

Serum Antibodies Against a Reactive Site of Equine Myelin Protein 2 Linked to Polyneuritis Equi Found in Horses Diagnosed with EPM

Siobhan P. Ellison, Pathogenes Inc.

Tom Kennedy, Eleven Bravo Inc.

Austin Li, Senior Scientist, Pathogenes Inc.

Lark Schweiss, Pathogenes Inc.

*Corresponding Author: Siobhan P. Ellison DVM PhD
15471 NW 112 Avenue,
Reddick, FL 32686
Phone: 352-591-3221
Fax: 352-591-4318
e-mail: sellison@pathogenes.com*

KEY WORDS: Equine protozoal myeloencephalitis, Polyneuritis equi, neurodegenerative disorder in horses, *Sarcocystis neurona*

ABSTRACT

Equine protozoal myeloencephalitis (EPM) is the most commonly diagnosed infectious neuro-degenerative condition in horses and neuroinflammation is present in all definitively diagnosed cases. Historically EPM is difficult to treat. Polyneuritis equi (cauda equina neuritis) is a rarely recognized non-infectious, chronic relapsing, peripheral neurodegenerative disorder in horses. We propose that polyneuritis equi may be associated with *Sarcocystis neurona* infections because we demonstrate the presence of antibodies against equine myelin protein and *S neurona* surface antigens in 78% (n=55) of serum from ataxic horses with a presumptive diagnosis of EPM. Serum antibodies against *S neurona* were detected in a horse with histopathologically confirmed immune mediated polymyositis and intramyofiber sarcocysts.

INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is the most commonly diagnosed infectious neuro-degenerative in horses. Little attention has been paid to the inflammatory component of disease despite the presence of neuroinflammation all definitively diagnosed cases of EPM. Histopathologic examination of a horse with EPM overwhelmingly reveals central nervous system (CNS) inflammation without the associated CNS presence of protozoa. It remains dogma that the clinical signs of EPM are due to presence of parasites in the CNS, and that the area of parasitization determines the signs observed.¹ It is more likely that clinical signs of EPM are due to a combination of parasitization and inflammation. Peripheral polyneuritis (polyneuritis equi) causes signs similar to EPM and is a rarely recognized, auto-immune, chronic relapsing, disorder in horses. We associate polyneuritis equi with *Sarcocystis neurona* infections by demonstrating the presence of serum antibodies against myelin proteins and *S neurona* surface an-

tigens in ataxic horses. Serum antibodies against *S neurona* were detected in a horse with histopathologically confirmed immune-mediated polymyositis and intramyofiber sarcocysts.

A large number of clinically normal horses have circulating antibodies to *S neurona*.¹ *Sarcocystis neurona* seropositive horses can have neurologic disease that is unrelated to *S neurona*. An unusual neurodegenerative disease with clinical signs similar to sarcocystosis is polyneuritis equi. Detection of anti-equine myelin P2 antibodies in horses with polyneuritis equi was first reported in 1981.⁵

⁶ The pathology and clinical finding of polyneuritis equi were initially described in 1897 in Germany, and has since been reported in Europe and the United States.⁷

Polyneuritis equi is a progressive, chronic, and often relapsing-remitting disease that affects the sacral and coccygeal nerves leading to paralysis of the tail, rectum, and bladder, with loss of sensation in the sacral dermatomes with surrounding hyperesthesia. This disease is often accompanied with cranial nerve paralysis, particularly the facial and trigeminal nerves. The cranial deficits may precede clinical signs associated with involvement of the sacral and coccygeal nerves. Clinical signs of polyneuritis equi can include behavior changes, ataxia, proprioceptive deficits, and possibly a side-winding gait.^{8,9} Antemortem diagnosis of polyneuritis equi by histopathology and circulating myelin protein serum antibodies have been described.^{6,8} Rostami and Gregorian indicated that anti-myelin serum antibodies may differentiate this T cell mediated peripheral neuritis from clinical signs associated with conditions that induce CNS lesions in horses.

The etiology of polyneuritis equi is unknown. The prevailing theory as to the

Figure 1. Amino acid sequence of MPP with putative epitope homology with *S neurona* and IL6 by colored lines beneath the sequence. The broken gray line above the sequence indicates non-disease inducing peptide and the solid black line indicates the putative T cell epitope for the clinical and histological disease.



Areas of amino acid homology between myelin protein peptide, *S neurona*, and equine IL6

Epitope	Amino acid sequence
SnSAG4	NTEISFKLG
SnSAG6	EFEETTAD
SnSAG5	SFKLG
SnSAG1	FEET
Equine IL6	TTAD

cause of polyneuritis equi is that it is immune mediated. Likely causes are an allergic neuritis in response to antigen released by trauma, infection, or a post-infectious allergic neuritis. Polyneuritis equi is comparable in its pathogenesis and lesions to the human Guillain-Barre syndrome and its laboratory model, experimental allergic (autoimmune) neuritis (EAN).⁷ Similar associations between anti-myelin proteins and disease are made in humans with multiple sclerosis (MS).

Sarcocystis neurona infections can result in signs of involvement of cauda equinae, thus the diseases look similar. A causal relationship between *S neurona* infections and polyneuritis equi has not been established. We hypothesize that immune reactions to *S neurona* infections may initiate inflammation that can develop into polyneuritis equi. Untreated neurologically abnormal horses may have serum antibodies against equine neurogenic myelin protein P2, (MP2), and *S neurona*.^{5,6,7,8,9}

Evidence suggests that levamisole can alter immune mediated disease and the clinical course of MS in humans.¹⁰ Levamisole HCl inhibits the production of IL6, a pleiotropic cytokine that can be pro-inflammatory, in mice, dogs, and humans.^{11,12,13}

¹⁴ A clinical response to levamisole HCl in horses with chronic, relapsing signs of EPM has been reported.² In this study, a clinical response to levamisole HCl was investigated.

MATERIALS AND METHODS

Sarcocystis neurona SnSAG 1, 5, 6 ELISA was performed as previously reported.¹⁵ A 22 amino acid peptide (myelin protein peptide, MPP) that corresponds to the neurotogenic site on equine myelin protein associated with peripheral demyelinating disease, Figure 1, was synthesized (United BioSystems, Herndon VA) and used as antigen (MPP). Antigen coating dilution was optimized using a checkerboard titration with equine sera positive for anti-myelin antibodies. Sera was assayed by indirect ELISA using a modification of the Fordyce procedure using MPP coated and blocked plates.⁶

The plates were incubated for 2 hours at 37°C, washed, and alkaline phosphatase conjugated anti-horse whole molecule (Sigma, St. Louis MO) at 1:3000 was added and incubated for 30 minutes at 37°C. The plates were washed and reacted with PMPP for 15 and 30 minutes. Plates were read on a Molecular Devices ELISA plate reader, the reciprocal of the last dilution positive (OD 0.518 or greater OD 405) was recorded as the titer. A positive titer was a value >8.

Positive Control Sera for MPP

One neurologically abnormal, 12-year-old, Warm Blood gelding (#10523) with serum antibodies against *S neurona* SAG 1, 5, 6 also had serum antibodies against MPP (titer 64) before any treatment. The horse was diagnosed with immune-mediated polymyositis and intramyofiber sarcocysts by histopathology (Oregon State University Veterinary Diagnostic Laboratory). Sera from this horse was used as a positive control in the assays.

Screen Sera for MPP Antibodies by ELISA

Sera from horses (172) with clinical signs of EPM and 10 neurologically normal horses

were tested by MPP ELISA.

Pooled sera from positive horses and sera from horse 10523 were used as a positive controls for each MPP ELISA.

Fifty five Sera from Ataxic Horses, pre- and post-EPM Treatment, Were Tested for SnSAG 1, 5, 6 and MPP Antibodies

Sera from horses that met study entrance criteria were selected for evaluation. Study entrance criteria included horses that were ataxic (grade 1-4), under a veterinarians care, treated with levamisole HCl (1mg/kg once daily for 10-24 days), an adjunct to anti-protozoal therapy, received a post-treatment exam, and had sera available for pre/post analysis. Horses were not included if there was no veterinarian exam, only one serum available, and horses had multiple therapies within 90 days. Fifty five neurologically abnormal horses were tested for serum antibodies against *S neurona* (SAG 1, 5, 6), C-reactive protein, and MPP by ELISA. Gait assessment scores (GAS) were assigned using the following criteria:

- GAS 0 no abnormalities;
- GAS 1 gait deficit just detected at a walk, drags limbs, circumducts limb(s) on tight circling, mild weakness noted on tail pull-easily pulled off track, but regains normal stride in 1-2 steps;
- GAS 2 gait deficit easily detected and exaggerated by backing, turning or neck extension, swaying at walk, displays a wide based stance after tight circling, weakness on tail pull-easily pulled off track, and does not return to normal stride for more than 3 steps.
- A GAS 3 was a gait deficit prominent on walking, turning or neck extension, unable to perform tight circles without falling backwards, weakness on tail pull easily pulled off track and almost falls, and doesn't regain normal stride.
- A GAS 4 was a horse that stumbles often, tripping spontaneously during exam, falls spontaneously and will fall

Table 1. Sera from 55 ataxic horses were examined for antibodies against *S. neurona* and myelin protein peptide (MPP). The majority of horses were positive for MPP.

	MPP + (78%)	MPP - (22%)
<i>S. neurona</i> SAG + (n=31, 56%)	24 (56%)	7 (58%)
<i>S. neurona</i> SAG - (n=24, 44%)	19 (44%)	5 (41%)

upon backing or tight circles, weakness on tail pull pulled off track, and could fall.

- A GAS 5 was a recumbent horse, unable to rise without assistance, may lean on a wall for support, spins to regain balance, and may have seizures.

The GAS assigned by a veterinarian were evaluated for success of treatment by evaluating a change in gait by the difference in score before and after treatment. Successful response to treatment was a change in GAS that was >0 and a treatment failure was a change in GAS that was < 0.

RESULTS

Screen Sera for MPP Antibodies by ELISA

One hundred seventy two sera were tested for serum MPP antibodies by MPP ELISA. Seventy seven of 172 (45%) sera obtained from neurologically abnormal horses were positive for antibodies (MPP ELISA titer > 8) establishing that MPP reactive antibodies are detected in some horses.

Fifty five Sera from Ataxic Horses, pre- and post-EPM Treatment, Were Tested for SnSAG 1, 5, 6 and MPP Antibodies

Thirty one (56%) of sera from 55 ataxic horses were seropositive for *S. neurona* and 24 (44%) were seronegative (Table 1). The majority of the horses (78%) were seropositive for MPP antibodies (Table 1). Twelve horses (22%) were MPP seronegative. Fifty-one sera were evaluated for serum C-reactive protein concentration (CRP) by capture ELISA. A serum CRP concentration >10 µgrams/ml was detected by capture ELISA in 66% (n=34) of the samples before treatment and a serum CRP concentration <10 µgrams/ml was detected in 33% (n=17)

of the samples (data not shown).

Successful response to treatment measured by a change in gait score was evident in 23 (86%) of horses that were positive by MPP ELISA and 25 (89%) of horses that were negative by MPP ELISA (Table 2).

DISCUSSION

Serum antibodies against *S. neurona* and MPP were detected in a horse with histologically confirmed polymyositis and intramyofiber sarcocysts. We detected antibodies against MPP in 45% of the serum samples obtained from ataxic horses in this study. We did not detect MPP antibodies in normal horse serum (data not shown). A slight majority of horses with antibodies against *S. neurona* were MPP positive in this study suggests that some of the clinical signs of EPM may be due to an autoimmune peripheral neuritis. Equine protozoal myeloencephalitis was suspected in horses with circulating *S. neurona* antibodies and an abnormal gait.

It is recognized, from molecular analysis and population genetics, that SnSAG1, SnSAG5, and SnSAG6 surface antigens will predominate in the *S. neurona* strains that

Table 2. A change in gait assessment score (GAS) was evaluated post treatment. A successful response to treatment was noted in the majority of horses. There was no difference between treatment successes in horses that were MPP seropositive versus MPP seronegative (MPP measured prior to treatment). This indicates that treatment was unassociated with location of neurological disease because MPP is detected in horses with peripheral neuropathy and not neuropathy in the central nervous system.

	MPP +	MPP -
(GAS) =0	4 (14%)	3 (11%)
(GAS) >0	23 (86%)	25 (89%)

circulate in nature and infect horses.¹ Highly specific bioassay using surface antigens SAG 1, 5, and 6 were useful to detect serum antibodies in horses with suspected EPM and were used in this study.^{12 13}

Generally, antibodies against *S neurona* and clinical neurological abnormalities are used to presumptively identify horses with EPM. Inflammatory lesions are often found in neural tissues postmortem from horses with EPM. Criteria for a histological diagnosis of EPM includes 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.¹⁴ The predominant microscopic lesion is reported as multifocal to coalescing areas of hemorrhage, nonsuppurative inflammation, and small foci of necrosis.¹ Perivascular cuffing by mononuclear cells can be evident in some areas. It is rare to find protozoa in neural tissues on post-mortem exam using immunohistochemistry, PCR, or culture. Polyneuritis equi is associated with the presence of equine myelin protein 2 auto-antibodies.^{5 6} Histological lesions associated with chronic polyneuritis equi are nonsuppurative inflammation of extradural nerve roots with perineural fibrosis and Wallerian degeneration. The inflammatory infiltrate can consist of lymphocytes, macrophages, and foci of neutrophils. Also, perineural fibrosis and perivascular lymphocytic cuffs with hemorrhage are found in affected areas.^{4 6} Demyelination is likely the primary pathophysiological event with degeneration and fibrous tissue replacement of affected nerves. No viral, bacterial, fungal, or protozoal agents are associated with the lesions in polyneuritis equi.

Several studies indicate that MPP is a reasonable assay antigen to detect peripheral neuropathy in the horse. Myelin proteins are largely conserved across species allowing comparison of studies from lab animals. Peripheral and CNS derived myelin P2 in the horse is practically identical to other myelin P2 binding structures.¹⁶ Induction of

experimental autoimmune neuritis (EAN) by whole peripheral nerve myelin P2 protein, or partial peptides derived from the whole protein, results in an inflammatory demyelinating disease in several species.¹⁷ The induction of EAN with myelin protein is dose-dependent.¹⁷ The neuritogenic site within the myelin protein was determined by inducing clinical and pathological signs of EAN in rats. A shorter synthetic peptide of myelin protein was ineffective at inducing pathological disease. The neuritogenic-containing myelin protein peptide is a logical choice for detecting anti-myelin antibodies in equine serum because this peptide contains the neuritogenic site that experimentally induces clinical signs of autoimmune neuritis.¹⁴

Anti-MMP antibodies are expected to preferentially detect peripheral neuropathies. Rats immunized with neuritogenic peptides of myelin protein develop extensive inflammatory demyelinating changes in ventral roots and sciatic nerves, but no central nervous system changes. Further, there was a positive correlation between the clinical severity of experimental disease, and the dose of the neuritogenic peptide in immunized rats. Lymph node cells from neuritogenic peptide-immunized rats generated reactive T cells specific for the MP2 peptide.¹⁷ These data demonstrated a cellular immune response by T helper cells to the peptide.

Rostami and co-workers concluded that the antigenic site for EAN induction in rats is located within the 53-78 amino acid residues of the myelin protein peptide and that the neuritogenic peptide may contain an important T cell epitope (8-11 amino acids) for the induction of experimental disease as shown by adoptive transfer of disease to naïve rats.¹⁸ They assert that at least one T cell epitope for the induction of disease resides within the 53-78 residues of myelin P2 protein, which may be the antigenic site for the disease process.¹⁸ Interestingly, they showed that the induced cellular immune response to the MP2 protein was milder than induced responses to the neuritogenic

peptide. The T cell line lost its proliferative activity to myelin protein activity, whereas the neuritogenic peptides activity was not affected on multiple challenges.

Based on the foregoing, it is logical to use the putative disease-inducing peptide to detect anti-myelin antibodies in horses with suspected peripheral neuropathy. The absence of MPP antibodies in some ataxic horses in this study may indicate that clinical signs are due to central neuroinflammation and not peripheral neuroinflammation. Detection of serum antibodies to MPP may differentiate a T cell mediated peripheral neuritis from clinical signs associated with conditions that induce central nervous system lesions in horses. However, it would be possible to have both central nervous system and peripheral neurodegenerative disease associated with EPM in the same animal.

Protozoa rarely remain in the CNS of horses that continue to exhibit progressive clinical signs of neurodegeneration, leading to speculation that the pathogenesis of disease is initiated by parasites, but disease progression is not parasite dependent. One may speculate as to the relationship of protozoa-mediated inflammation and cytokine mediated encephalomyelitis by molecular analysis. A molecular (BLAST, Basic Logical Alignment Tool NCBI) analysis of the amino acids 57-78 (MPP) of the neuritogenic peptide against the *Sarcocystis neurona* NCBI data bank reveals homology with amino acids from surface antigens (SnSAG) 4, 5, 6, 1 of *S neurona* and equine IL6. Surface antigens 6, 1, and equine IL6 show sequence identity to amino acids 69-77, 71-74, and 74-77, respectively. The sequence homology is within the neuritogenic region of the protein.

Based on our study results, homology with the reactive antigenic sites for myelin protein-associated autoimmune polyneuritis supports the association of the pathogenesis of neuritis in EPM horses with *S neurona* antigens. Further, our results support that IL6 may be the reactive T cell epitope in horses with autoimmune polyneuritis. Sur-

face antigen SAG 4, a conserved *Sarcocystis* surface antigen, is identical to amino acids of MPP at residues 60-68. This homology is within the non-disease inducing 61-72 residues of myelin protein. The homologous amino acids of surface antigen SnSAG 5 also is inside the non-reactive part of the peptide at residues 64-68. It remains to be determined if the three dimensional structure of myelin P2 protein grants significance for SAG 4 and SAG 5 antigens, or a combination of these antigens, in the inflammatory component of EPM. *Sarcocystis neurona* display major surface antigens during infection, and it is possible that these *S neurona* antigens or antibodies against them react with T cell epitopes that induce neurodegenerative disease in horses. A possible mechanism of the neuroinflammatory component of EPM syndrome can be speculated if clinically ill horses have serum antibodies against SAG 1, 5, 6 and MPP as we demonstrated.

Supporting the association of the mechanism of disease with equine cytokine IL6 is the clinical response to levamisole HCl because one known action of levamisole HCl is the inhibition of the production of IL6. (9) A clinical response was apparent in 48 of 55 treated horses in this study. It would be beneficial to differentiate horses with IL6 mediated peripheral neuropathies versus central neurodegenerative conditions because treatment may differ.

Our data supports that some, but not all horses may have peripheral neuritis and at least one etiology of polyneuritis equi may be *Sarcocystis neurona* infections (Table 1). It was not determined how long MPP antibodies remain in the successfully treated horses. Our data indicates that levamisole HCl may treat both peripheral and central neuroinflammation associated with protozoal infections. (12) There was little difference between horses with and without serum MPP antibodies that show a positive response to treatment (Table 2) indicating another condition, central neuroinflammation, may be present in some horses. Levamisole HCl

is potentially beneficial to alleviate clinical signs of peripheral neuritis equi associated with *S. neurona* infections, alleviating clinical signs in more than 85% of the treated horses (Table 2).

REFERENCE

1. An update on *Sarcocystis neurona* infections in animals and equine protozoal myeloencephalitis (EPM). Dubey JP, Howe DK, Furr M, Saville WJ, Marsh AE, Reed SM, Grigg ME. 2015, *J Parasitol*, pp. 637-43.
2. Circulating antibodies to the neuritogenic myelin protein 2, in neuritis of the cauda equina of the horse. Kadlubowski M, Ingram PL. 1981, *Nature*, Vol. 293, pp. 299-300.
3. Use of an ELISA in the differential diagnosis of cauda equina neuritis and other equine neuropathies. Fordyce S, Edington N, Bridges GC, Wriht JA, Edwards GB. 1987, *Equine Veterinary Journal*, Vol. 19, pp. 55-59.
4. Cauda Equina Neuritis: A Chronic Idiopathic Polyneuritis in Two Horses. Rousseaux CG, Fletcher KG, Clark EG, Naylor JM. 1984, *Can Vet J*, Vol. 25, pp. 214-218.
5. Antemortem diagnosis of polyneuritis equi. Aleman M, Katzman SA, Vaughn B, Hodges J, Crabbs TA, Christopher GC, Shelton GD, Higgins RJ. 2009, *J Vet Intern Med*, Vol. 23, pp. 665-668.
6. Differential diagnosis of polyneuritis equi. Vatisas N, Mayhew J. January 1995, In *Practice*, pp. 26-29.
7. Four year double-blind controlled study of levamisole in multiple sclerosis. Massaro AR, Cioffi RP, Laudisio A, Schiavino D, Maraini. 1990, *Italian Journal of Neurological Sciences*, pp. 595-9.
8. Levamisole enhances immune response by affecting the activation and maturation of human monocyte-derived dendritic cells. Chen L, Ln Y, Chiang B. 2008, *Clin Exp Immunol* 151, pp. 174-181.
9. Levamisole causes differential cytokine expression by elicited mouse peritoneal macrophages. Kimball E, Schneider CK, Fisher C, Clark C. 1992, *J Leukoc Biol Sept*. Vol. 52, pp. 349-356.
10. Levamisole induces interleukin-18 and shifts type 1/type 2 cytokine balance. Szeto C, Gillespie KM, Mathieson PW. 2000, *Immunology*, Vol. 100, pp. 217-224.
11. IL6 and TNF alpha production during active canine visceral leishmaniasis. de Lima v, Perio J, Vasconcelos R. 2007, *Vet Immunol Immunopathol*, pp. 189-193.
12. Decoquate combined with levamisole reduce the clinical signs and serum SAG 1, 5, 6 antibodies in horses with suspected equine protozoal myeloencephalitis. Ellison SP, Lindsay DS. 2012, *Intl J Appl Res Vet Med*, pp. 1-7.
13. Development of an ELISA to detect antibodies to rSAG1 in the horse. Ellison SP, Kennedy T, Brown KK. 2003, *J App Res Vet Med*, pp. 318-327.
14. Accurate Antemortem Diagnosis of Equine Protozoal Myeloencephalitis (EPM) Based on Detecting Intrathecal Antibodies against *Sarcocystis neurona* Using the SnSAG 2 and SnSAG 4/3 ELISAs. Reed SM, Howe DK, Morrow JK, Graves A, Yeorgan MR, Johnson AL, MacKay RJ, Furr M, Saville WJA, Willimas NM. 2013, *J Vet Intern Med*, pp. 1193-1200.
15. Structure of myelin P2 protien from equine spinal cord. Hunter DJB, Macmaster R, Roszak AW, Riboldi-Tunncliffe A, Griffiths IR, Freer AA. 2005, *ACTA Crystallographica*, pp. 1067-1071.
16. Induction of severe experimental autoimmune neuritis with a synthetic peptide corresponding to the 53-78 amino acid sequence of the myelinating P2 protein. Rostami A, Gregorian SK, Brown MJ, Pleasure DE. 1990, *Journal of neuroimmunology*, pp. 145-151.
17. Peptide 53-78 of myelin P2 protein is a T cell epitope for the induction of experimental autoimmune neuritis. SK, Rostami AM and Gregorian. 1990, *Cell Immunol*. pp.433-41.
18. Equine Herpes Virus-1 Consensus Statement. Lunn DP, Davis-Poynter N, Flaminio MJ, Horohov DW, Osterrieder K, Pusterla N, Townsend H. 2009, *J Vet Intern Med*, Vol. 23, pp. 450-461.
19. Prophylactic administration of ponazuril reduces signs and delays seroconversion in horses challenged with *Sarcocystis neurona*. Furr M, McKenzie H, Saville WJ, Dubey JP, Reed SM Davis W. 2006, *J Parasitol*, Vol. 92(3), pp. 637-43.
20. Clinical diagnosis of equine protozoal myeloencephalitis (EPM). Furr M, MacKay R, Granstrom D, Schott H, Andrews F. 2002, *J Vet Intern Med*, pp. 618-621.
21. Advancements in equine lyme disease testing. E, Larson. April 2015, *The Horse*.
22. Choroid plexus involvement in dogs with spontaneous visceral leishmaniasis: a histopathological investigation. Melo G, Machado G. 2009, *Brazilian Journal of Veterinary Pathology*, pp. 69-74.
23. Current therapeutic approaches to equine protozoal myeloencephalitis. Dirikolu L, Foreman JH, Tobin T. 2013, *JAVMA*, pp. 482-491.