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Phosphorylated neurofilament H (pNF-H) as a potential diagnostic marker for neurological disorders in horses

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ABSTRACT

The current study aimed at the investigating the potential use of phosphorylated neurofilament H (pNF-H) as a diagnostic biomarker for neurologic disorders in the horse. Paired serum and cerebrospinal fluid (CSF) samples (n = 88) and serum only (n = 30) were obtained from horses diagnosed with neurologic disorders and clinically healthy horses as control. The neurologic horses consisted of equine protozoal myeloencephalitis (EPM) (38 cases) and cervical vertebral malformation (CVM) (23 cases). Levels of pNF-H were determined using an ELISA. The correlation between CSF and serum concentrations of pNF-H was evaluated using Spearman's Rank test and the significance of the difference among the groups was assessed using a nonparametric test. Horses had higher pNF-H levels in the CSF than serum. Horses afflicted with EPM had significantly higher serum pNF-H levels in comparison to controls or CVM cases. The correlation between CSF and serum pNF-H levels was poor in both the whole study population and among subgroups of horses included in the study. There was significant association between the likelihood of EPM and the concentrations of pNF-H in either the serum or CSF. These data suggest that pNF-H could be detected in serum and CSF samples from neurologic and control horses. This study demonstrated that pNF-H levels in serum and CSF have the potential to provide objective information to help in the early diagnosis of horses afflicted with neurologic disorders.

1. Introduction

The timely and accurate diagnosis of equine spinal cord disorders, such as equine protozoal encephalomyelitis (EPM), neuroaxonal dystrophy (NAD)/equine degenerative myeloencephalopathy (EDM), and equine motor neuron disease (EMND) could lead to early intervention and a more favorable prognosis. Although spinal ataxia and weakness appear to be the most common presentation of spinal cord disorders in horses, signs are variable and can mimic many other diseases (Furr et al., 2002). Current diagnostic methods are based mainly on the observation of overt clinical signs and confirmation with laboratory tests (Dubey et al., 2001; Reed et al., 2013) that are either invasive, i.e., biopsies of relevant tissues or cerebrospinal centesis to obtain samples of the cerebrospinal fluid (CSF), myelography or costly, i.e. magnetic resonance imaging (MRI). Additionally, clinical examination and aforementioned diagnostic testing occasionally do not confirm that neurologic disease exists. However, a definitive diagnosis may be occasionally determined by autopsy, and the ante mortem diagnosis of these conditions may remain a clinical challenge. Furthermore, those screening tests reflect exposure to the insult to the nervous system but not the amount of damage to the nervous tissues. Detection of specific markers for axonal damage such as phosphorylated neurofilament H (pNF-H) in serum and CSF are likely to reflect damage to neurons or axons, which would be reflected in the clinical signs of a particular disorder.

The neurofilaments (NFs) are the structural proteins of the neuron and are particularly concentrated in axons (Gresle et al., 2011). These proteins consist of at least three major subunits—light, medium, and heavy (NF-L, NF-M, and NF-H), which are released in relatively large amounts in the CSF or blood following neuronal and axonal degeneration. NFs are mostly synthesized within the cell body and travel along the axons to reach their sites of function (Yuan et al., 2012). Thus, detection of NFs as specific markers in serum and CSF has significant value for diagnostic or monitoring purposes (Norgren et al., 2003). Furthermore, NFs have been identified as marker for axonal damage (Trapp and Peterson, 1998; Newcombe, 1986; Donato, 2001; Petzold

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et al., 2002, 2003). Abnormal accumulations of NFs occur in many neurodegenerative diseases including motor neuron diseases and sensorimotor neuropathies. Few studies done in humans with neuropathy revealed that the NF proteins are the most reliable protein biomarker validated for quantification of neurodegeneration (Petzold, 2005; Teunissen et al., 2005). Among the three subunits, pNF-H is most resistant to calpain-mediated degradation. Hence, due to their properties and stability, pNF-H is an excellent target for antibody-based assays. To our knowledge, only three studies have assayed pNF-H in domestic animals; to measure the spinal cord injury in dogs (Nishida et al., 2014), hypoxic-ischemic encephalopathy in foals (Ringger et al., 2011) and equine grass sickness (Stradford et al., 2013). The aims of this study were to determine whether pNF-H would be detected in serum and CSF samples from neurologically afflicted horses; assess if afflicted horses had different concentrations than controls; and to examine whether there was a correlation between pNF-H levels in CSF and serum samples. Our overall hypothesis was that pNF-H would be detectable in serum and CSF from healthy horses and horses with neurologic conditions. The successful detection of pNF-H in serum samples would provide non-invasive and accurate biomarkers for neuronal and axonal degeneration.

2. Materials and methods

2.1. Study design and source of samples

We carried out a cross-sectional study to address the stated objectives. Inclusion criteria were any horses admitted to Cornell Equine and Farm Animal Hospital for evaluation of neurological disease during 2014 with paired CSF and serum samples and a thorough description of the neurological examination and confirmed diagnosis of either EPM or CVM. In addition, paired CSF and serum samples of confirmed EPM and CVM cases with details of the clinical examination and diagnostic imaging were obtained from Equine Diagnostic Solutions Laboratory[1] and Additional samples of EPM cases from New Bolton Center[2]. All the samples were frozen at − 80 °C until assayed. The horses were assigned to two groups; clinically normal horses as control (n = 57) and neurological group which further subdivided into EPM (n = 38) and CVM (n = 23). All the CSF samples were clear thus indicating absence of gross blood contamination.

2.2. Inclusion criteria

Horses that had clinical suspicion of neurologic disorder and confirmed with histopathological examination of the central nervous system consistent with EPM were included in this category. The criteria for a postmortem diagnosis of EPM included the presence of multifocal, asymmetric myelitis with or without encephalitis consisting of infiltrates of lymphocytes, macrophages, and occasional eosinophils forming wide cuffs of cells around blood vessels (Reed et al., 2013). In addition, horses with clinical suspicion of EPM and confirmation with evidence of intrathecal production of antibody for *Sarcocytis neurona* were also included in this group. Horses with clinical neurologic signs including ataxia and lack of coordination, myelogram showing compression, and/or postmortem examination demonstrating pathologic changes were included in the CVM category. Horse without evidence of clinical neurologic disease and with a confirm diagnosis that such as colic or other non-neurologic condition were included in the category of normal.

2.3. pNF-H assay

The pNF-H assay was performed using a sandwich ELISA[3] as described previously (Anderson et al., 2008). The antibodies used in this ELISA are specific for pNF-H from human and other mammalian species. Frozen serum and CSF samples were thawed prior to assay. The assay was performed according to the manufacturer's protocol. The standard curve was constructed by plotting mean absorbance of the standards provided by the manufacturer on a logarithmic scale. The concentration of each sample was calculated from the standard curve and the limit of detection was 0.058 ng/ml. Briefly, the chicken polyclonal antibodies generated against pNF-H are precoated onto 96-wells plate and are used to capture pNF-H from the samples. Captured pNF-H was detected using pNF-H specific rabbit polyclonal antibodies and a goat anti-rabbit alkaline phosphatase conjugate. After the addition of substrate solution the amount of pNF-H was determined. The standard curve demonstrates a direct relationship between optical density (OD) and pNF-H concentration. All samples were tested in duplicate, and average value of each sample was calculated. The person who performed the assays was completely blinded to the clinical information.

2.4. Statistical analysis

Measures of central tendency and dispersion were reported and stratified by the source (neurological or control group) of the sample. The significance of differences in the pNF-H concentration in serum and CSF samples in serum and CSF samples from the same horse was evaluated using the paired t-test. Differences in concentration of pNF-H, between horses afflicted with neurologic disorders and controls was evaluated using the two-sample t-test. Differences in mean values of pNF-H among neurologic disorders groups and controls were evaluated using Kruskal-Wallis one-way analysis of variance and Dunnett T3 as a post-hoc test for pairwise comparison. Spearman's ranked correlation was employed to evaluate the correlation between the CSF and serum concentrations of pNF-H. The relationship between serum or CSF levels and the likelihood of EPM was evaluated using the logistic regression analysis. All statistical analyses were performed using the SPSS v.23[4] and significance was considered at type-I error protection of $\alpha < 0.05$, unless stated otherwise.

3. Results

The inter- and intra-assay coefficients of variation for pNF-H in both serum and CSF samples were 8.6% and 5.9% respectively, which implies the reproducibility of the test. Our analyses showed that the pNF-H level was successfully detected in both serum and CSF from horses afflicted with EPM, CVM, and normal horses without clinical evidence of neurologic disease. Table 1 shows the descriptive statistics of the pNF-H concentration for each subgroup of horses by type of samples. The levels of the pNF-H in CSF tend to be significantly higher than those of the serum. There was high variability in the levels of these proteins within the different groups as indicated by the range values and the values were not normally distributed. Fig. 1A and B shows the frequency distribution of the number of horses by the CSF and serum levels of pNF-H. The majority of the controls had pNF-H levels of CSF and serum around 0 values and none of the controls had values > 2.0 ng/ml.

None of the CVM horses had serum pNF-H values > 4.77 ng/ml (Fig. 2). Serum levels of pNF-H varied by group. Horses afflicted with EPM had significantly higher serum levels of pNF-H in comparison to horses diagnosed with CVM and control group, however, horses afflicted with EPM had significantly higher levels than either controls or

[1] Equine Diagnostic Solutions, Kentucky, USA.
[2] New Bolton Center, Univ. of Pennsylvania, PA.
[3] EMD Millipore Corporation, Billerica, MA.
[4] IBM-Statistical Software, White Plains, NY.