Published in final edited form as:

Vet Immunol Immunopathol. 2014 October 15; 161(0): 161–169. doi:10.1016/j.vetimm.2014.08.001.

Epitope shifting of gp90-specific cellular immune responses in EIAV-infected ponies

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Abstract

Unlike other lentiviruses, EIAV replication can be controlled in most infected horses leading to an inapparent carrier state free of overt clinical signs which lasts for many years. While the resolution of the initial infection is correlated with the appearance of virus specific cellular immune responses, the precise immune mechanisms responsible for control of the infection are not yet identified. Since the virus undergoes rapid mutation following infection, the immune response must also adapt to meet this challenge. We hypothesize that this adaptation involves peptide-specific recognition shifting from immunodominant variable determinants to conserved immunorecessive determinants following EIAV infection. Forty-four peptides, spanning the entire surface unit protein (gp90) of EIAV, were used to monitor peptide-specific T cells responses *in vivo* over a six month period following infection. Peptides were injected intradermally and punch biopsies were collected for real-time PCR analysis to monitor the cellular peptide-specific immune responses *in vivo*. Similar to the CMI response to HIV infection, peptide-specific T cell recognition patterns changed over time. Early post infection (1 month), immune responses were directed to the peptides in the carboxyl-terminus variable region. By six months post infection, the peptide recognition spanned the entire gp90 sequence. These results indicate that peptide recognition broadens during EIAV infection.

Keywords

EIAV; lentivirus; cellular immune responses; envelope; immunodominant shifting

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Conflict of Interest Statement

The authors declare no conflict of interest in this work.

Introduction

Equine infectious anemia virus (EIAV) has been used as a model for investigating the underlying protective immune mechanisms against lentiviruses (Craig and Montelaro, 2011). Following infection, most horses control their initial EIAV infection leading to an inapparent carrier state (Hammond et al., 2000; Harrold et al., 2000). Immune responses play an important role in controlling EIAV infection (Mealey et al., 2001; Perryman et al., 1988) since the administration of immunosuppressive drugs to inapparent carriers can induce the recurrence of disease (Craig et al., 2002). This development of protective immunity is protracted and often requires 6 – 12 months to become established (Hammond et al., 2000; Harrold et al., 2000). Based on this evidence of protective immunity in EIAV inapparent carriers, it has been proposed that a successful vaccine should induce similar immune responses (Tagmyer et al., 2007). An attenuated strain (D9) of EIAV provided protection against homologous virus challenges (Craig et al., 2007), but optimum protective immunity was not seen until six months post vaccination. Thus, protective immunity against EIAV infection is a lengthy process that likely involves the development of immunity to viral variants (Hammond et al., 2000). Analyses of longitudinal serum samples from EIAV infected horses displayed an evolution of envelope-specific antibodies responses as measured by changes in avidity, conformational recognition, and neutralization titers in EIAV infected horses (Hammond et al., 1997; Hammond et al., 1999).

Cellular immune responses also appear important in controlling EIAV infections as the appearance of EIAV-specific cytotoxic T lymphocytes (CTL) correlates with the control of the initial viremia (McGuire et al., 2000). Additionally, CTL responses are detected in inapparent carriers during the chronic phase of EIAV infection (Chung et al., 2005; Mealey et al., 2003; Zhang et al., 1999). Both T helper (Th) and CTL epitopes have been identified in the EIAV envelope protein of EIAV-infected horses (Tagmyer et al., 2008). While various studies have demonstrated EIAV Gag and Pol specific CTL responses (Lonning et al., 1999; Mealey et al., 2005) and identified broadly reactive T-cell epitopes (Chung et al., 2004, 2005; McGuire et al., 2000), the identification of epitope-specific cellular immune responses that correlate with protection has proven elusive (Mealey et al., 2009).

Not all potential epitopes in an antigenically complex viral antigen can be recognized by CD4 or CD8 T cells (Gallimore et al., 1998). Also, not all recognized epitopes elicit equal responses. Typically, T cells responses focus on one or a few epitopes (immunodominant epitopes) and the remainder of the epitopes (immunorecessive epitopes) elicit limited responses (Yewdell and Bennink, 1999). Most acute viral infections involve the early recognition of immunodominant epitopes leading to the eradication of the infection (Chisari and Ferrari, 1995; Perelson et al., 1993). By contrast, there is a shift in T cell recognition from immunodominant to immunorecessive epitopes during chronic viral infections (Fuller et al., 2004; Turner et al., 2005; Wherry et al., 2003). This epitope shifting is evidenced by increases in the specificity, breadth and magnitude of T cell responses over time (Moskophidis et al., 1993). Thus, initial HIV-specific T cell responses are skewed towards immunodominant epitopes in the antigenically variable regions of the virus in the initial stages of the infection, whereas immunorecessive epitopes in the more conserved regions are recognized during the later stages of infection (Goulder et al., 2001; Jamieson et al., 2003;

Liu et al., 2013). It has been proposed that protective immunity likely requires T cell recognition of these immunorecessive epitopes in the antigenically conserved regions of HIV (Frahm et al., 2006; Goonetilleke et al., 2009).

Here, we show that EIAV envelope-specific T cell recognition also undergoes a shift in epitope recognition. Initially, the T cell response is directed towards the more variable regions of the viral envelope protein, gp90, and then shifts to more conserved regions later in the infection. This shift in epitope recognition is associated with the control of viral replication and the establishment of the inapparent carrier state.

Materials and Methods

Ponies, virus and experimental challenge

Sixteen ponies of mixed age and gender, seronegative for EIAV, were used in this study. All the ponies were also pre-screened by intradermal inoculation of peptide pools A to G prior to EIAV infection (see below) and no responses were detected. MHC haplotypes were determined using polymorphic microsatellites, as previously described (Tseng et al., 2010). Five ponies (G35, L132, H46, H38 and H32) shared the same MHC microsatellite allele 219–252–280–172–247, three of the ponies (L126, L132 and I28) shared the allele 211–260–268–166–247, and another three ponies (D49, H36 and H32) shared the same MHC microsatellite allele 211–260–278–174–241 (Table 1). An inapparent carrier D64 was also included for comparisons. This horse was infected with EIAVPV strain in a previous study (Tagmyer et al., 2007). Throughout this study period, D64 remained in an inapparent state exhibiting no clinical signs of EIA.

The D9 strain of EIAV (EIAV_{D9}) was used as the challenge virus. The EIAV_{D9} strain was derived from the infectious molecular clone EIAV_{UK} and attenuated by the insertion of two stop codons and a nine base pair deletion into the S2 gene (Craig et al., 2007). Previous work has shown that ponies infected with EIAV_{D9} develop protective immunity against EIAV strains with a homologous gp90 sequence (Craig et al., 2007).

Ponies were inoculated intravenously with 10^3 TCID₅₀ of EIAV_{D9} (Craig et al., 2007). All ponies were monitored for clinical symptoms of EIA throughout the course of the 6 months study. Rectal temperatures were obtained daily and platelet numbers analyzed weekly using an IDEXX QBC Vet Autoreader. Plasma samples were also collected weekly and stored at –80°C for later detection of viral RNA. Gag-specific primers were designed and a quantitative real-time multiplex RT-PCR assay was used for the quantitation of viral RNA (Cook et al., 2002).

All animals were handled under the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture, according to protocols approved by the University of Kentucky Institutional Animal Care and Use Committee.

Production of synthetic peptides and construction of peptide pools matrix

Forty-four peptides of 20 amino acids in length (Table 2), spanning the entire gp90 of EIAV_{D9} strain, were synthesized and used to construct peptide pools 1–7 and A–G (Figure

1). All peptides were HPLC-purified and dissolved in 100% dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, MO) at a stock concentration of 2 mg/ml. (Tagmyer et al., 2007).

Determination of gp90 specific cellular immune responses over time *in vivo*

Identification of gp90-specific cellular immune responses *in vivo* was performed as recently described (Liu et al., 2012). The optimal working concentration for individual peptides was determined by serially diluting a known positive peptide (Liu et al., 2012). Initially peptide pools (Table 2) were injected intradermally into the neck of each pony. Palpable infiltrations (> 5mm) were measured 48 hours later at each injection site and both a 2 and 4 mm skin biopsy were collected from each reactive site. To confirm the identity of the specific peptide response, individual peptides were similarly injected intradermally and biopsied 48 hours later.

The 4 mm biopsies were placed into 10% formalin prior to processing for immunohistochemistry staining, as previously described (Liu et al., 2012). The 2 mm skin biopsies were stored in RNALater® (Ambion, Austin, TX) and later homogenized using a Retsch MM301 Ball Mill (Newtown, PA). The suspended cell-lysates were transferred to a 1.5ml eppendorf tube containing RNA-STAT 60 (Tel-Test, Friendswood, TX)). Total RNA was isolated according to the manufacturer's directions and 1 µg of RNA was suspended in 41.5µl nuclease-free water (Qiagen, Valencia, CA) and reverse transcribed into cDNA (Liu et al., 2012).

Intron-spanning TaqMan primers and probes for equine IFN γ were used for real-time PCR, as previously described (Liu et al., 2011a). PCR amplification efficiencies were determined using Linreg (Ramakers et al., 2003) and only samples with amplification efficiencies above 95% were included for further analyses. Beta-glucuronidase (β -GUS) was used as the housekeeping gene and the Δ CT method (Livak and Schmittgen, 2001) was used to determine relative gene expression with the saline injection site for each horse serving as the calibrator and RQ values greater than 2 were considered positive.

Determination of EIAV_{D9} strain gp90 variation

Virus sequencing was performed at the Department of Microbiology and Molecular Genetics, University of Pittsburgh. The cloning and sequencing of EIAV_{D9} gp90 were conducted as previously described (Craig et al., 2010). In total 87 EIAV_{D9} gp90 clones from 25 ponies were analyzed at six months post infection. Multiple sequence alignments were conducted (<http://www.genome.jp/tools/clustalw/>) and gp90 amino acid sequence variability was determined as previously described (Bansal et al., 2005; Rodriguez et al., 2004). Normalized Shannon entropy scores were calculated for each amino acid position in the protein alignment using the program Scorecons (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl). Single peptide variability was calculated as the mean of normalized Shannon entropy scores of the amino acids located in the peptide.

Data Analysis

One way analysis of variance (one way ANOVA) was used to determine differences of normalized Shannon entropy scores over time. Unpaired t test was used to determine

differences between the normalized Shannon entropy scores of early and later recognized peptides. Statistical significance was set at $p < 0.05$.

Results

Clinical Response to infection

Each pony seroconverted by 35 days post infection as determined by a commercial ELISA (Centaur, Overland Park, KS). No clinical signs associated with EIA were detected during the course of the 6 month study. Post-infection rectal temperatures and platelet counts for the ponies are shown in Figure 2. A low level of EIAV_{D9} replication, 10^3 – 10^4 RNA copies/ml plasma, was detected during the six months period of observation.

Determination of gp90-specific cellular immune responses in EIAV_{D9} infected ponies *in vivo*

Over the course of this study, 11 of the 16 ponies tested showed gp90-specific evidence of T lymphocyte infiltration and IFN γ expression at the injection site. Screening all ponies with 44 EV0 peptides indicated that not all peptides were equally recognized (Table 3). In total, 19 single peptides elicited a response. Peptides #36 and #38 were the most widely recognized peptides, seen by 4 and 6 ponies, respectively. It was found that ponies sharing ELA haplotypes recognized the same peptide, such as #36 being recognized by ponies G35 and H46. But individual peptides could also be recognized by ponies with different haplotypes, such as #38 being recognized by ponies G35 and I28. Additionally, not all of the peptides were recognized and peptide recognition changed over time. One month post infection, 7 ponies exhibited an *in vivo* IFN γ response to multiple peptides in the carboxyl end of gp90 (Figure 3A). Five of the ponies recognized only one peptide and two ponies recognized two peptides with peptides #36 and #38 being recognized by multiple ponies. Three months post infection, peptides from the middle and carboxyl terminus of gp90 were being recognized (Figure 3B). By six months post infection, most ponies recognized more than one peptide and the recognized peptides spanned the entire length of gp90 (Figure 3C).

By contrast, an inapparent carrier, D64, infected for more than six years with EIAV showed identical T cell recognition patterns at 3 and 6 years post-infection (Figure 4A). Peptide pools C, E, F, G, 3, 4, and 6 elicited palpable infiltrations at both sample times. Likewise, injection of the predicted single peptides (Grey boxes) confirmed that this inapparent carrier recognized peptides 4, 12, 37, 38 and 40 at both times it was tested (Figure 4B)

Peptides recognized early in EIAV infection were located in more variable regions of gp90

Approximately 87 virus clones from ponies infected with the EIAV_{D9} for six months were collected and the hypervariable regions of gp90 (V3 to V8) sequenced (Leroux et al., 2001; Leroux et al., 1997). The most variable peptides (#18, #19, #20) were found to be located within the V3 region which contains a known principal neutralizing domain (PND) (Leroux et al., 1997). When the normalized Shannon entropy scores for each of the corresponding gp90 peptides were calculated it was determined that there was a trend for an increase of the entropy scores over six months ($p=0.07$), indicating that early in the infection the recognized peptides were located in the more variable regions of gp90. Later in the infection, by

contrast, more peptides located in the conserved regions were being recognized. A significant ($p=0.03$) difference in entropy score was also seen when the peptides were classified in regards to whether they were recognized early or later in the infection for each pony (Figure 5B) again indicating that more conserved regions were being recognized later in the infection.

Discussion

The ability of lentiviruses to undergo antigenic variation is the primary mechanism of immune evasion in the infected host (Burns and Desrosiers, 1994; Craig and Montelaro, 2010; Payne et al., 1987). During infection, EIAV also mutates within distinct conserved and variable regions (Leroux et al., 2001; Leroux et al., 1997; Zheng et al., 1997). A previous investigation of antigenic variation in EIAV_{D9} indicated that this virus undergoes similar mutation following infection with the median divergence being around 1.89% from the initial strain six months post inoculation (Craig et al., 2010). To counter this antigenic variation in lentiviruses, the immune responses must also adapt in order to recognize the newly arising viral variants (Nowak et al., 1995). Thus, in EIAV-infected horses, antigenic variation in the envelope gp90 results in escape variants that evade the neutralizing antibody response forcing the host to develop antibodies against the newly arising epitopes in order to control viral replication (Montelaro et al., 1998). While there is evidence that CTL responses also play a role in restricting EIAV replication (McGuire et al., 2004), there have been no studies investigating the effect of antigenic variation in cellular immune responses. Indeed, most studies investigating T cell responses in EIAV-infected horses have involved few or single time points post-infection (McGuire et al., 2004; Tagmyer et al., 2008).

Here, we analyzed the cell mediated immune response to specific peptides of gp90 over the first six months of the infection in ponies infected with EIAV_{D9}. Our results indicate the T cell response undergoes expansion and contraction in terms of gp90 epitope recognition. By one month post infection, peptides in the more variable carboxyl-terminus of gp90 were recognized. By six months post infection, peptide recognition included those found in the more conserved amino-terminus regions of gp90. Normalized Shannon entropy scores of the corresponding amino acid sequences confirmed that those peptides recognized early in the infection were located in the more variable regions of gp90 whereas those recognized later in the infection were located in more conserved regions. This shift in peptide recognition in newly infected horses may be the result of mutations in the variable region epitopes leading to an escape from T cell recognition. Indeed, we observed the loss of recognition of these more variable peptides in many of the ponies over time. By contrast, the peptide recognition pattern in an inapparent carrier (D64) was much more stable over a three year time period. This persistent recognition of conserved peptides likely reflected a more effective T cell response where virus replication was tightly controlled and the carrier state maintained.

Similar changes in peptide recognition have been reported for HIV infections with specific T cell responses being skewed initially towards variable region peptides early in the infection and then towards more conserved peptides in chronic infections (Bansal et al., 2005; Goulder et al., 2001; Liu et al., 2011b). While the mechanisms responsible for this shift in recognition from immunodominant to immunorecessive peptides are still unknown, early T

cell recognition of peptides in the variable region likely provides selective pressure for mutations which escape T cell detection (Cao et al., 2003; Kelleher et al., 2001; Mealey et al., 2004). Repeated exposure to conserved epitopes in new viral variants is likely the major reason for the recognition shifting from immunodominant variable peptides to immunorecessive conserved peptides (Goonetilleke et al., 2009). Our findings are thus consistent with others indicating that immunodominant epitopes recognized by the T cells in the acute phase of the immune response likely serve as immunodecoys, whereas immunorecessive epitopes may be more important for protection (Frahm et al., 2006; van der Most et al., 1997).

Not all ponies developed gp90 specific cellular immune responses, as measured here, although all seroconverted one month post infection. This may have been due to our use of a live attenuated virus whose replication is significantly reduced in horses compared to more virulent strains (Craig et al., 2005). It has been proposed that continued exposure to viral antigen is needed to maintain virus-specific T cells during chronic infection (Shin et al., 2007). The reduced replication of EIAV_{D9} in some of the ponies may have failed to stimulate an immune response robust enough for our detection (Peng et al., 2005). Our outbred ponies exhibited diversity in their MHC antigens that would likely impact peptide recognition by T cells. Indeed, comparison of the MHC haplotypes shared amongst those ponies which recognized the same peptide indicated that they shared the same MHC, though others with different haplotypes could also recognize the same peptide. Broadly recognized T cell epitopes have previously been reported in EIAV infected ponies with different MHC haplotypes (Chung et al., 2004). Another possibility for this cross-reactivity is that some of our peptides may have contained multiple epitopes. Since the typical length of a T cell epitope is 8 to 10 amino acids, we cannot exclude the possibility that our peptides (20 amino acids in length) might include multiple T cell epitopes. It is also important to emphasize that we were only detecting immune responsiveness to those peptides present in gp90 of EIAV_{D9}. Cell-mediated immune responses are also directed against other EIAV proteins including gag and capsid proteins (Chung et al., 2004; McGuire et al., 1994; Mealey et al., 2005). The true breadth of the T cell response post-infection is likely greater than what we have detected.

Conclusions

Similar to the CMI response to HIV infection, equine peptide-specific T cell recognition patterns to EIAV changed over time. Immune responses were directed towards peptides in the carboxyl-terminus variable region of the major envelope protein early in the infection. By six months post infection, peptide recognition spanned the entire gp90 sequence indicating peptide recognition broadens during EIAV infection. Importantly, our data did not show T cells exclusively targeting variable peptides during the acute phase of the infection, but rather an expansion of responses to the more conserved peptides during the chronic stage of the infection.

Acknowledgements

The authors wish to thank Seth Hatfield and farm staff for their technical support and care of all ponies. This work was supported by NIH R01 AI25850-23 and CTSA UL1RR033173 (NCRR).

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	A	B	C	D	E	F	G
1	1	3	5	7	9	11	13
2	15	17	19	21	23	25	27
3	29	31	33	35	37	39	41
4	43	2	4	6	8	10	12
5	14	16	18	20	22	24	26
6	28	30	32	34	36	38	40
7	42	44					

Figure 1.

Matrix of gp90 peptides. Forty-four original autologous peptides from EIAV_{D9} were synthesized. In total, 14 peptides pools were constructed and each single peptide could be found in two different peptides pools.

Figure 2.

Clinical and virological profiles of EIAV_{D9} infected ponies. A) Rectal temperature (°C) was measured daily in all EIAV_{D9} infected ponies. The average rectal temperature was calculated and plotted. B) Viral p26 copies in the plasma (right axis) and platelet counts (left axis) were determined at 3 week intervals. The average (+ SE) p26 copies and platelet counts for the 16 experimentally infected ponies are shown.

Figure 3.

Summary of peptide recognition in EIAVD9 infected ponies. Eleven of the 16 ponies showed gp90 peptide-specific responses during the six month period of observation. The figure shows the accumulated recognition pattern for each of the peptides tested.

A

	A	B	C	D	E	F	G	Year 2010	Year 2013	Areas of palpable infiltrations(mm ²)
1	1	3	5	7	9	11	13	0	0	
2	15	17	19	21	23	25	27	0	0	
3	29	31	33	35	37	39	41	49	100	
4	43	2	4	6	8	10	12	50	100	
5	14	16	18	20	22	24	26	0	0	
6	28	30	32	34	36	38	40	625	500	
7	42	44						0	0	
Year 2010	0	0	150	0	100	150	300			
Year 2013	0	0	100	0	150	400	225			

Areas of palpable infiltrations(mm²)

B **Figure 4.**

IFN γ expression in peptides· intradermal injection sites in EIAV_{D9} infected ponies. Ponies were tested at 1 (A), 3 (B) and at 6 (C) months post-infection. IFN γ gene expression was determined using relative quantitation (RQ) and the results were log transformed. Each point represents an individual pony's response to the peptide.

Figure 5.

Stable peptide recognition in an inapparent EIAV carrier horse. A) Peptide pools were screened in 2010 and 2013 approximately 3 and 6 years post infection. B) The peptides injection sites eliciting a positive response were biopsied and $\text{IFN}\gamma$ gene expression determined using Real time-PCR.

Figure 6.

Comparison of the normalized Shannon entropy scores among recognized peptides over time post infection with EIAV_{D9}. A) 1, 3 and 6 months post infection. One way analysis of variance (one way ANOVA) was used to determine differences in scores ($p < 0.07$). B) Early (1 differences between the scores ($p = 0.03$). Statistical significance was set at $p < 0.05$."

Table 1**MHC haplotypes**

Horse ID	Virus	UMN-JH34-2 ¹	COR112-COR113-UM011-COR114 ²
G35	EIAV _{D9}	219-252-280-172-247	207-254-268-174-235
L126	EIAV _{D9}	211-260-268-166-247	211-260-268-166-247
L132	EIAV _{D9}	219-252-280-172-247	211-260-268-166-247
F31	EIAV _{D9}	205-252-274-168-243	203-260-266-168-249
I28	EIAV _{D9}	215-252-260-168-243	211-260-268-166-247
H46	EIAV _{D9}	221-268-278-174-241	219-252-280-172-247
D49	EIAV _{D9}	211-260-278-174-241	211-258-274-164-237
H38	EIAV _{D9}	219-252-280-172-247	223-252-268-166-247
H36	EIAV _{D9}	211-260-278-174-241	205-252-274-168-243
H32	EIAV _{D9}	211-260-278-174-241	219-252-280-172-247
D55	EIAV _{D9}	215-252-260-168-243	211-258-274-164-237
I35	EIAV _{D9}	205-262-270-184-245	221-262-270-172-237
J30	EIAV _{D9}	197-248-270-184-245	219-244-268-172-249
L124	EIAV _{D9}	221-262-270-172-237	221-238-264-180-243
I33	EIAV _{D9}	219-236-266-168-249	203-260-266-178-241
L128	EIAV _{D9}	219-250-266-170-245	211-262-270-184-245
D64	EIAV _{PV}	219-238-266-168-249	211-238-266-178-241

¹UMN- JH34-2 is an equine MHC microsatellite constellation corresponding to ELA-I.

²COR112, COR113, UM011 and COR114 represent microsatellite constellations corresponding to ELA-II haplotypes.

Table 2**Gp90 peptide Sequences**

Peptide No.	Peptide sequence
1	MVSIAFYGGIPGGISTPITQ
2	PGGISTPITQQSEKSKCEEN
3	QSEKSKCEENTMFQPYCYNN
4	TMFQPYCYNNDSKNSMAESK
5	DSKNSMAESKEARDQEMNLK
6	EARDQEMNLKEESKEEKRRN
7	EESKEEKRRNDWWKIGMFL
8	DWWKIGMFLCLAGTTGGIL
9	CLAGTTGGILWWYEGLPQQH
10	WWYEGLPQQHYIGLVAIGGR
11	YIGLVAIGGRLNGSGQSNAI
12	LNGSGQSNAI ECWGSFPGCR
13	ECWGSFPGCRPFQNYFSYET
14	PFQNYFSYETNRSMHMDNNT
15	NRSMHMDNNTATLLEAYHRE
16	ATLLEAYHREITFIYKSSCT
17	ITFIYKSSCTSDHCQEYQC
18	DSDHCQEYQCKKVNLSDDS
19	KKVNLSDDSSNPVRVEDVM
20	SNPVRVEDVMNTTEYWGFKW
21	NTTEYWGFKWLECNQTENFK
22	LECNQTENFKTILVPENEMV
23	TILVPENEMVNINDTDWIP
24	NINDTDWIPKGCNETWARV
25	KGCNETWARVKRCPIDILYG
26	KRCPIDILYGIHPIRLCVQP
27	IHPIRLCVQPPFFLVQEKGI
28	PFFLVQEKGIANTSIRIGNCG
29	ANTSIRIGNCGPTIFLGVLED
30	PTIFLGVLEDNKGVVRGNYT
31	NKGVVRGNYTACNVSRLKIN
32	ACNVSRLKINRKDYTGIIYQV
33	RKDYTGIIYQVPIFYTCNFTN
34	PIFYTCNFTNITSCNNEPII
35	ITSCNNEPIISVIMYETNQV

Peptide No.	Peptide sequence
36	SVIMYETNQVQYLLCNNNNS
37	QYLLCNNNNSNNYNCVVQSF
38	NNYNCVVQSFQVIGQAHLEL
39	GVIGQAHLELPRPNKRIRNQ
40	PRPNKRIRNQSFNQYNC SIN
41	SFNQYNC SINNKTELETWKL
42	NKTELETWKL VKTSGITPLP
43	VKTSGITPLPISSEANTGLI
44	ISSEANTGLIRHKRDFGISA

Table 3

		One Month post infection																		
		2	8	11	14	16	21	22	23	26	28	30	33	34	35	36	38	41	42	44
Peptides	Horse ID																			
	G35																			
	L126																			
	L132																			
	F31																			
	I28																			
	H46																			
	D49																			
	H38																			
	H36																			
	H32																			
	D55																			
		Three months post infection																		
		2	8	11	14	16	21	22	23	26	28	30	33	34	35	36	38	41	42	44
Peptides	Horse ID																			
	G35																			
	L126																			
	L132																			
	F31																			
	I28																			
	H46																			
	D49																			
	H38																			
	H36																			
	H32																			
	D55																			
		Six months post infection																		
		2	8	11	14	16	21	22	23	26	28	30	33	34	35	36	38	41	42	44
Peptides	Horse ID																			
	G35																			
	L126																			
	L132																			
	F31																			
	I28																			
	H46																			
	D49																			
	H38																			
	H36																			
	H32																			
	D55																			

Shaded box indicates peptide reactivity.