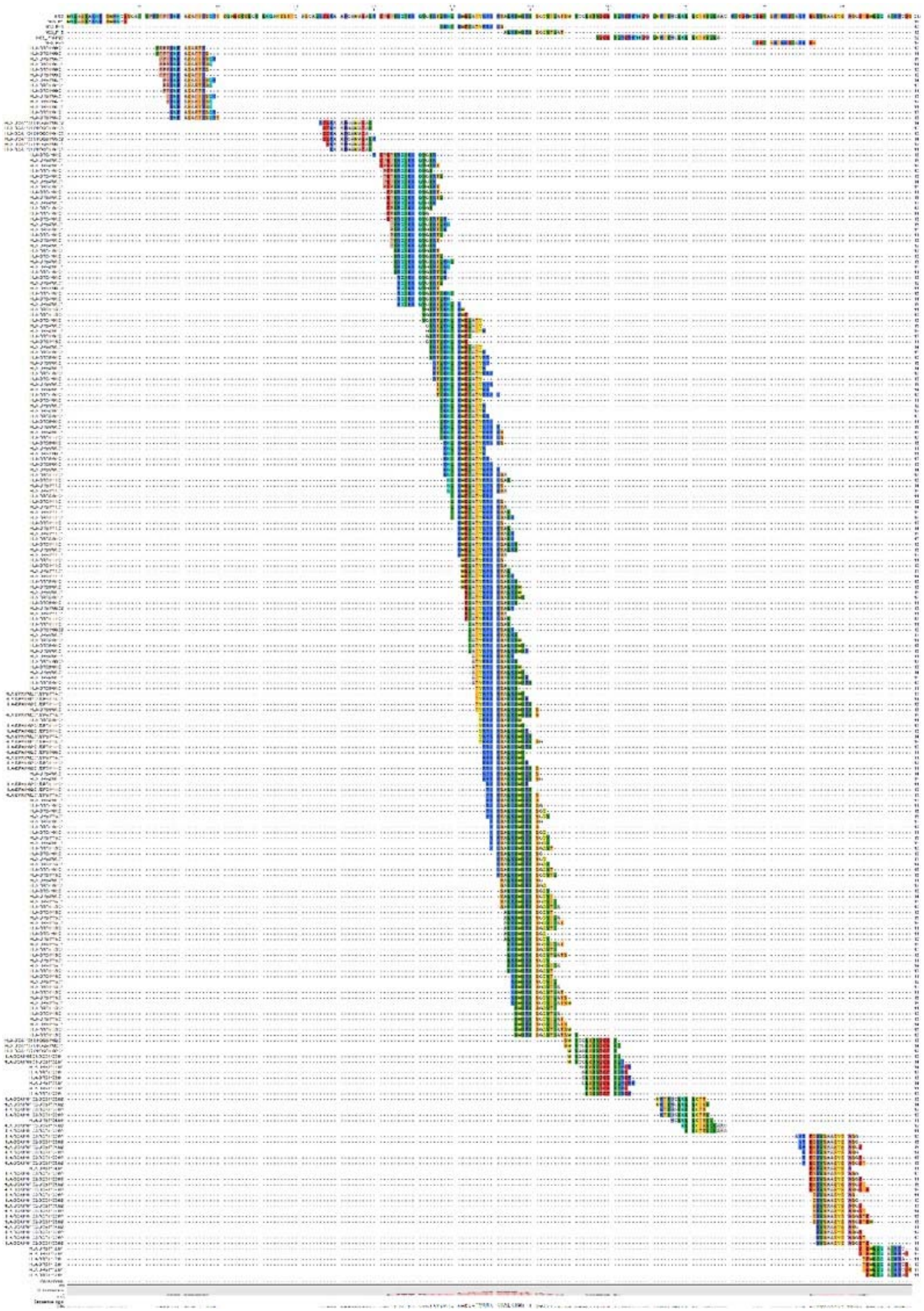

Fig. S3. CD4+ T cell epitopes. Individual peptides NS3_P1, NS3_P13, NS3_P15, NS3_P18-P20 and NS3_P23 from this study and all the significant predicted human HLA reference set (HLA-DRB*, HLA-DQA*, HLA-DQB*, HLA-DPA* and HLA-DPB*) epitopes are mapped to the AHSV4 protein NS3 sequence.

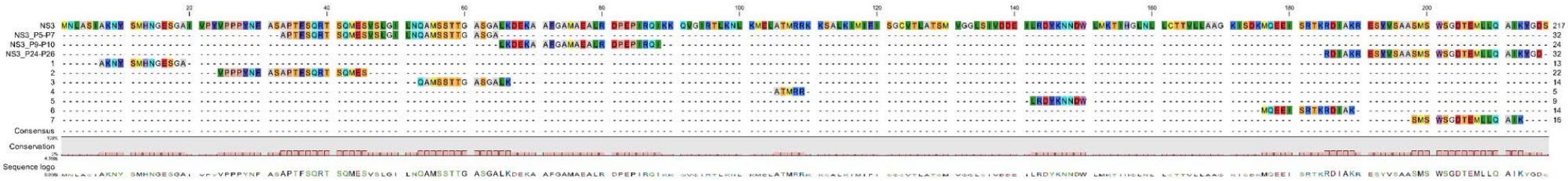

Fig. S4. Linear B cell epitopes. Individual peptides NS3_P5-P7, NS3_P9-P10 and NS3_P24-P26 from this study and the seven predicted linear B cell epitopes are mapped to the AHSV4 protein NS3 sequence.

Table S5. The selected individual peptides from the CD4, CD8 and B cell groups with their amino acid sequences. The total numbers of sequences obtained from GenBank used to generate the segment specific alignments are indicated per protein. Based on the sequence alignment, the percentage sequence identity per amino acid were calculated and these were subsequently used to determine the average percentage sequence identity per peptide.

CD4 group-peptides				
Peptide	**Sequence**	**AHSV protein**	**Number of sequences obtained from GenBank**	**Average percentage sequence identity (%)**
VP1-1_P8	YENVHTAELDIYVRSI	VP1	245	95.4
VP1-1_P11-P12	EEEFLRNYAVCDGVHPLKDFVEMR			94.3
VP1-1_P17	RETKHKPLGLLVASDF			92.7
VP1-1_P21-P23	QITEYGYCYSSPLLFEMCVTESILEFNMWYRM			96
VP1-1_P43	HVILASLCLSIQTITG			96
VP1-1_P73	TPYNFRNGMLDGIREA			96.2
VP1-1_P77-P78	LIEFGYGEGRVLNTLWNGKR			92.8
VP2-1_P1	VIVTKKGRVKHKEVDG	VP2	277	67.8
VP2-1_P4	MASEFGILMTNEKFDP			65.7
VP2-1_P7-P8	TNHRFGLCEVEHDMSISEFMYNEI			61.3
VP2-1_P13	RNDHQIRVDRDDNEMR			78.2
VP2-1_P17-P19	CYPSVFLRREARSQKLDRIRNYIGKRVEFYEE			53.1
VP2-1_P22	DQNKMSKVEQWRDAVN			55
VP2-1_P25-P26	KRGECYDHGTDIIYQFIKKLRFGM			61.7
VP2-2_P4-P6	DYGEILTEKVEDLYKSVLLERKWEDEVDDPES			61
VP2-2_P9	NEPHRVFLSAGKDVDN			45.3
VP2-2_P42	STAKGLGVIGVDIFNS			76.6
VP2-2_P44-45	QLSISMSGWIPYVERMCAESKVQT			64.1
VP2-2_P43	IGVDIFNSQLSISMSG			65.9
VP2-2_P48	LKRWFISYYTTLKLDR			65.9
VP2-2_P50-P51	RAEPRMSFKFEGLSTWIGSNCGGV			82.8
VP7_P14	AMNDIVRITGQMQTFG	VP7	284	99
VP7_P17-P18	YAGAVEVQQSGRYYVPQGRTRGGY			97.3
VP7_P42	MNGVVAPVGQINRALV			98.9
NS3_P1	MNLASIAKNYSMHNGE	NS3	519	69.7
NS3_P13	LKNLKMELATMRRKKS			80.3
NS3_P15	ALKIMIFISGCVTLAT			79.1
NS3_P18-P20	VDDEILRDYKNNDWLMKTIHGLNLLCTTVLLA			71.9
NS3_P23	QEEISRTKRDIAKRES			95.7
CD8 group-peptides				
Peptide	**Sequence**	**AHSV protein**	**Number of sequences obtained from GenBank**	**Average percentage sequence identity (%)**
VP1-1_P10-P12	LKLEDLEPEEEFLRNYAVCDGVHPLKDFVEMR	VP1	245	94.8
VP1-1_P43	HVILASLCLSIQTITG			96
VP1-1_P51-P52	EIVMPEDMYTSILRLAKNTSSGFS			96.1
VP1-1_P71-P72	LNLTIAIDYSEFDTHLTPYNFRNG			96.2
VP1-1_P76-P78	YEGYTLDELIEFGYGEGRVLNTLWNGKR			92.8
VP2-1_P3	SLEKTICDVIVTKKGR	VP2	277	70.7
VP2-1_P6-P8	VCGYEWDETNHRFGLCEVEHDMSISEFMYNEI			63.7
VP2-1_P9-P10	SEFMYNEIRCEGAYPIFPRYIIDT			68.2
VP2-1_P11-P12	FPRYIIDTLKYEKFIDRNDHQIRV			74.1
VP2-1_P16-P18	GEMYFSPECYPSVFLRREARSQKLDRIRNYIG			60.5
VP2-1_P24-P26	ERIVSIEPKRGECYDHGTDIIYQFIKKLRFGM			57.8
VP2-1_P29-P30	HSDYCIVPNKGGTSIGSWHIRKRT			45.2
VP2-1_P42-P44	DTARQEIRKAWVKGMPYMDFSKPMKIARGFNR			58.9
VP2-1_P45-P46	KIARGFNRNMLFFAALDSFRKRNG			55.8
VP2-2_P36-P37	LNTFTDFQRCVQSELLLPTLKLNF			59.2
VP2-2_P41-P42	DKRHPLLISTAKGLGVIGVDIFNS			76.7
VP2-2_P44	QLSISMSGWIPYVERM			68.5
VP2-2_P43	IGVDIFNSQLSISMSG			65.9
VP2-2_P53-P54	RDYVIQMLPTRKPKPGALMVAYAR			69.5
VP2-2_P55-P56	ALMVAYARDSRIEWIEAELSQWLQ			53.9
VP2-2_P60-P62	NKSVLRARTLKIYNRGSMDTLILISSGVYTFG			77.8
VP4_P55-P56	LWVSNRQNFTYDDVPVNRNFITLR	VP4	239	96.7
VP7_P39	NEIVAYLLVASLADVY	VP7	284	99
NS3_P15	ALKIMIFISGCVTLAT	NS3	519	79.1
NS3_P17-P19	SMVGGLSIVDDEILRDYKNNDWLMKTIHGLNL			71.2
B cell epitopes				
Peptide	**Sequence**	**AHSV protein**	**Number of sequences obtained from GenBank**	**Average percentage sequence identity (%)**
VP2-1_P41-P43	KKKEEGEDDTARQEIRKAWVKGMPYMDFSKPM	VP2	277	61.5
VP2-2_P49-P51	YTTLKLDRRAEPRMSFKFEGLSTWIGSNCGGV			65.9
VP4_P17-P18	TVLHGSEAETLSYADPKRHVVKGQ	VP4	239	98.3
VP4_P77	DLLAALFEFMYFAKHF			98.6
VP4_P80	SWIQYLRNA			98.5
NS3_P5-P7	APTFSQRTSQMESVSLGILNQAMSSTTGASGA	NS3	519	84.7
NS3_P9-P10	LKDEKAAFGAMAEALRDPEPIRQI			96.7
NS3_P24-P26	RDIAKRESYVSAASMSWSGDTEMLLQAIKYGD			85.4