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**Identification and analysis of biomarkers associated with infection with *Sarcocystis neurona.***

*Sarcocystis neurona* is the causative agent of Equine Protozoal Myeloencephalitis (EPM). Though in some states in the US, over 50% of horses are seropositive for *S. neurona*, prevalence of clinical EPM is estimated at 0.5–1%. The reason for these different outcomes of infection is poorly understood. In order to identify biomarkers indicative of the disease we have analyzed sera from horses using SELDI ToF mass spectrometry, before and after infection with *S. neurona*. All the horses presented clinical disease. We have identified several proteins in the range of 8-11 kDalton that show a significant increase/decrease 11 weeks after infection. These biomarkers may help in the development of new diagnostics and provide a better understanding of the different outcomes of disease.

**Identification et analyse de marqueurs biologiques associés à l'infection avec *Sarcocystus neurona.***

*Sarcucystis neurona* est l'organisme causatif de la myeloencéphalite protozoaire équine (EPM). Bien que plus de 50% des chevaux soient testés séropositifs pour *S. neurona* dans certains états des États-Unis, la prévalence de l'EPM clinique n'est estimée qu'à 0.5 – 1%. La cause de cette différence est incomprise. Afin d'identifier des marqueurs biologiques associés à la maladie, nous avons analysé, à l'aide de spectrométrie de masse SELDI-ToF, des sérums de chevaux pré- et post-infection avec *S. neurona*. Tous les chevaux testés démontraient des signes cliniques de la maladie. Nous avons identifié plusieurs protéines entre 8 et 11 kDa qui présentent une augmentation ou une diminution d'expression onze semaines post-infection. Ces marqueurs biologiques pourraient aider au développement de nouveaux outils de diagnostique pour l'EPM et à une meilleure compréhension des différentes issues de la maladie.

**Analysis of equine serum samples**

The objective of this study was to identify candidate biomarkers associated with infection with *Sarcocystis neurona* in horses. We received serum samples from 8 infected horses and two control animals. Samples were taken 1 week prior and at 9 times over 12 weeks following experimental infection, with samples from 6-8 horses taken at each time point (Figure 1). All sera were aliquoted into 50 μl batches and stored at–80$^{o}$C until analyzed.

Sera were separated into 6 fractions using anion-exchange chromatography. Fractions 1, 3, 5 and 6 were then bound to weak cationic (CM-10) protein chip arrays. in Sinaptic acid was applied to generate an energy-absorbing matrix to assess proteins of intermediate (10-30 kDa) and large (30-100 kDa) mass. Chips were read using SELDI-TOF mass spectrometry (MS) with a PBSII protein chip reader. Each chip underwent two series of MS analysis to obtain reproducible spectra for these two mass ranges. The laser was set at a sensitivity of 8 and an intensity of 210.

To analyze peptides (2-15 kDa), samples were bound to CM10 chips and an α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix was applied. Spectra were obtained with a PBSII protein chip reader with a laser sensitivity of 7 and an intensity of 190. We repeated our analysis on two different PBSII protein chip readers.

An example of these spectra is shown in Figure 2. The same spectra represented in gel format can be seen in Figure 3. Serum peptide and protein profiles were obtained for all horses. All analyses were done using Ciphergen Express Software. Peaks with a signal/noise ratio higher than 6 were selected and profiles were normalized to the total ion current. Significant differences ($P$<.05) in peak intensity (protein abundance) between time points were noted.

Minimal differences were observed in the intermediate and large protein ranges over the course of infection, so we concentrated on small protein and peptide profiles. Differences between samples prior to infection and 11 weeks post-infection were observed in fraction 1, but in none of the other fractions. Analysis from the two different machines yielded similar serum protein profiles.

Figures 4-6 show intensities of peaks that were significantly different (p= 0.05) at week 11 post infection compared to pre-infection.

Week 1 and week 11 samples from two horses were fractionated using anion exchange chromatography, desalted using acetone precipitation and electrophoresed through a Novex 4-16% Bis-Tris gel (Invitrogen; Figure 7), as well as a 16% Tris-Tricine gel (Invitrogen). Bands of interest were excised for MS-MS sequence analysis at Genome Quebec. A band of 10-12 kDa appeared to be more abundant in week 11 samples and was identified to be serum amyloid A. Confirmation of whether this was the bands identified by SELDI-ToF analysis in this size range was not possible. Other bands sent for sequencing contained degraded products of albumin, γ-immunoglobulin and α-chain immunoglobulin E.

None of the candidate biomarkers can be postulated as specific indicators of *S. neurona* infection in horses, as all have been previously associated with inflammation or infection. Furthermore, none of the candidate biomarkers appeared to be detectable prior to the onset of symptoms in these horses, which is a necessary goal for an early diagnostic. Additional SELDI-ToF analyses that would generate more information are not readily apparent at this point. Considering the cost, we propose that the project be shelved at this point.

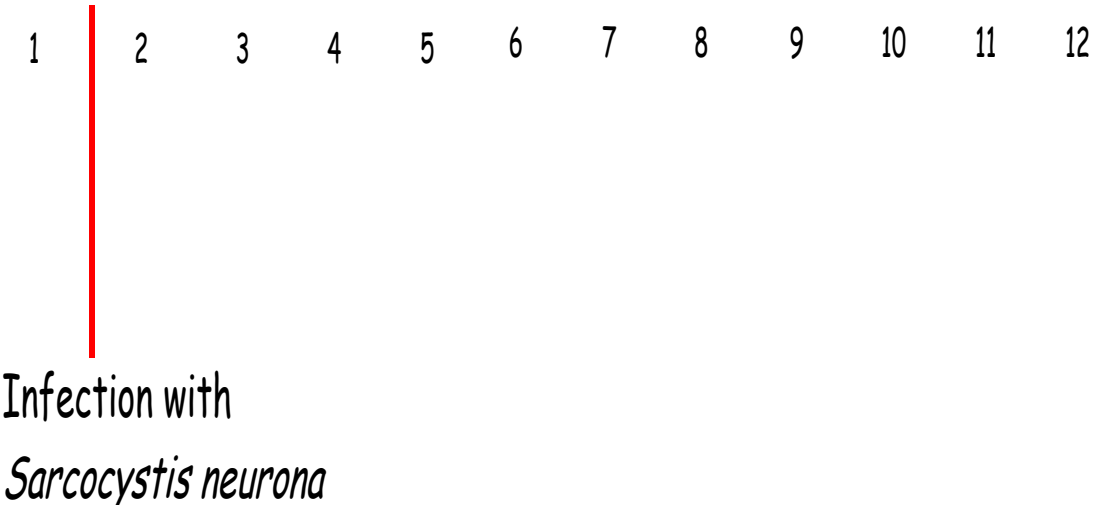



**Figure 1: Serum samples from horses infected with *S. neurona*.** 8 horses (1, 3, 7, 8, 9, 11, 12, 14) were infected with *S. neurona* and there were two uninfected controls (6, 13) Arrows indicate time points at which serum samples were collected from 6-8 animals. Control horse 6 was sampled on week 9 and control horse 13 was sampled on weeks 10 and 11.

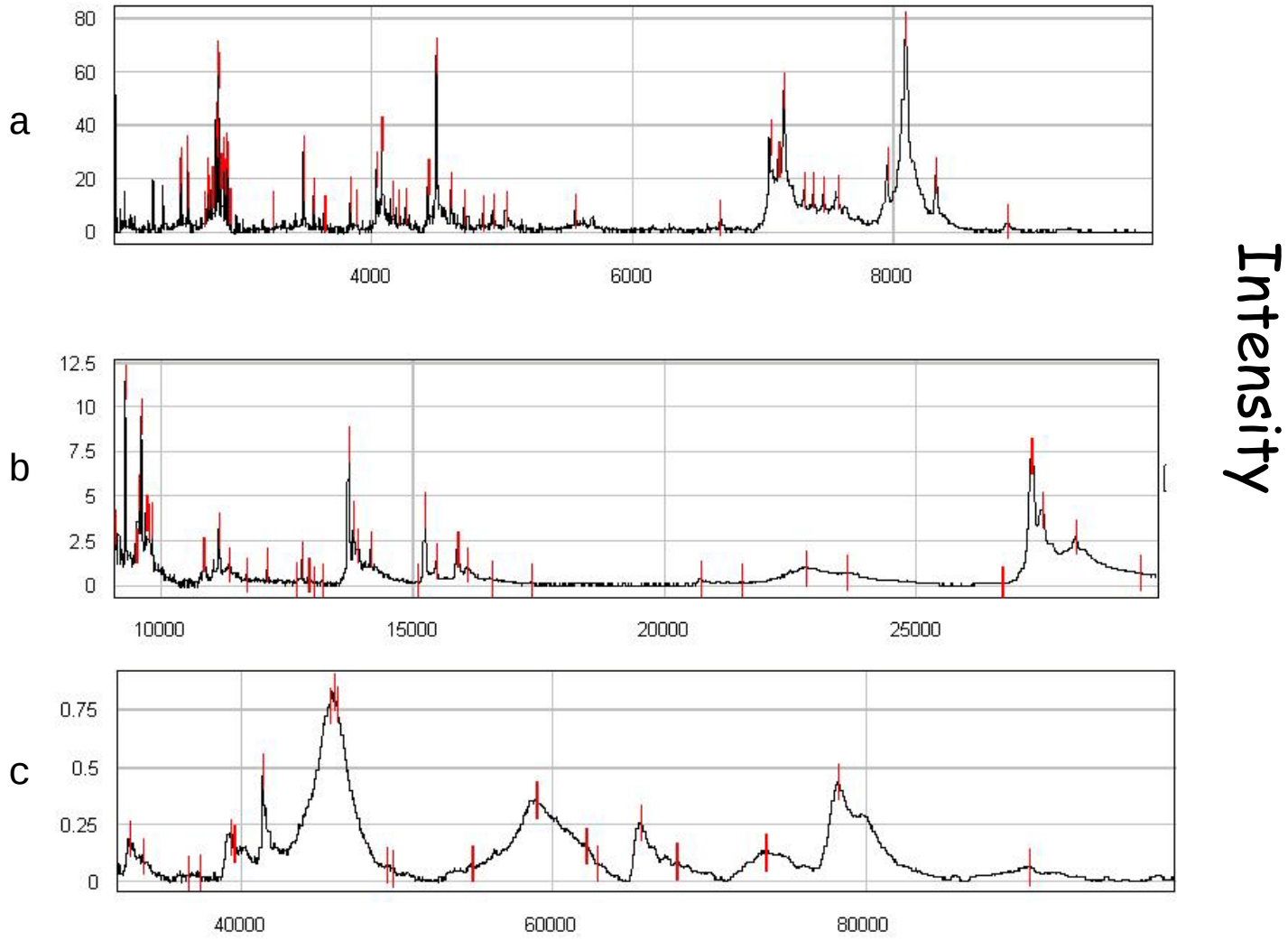



**Figure 2: Examples of Serum Protein Profiles.** a) peptides (3-9 kDa); b) proteins in the low molecular weight range (10-30 kDa); c) proteins in the high molecular weight range (30-150 kDa).

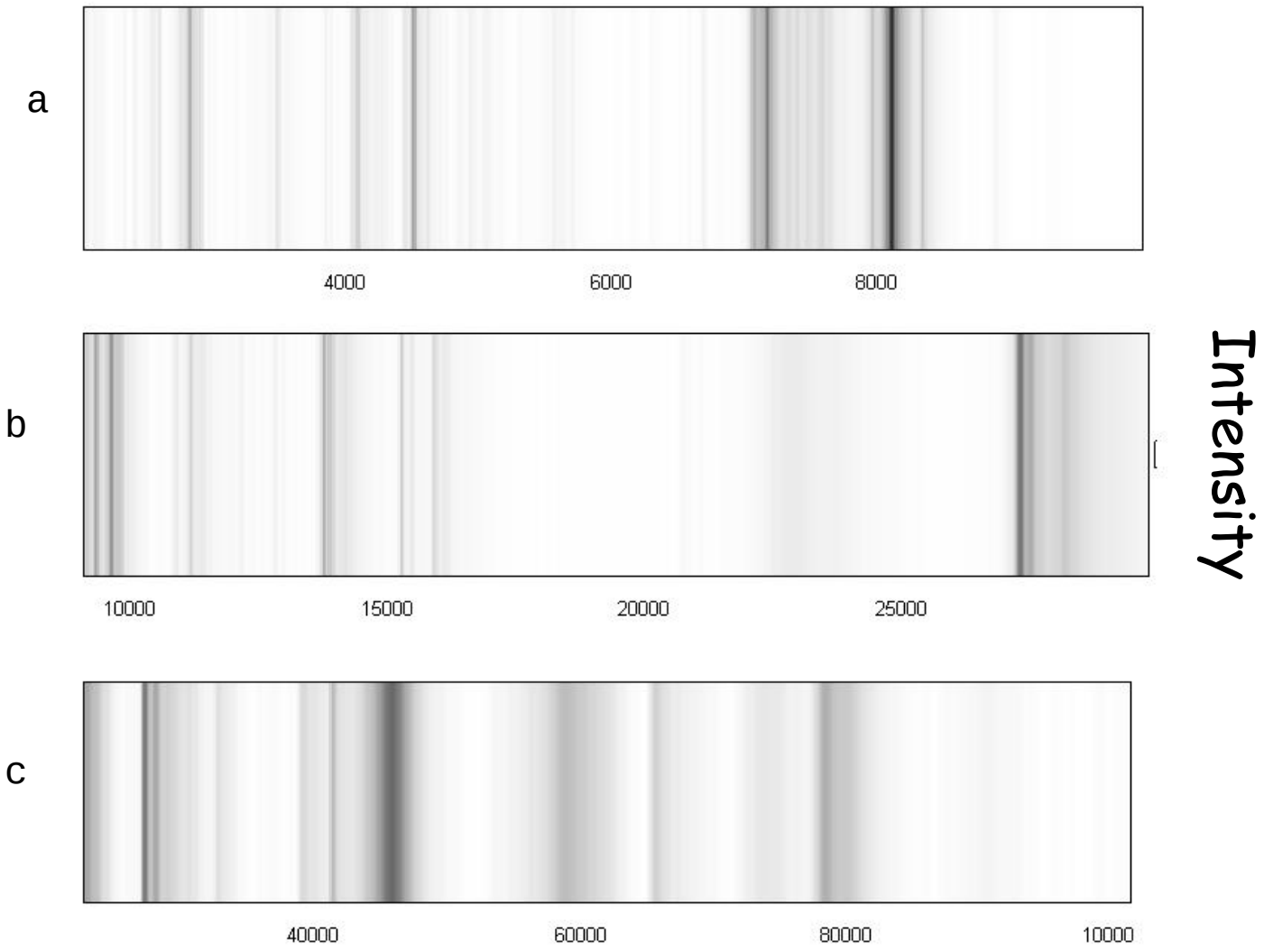



**Figure 3: Examples of Serum Protein Profiles in gel view.** a) peptides (3-9 kDa); b) proteins in the low molecular weight range (10-30 kDa); c) proteins in the high molecular weight range (30-150 kDa)

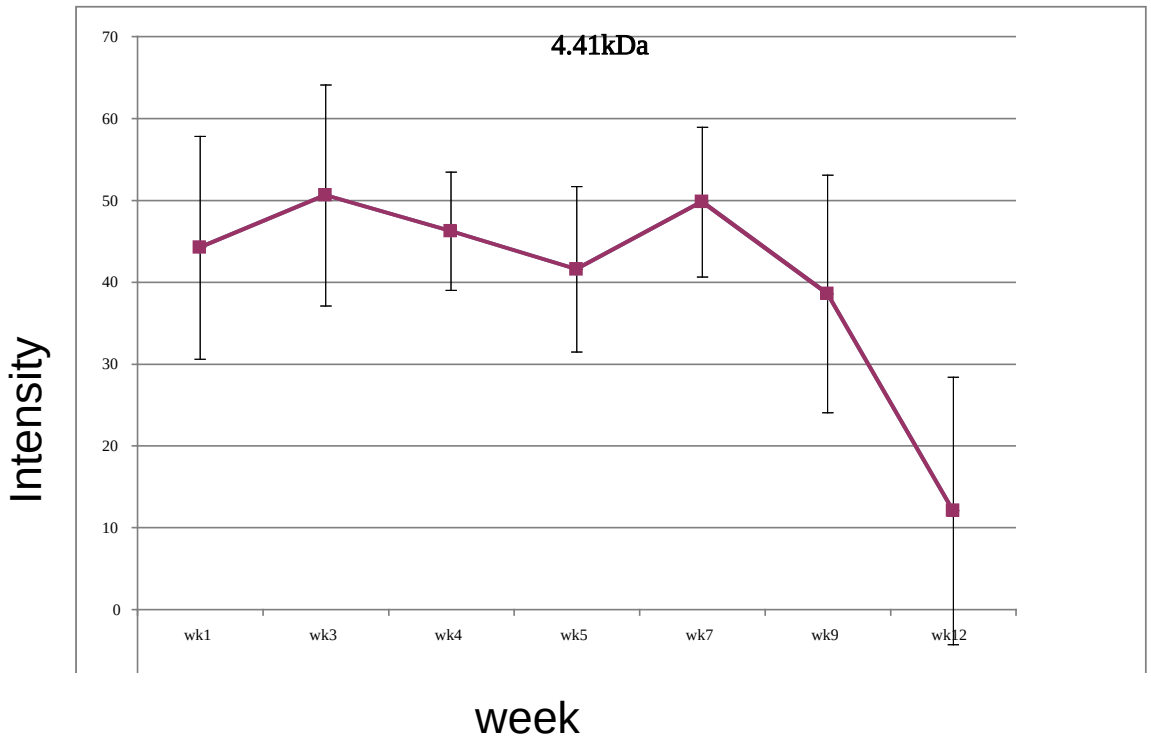



**Figure 4: Average intensity of a peak at 4.41kDa .**The intensity of this peak was significantly lower 11 weeks post infection.

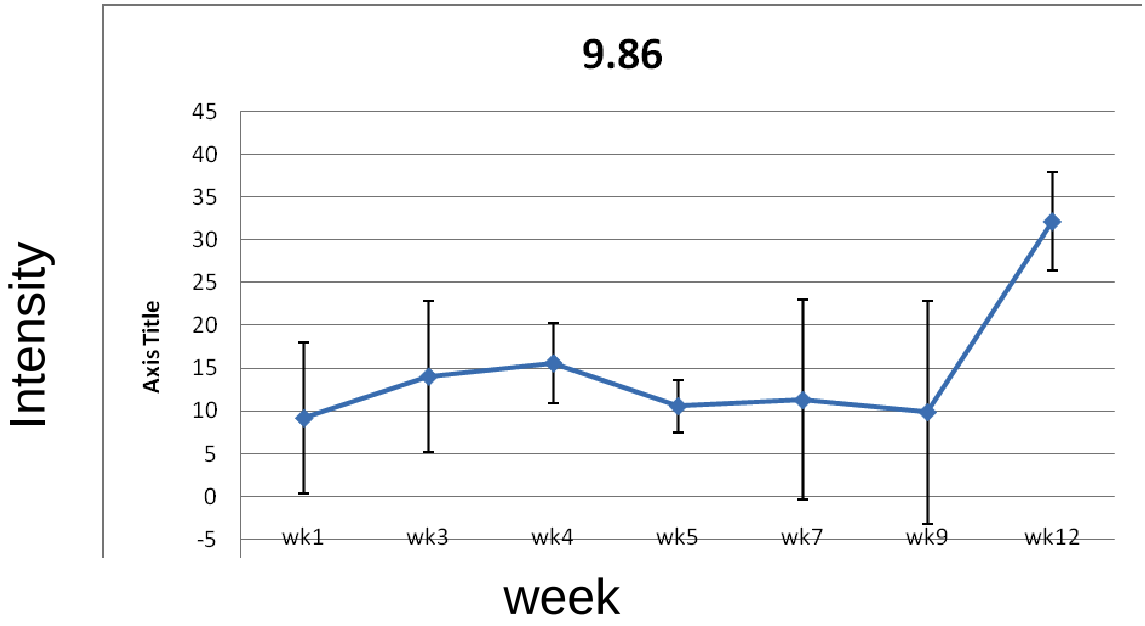
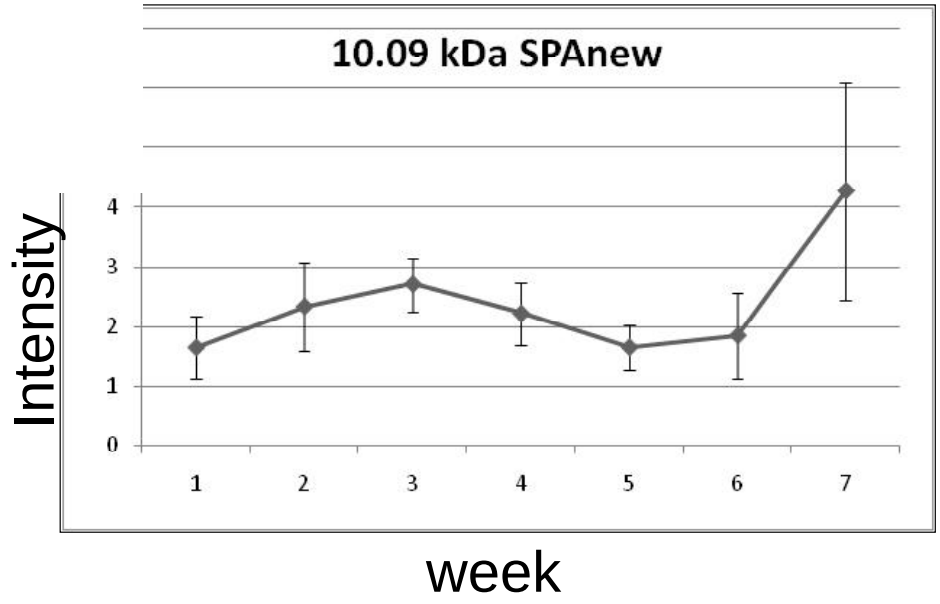



**Figure 5: Average intensity of a peak at 9.86 kDa .**The intensity of this peak was significantly lower 11 weeks post infection.

**a)**




**b)**

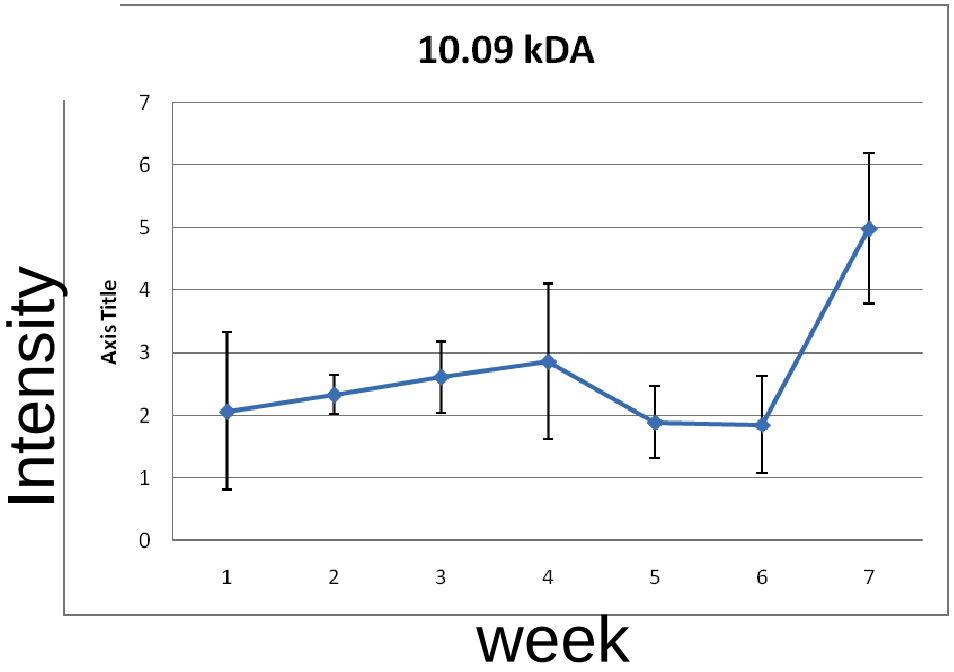



**Figure 6: Average intensity of a peak at 10.09 kDa .**a) profile obtained from PBSII reader 1; b) profile obtained from PBSII reader 2**.** The intensity of this peak was significantly higher 11 weeks post infection.

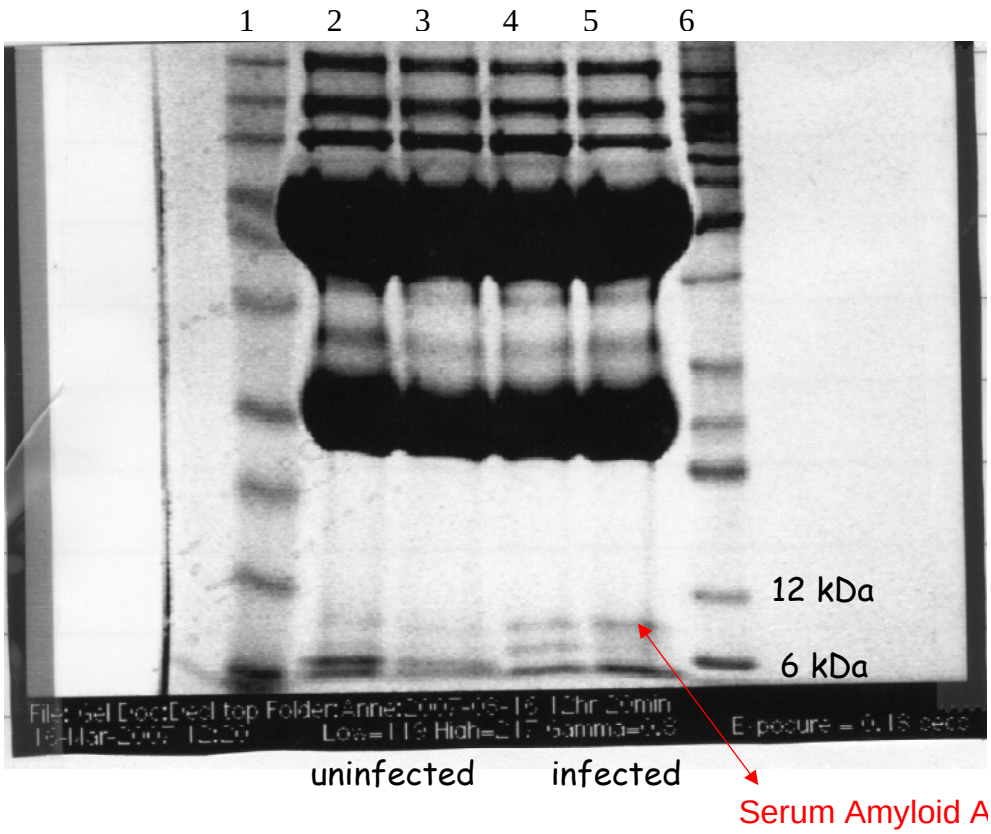



**Figure 7: 4-16% Bis-Tris gel of samples from 2 horses before and 11 weeks post infection.** Lanes 1 and 6 are the molecular mass markers. Lanes 2 and 3 are samples from horses before infection; lanes 4 and 5 are samples from horses 11 weeks post infection. The band marked by a red arrow was identified as serum amyloid A.