2nd EPM Society Workshop

October 25 - 27, 2017
Granlibakken Resort
Tahoe City, California

The EPM Society
c/o 1501 Bull Lea Road,
Suite 104
Lexington, KY 40511

Committee

Stephen Reed
Society President,
Rood and Riddle Equine Hospital

Nicola Pusterla
Society Host Organizer,
University of California, Davis

Jennifer Morrow
Society Secretary/Treasurer,
Equine Diagnostic Solutions

Jenny Evans
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Martin Furr
Oklahoma State University

Daniel Howe
University of Kentucky

Amy Johnson
University of Pennsylvania

Robert MacKay
University of Florida

Sharon Witonsky
Virginia Tech

Index

Granlibakken Resort Information

Venue Address
Granlibakken Resort
725 Granlibakken Road
Tahoe City, CA 96145
530-583-4242

Badges
Badges will be provided by Granlibakken and can be picked up at the resort's registration desk. The badge should be worn at all times so the resort can identify that you are with the workshop. The workshop is in Mountain Room.

Internet
Free Wi-Fi is complimentary and does not require a password in the meeting spaces and common areas. In the hotel rooms each person will get individual Wi-Fi codes for their room upon check in.

Meals and Breaks
Breakfast, Thursday and Friday from 7:30 - 8:30 a.m., and Lunch, Thursday from 12:45 - 1:45 p.m., is in Granhall. The Morning Break (10:45 - 11:00 a.m.) and Afternoon Break (4:00 - 4:15 p.m.) on Thursday and Morning Break (10:00 - 10:15 a.m.) on Friday is in Mountain Room. Dinner on Wednesday (6:30 - 9:00 p.m.) and Thursday (7:30 - 10:00 p.m.) is in Cedar House.

Information for Speakers
A laptop and microphone will be available. Please have your presentations, if applicable, loaded prior to your scheduled session. Due to the tight schedule, we ask you to use the laptop provided and bring your presentation on a USB drive.

Sponsor Contact Information

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Program Overview

WEDNESDAY, OCTOBER 25		
6:30 - 9:00 p.m.	*Welcome Dinner in Cedar House*	
THURSDAY, OCTOBER 26		
7:30 - 8:30 a.m.	*Breakfast in Granhall*	
8:30 - 9:00	**Greetings, goals, and an overview of the 2014 EPM Workshop and the 2016 EPM consensus statement**	**Nicola Pusterla and Stephen Reed**
9:00 - 10:45	**Biology of *Sarcocystis neurona* and *Neospora hughesi***	
9:00 - 9:15	OVERVIEW	Daniel Howe
9:15 - 9:30	Size does matter: *Sarcocystis neurona* - The trickster	Antoinette Marsh
9:30 - 9:45	Seroprevalence of *Sarcocystis neurona* and *Neospora hughesi* among healthy equines in the United States	Kaitlyn James
9:45 - 10:00	*Sarcocystis fayeri* infection associated with neuromuscular disease in horses	Monica Aleman
10:00 - 10:15	Proportional morbidity rate (incidence) of equine protozoal myeloencephalitis (EPM) in North America	Frank Andrews
10:15 - 10:30	Molecular epidemiology of *Sarcocystis neurona* from land-to-sea: detection and molecular characterization in opossums and marine mammals from western Washington	Alice O'Byrne
10:30 - 10:45	BIOLOGY DISCUSSION	
10:45 - 11:00	*Morning Break in Mountain Room*	
11:00 - 11:30	**Genetics, Immunology and Vaccine**	
11:00 - 11:15	OVERVIEW	Sharon Witonsky
11:15 - 11:30	Identifying the immune phenotype in EPM horses	Sharon Witonsky
11:30 - 12:45	**Co-morbidity Between Apicomplexan protozoa**	
11:30 - 11:45	OVERVIEW	Martin Furr and Patricia Conrad
11:45 - 12:00	Immunological investigation of protozoal co-infection in horses with equine protozoal myeloencephalitis in the eastern United States	Sarah Schale
12:00 - 12:15	*Sarcocystis fayeri* associated anti-toxin in serum from horses with neuromuscular disease	Siobhan Ellison
12:15 - 12:30	*Toxoplasma gondii* seroprevalence and association with equine protozoal myeloencephalitis: a case-control study amongst California horses	Kaitlyn James
12:30 - 12:45	GENETICS, IMMUNOLOGY AND VACCINE AND CO-MORBIDITY DISCUSSIONS	
12:45 - 1:45	*Lunch in Granhall*	
1:45 - 4:00	**Laboratory Diagnostics**	
1:45 - 2:00	OVERVIEW	Jennifer Morrow and Amy Johnson
2:00 - 2:15	C-reactive protein and serum amyloid A in the diagnosis of equine protozoal myeloencephalitis and other equine nervous system diseases	Amy Johnson
2:15 - 2:30	Evaluation of serum amyloid A as a biomarker for EPM diagnosis	Stephen Reed

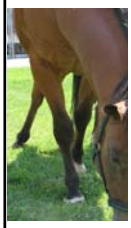
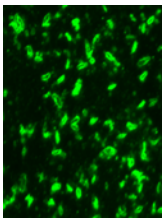
2:30 - 2:45	Comparison of specific antibody index and Goldmann-Witmer coefficient (C-value) to evaluate intrathecal immunoglobulin G production in equine protozoal myeloencephalitis	Amy Graves
2:45 - 3:00	Phosphorylated neurofilament H (pNF-H) as a potential diagnostic marker for neurological disorders in horses	Amy Johnson
3:00 - 3:15	Performance assessment of different diagnostic assays to identify EPM-affected horses in a clinical setting	Rodney Belgrave
3:15 - 3:30	Pathology of cases of equine protozoal myelitis submitted to the California Animal Health and Food Safety Laboratory between 1990 and 2016	Akinyi Nyaoke
3:30 - 3:45	Development and validation of four assays offered by Pathogenes	Siobhan Ellison
3:45 - 4:00	LABORATORY DIAGNOSTICS DISCUSSION	
4:00 - 4:15	*Afternoon Break in Mountain Room*	
4:15 - 5:15	**Relevance and Future Needs in the Field of EPM**	
4:15 - 4:30	OVERVIEW	Nicola Pusterla
4:30 - 4:45	Assessment of the diagnostic value of neurological signs in the clinical diagnosis of equine protozoal myeloencephalitis	Kaitlyn James
4:45 - 5:00	Standing cervical spinal tap: an alternative to standing lumbosacral CSF tap for EPM diagnosis	Pilar Camacho-Luna
5:00 - 5:15	RELEVANCE AND FUTURE NEEDS DISCUSSION	
5:15 - 7:00	**Treatment and Prevention**	
5:15 - 5:30	OVERVIEW	Stephen Reed and Rob MacKay
5:30 - 5:45	*Sarcocystis neurona* and antiprotozoal bumped kinase inhibitors	Daniel Howe
5:45 - 6:00	Diclazuril treatment ineffective at preventing *Sarcocystis neurona* induced myeloencephalitis relapse in established mouse model	Alayna Hay
6:00 - 6:15	Diclazuril nonlinear mixed-effects pharmacokinetic modeling of plasma concentrations after oral administration to adult horses every 3 to 4 days	Nicola Pusterla
6:15 - 6:30	Defining relapses attributed to equine protozoal myeloencephalitis update	Siobhan Ellison
6:30 - 6:45	Novel high-throughput screen of drug compound library identifies inhibitors of *Sarcocystis neurona* growth	Heather Fritz
6:45 - 7:00	TREATMENT AND PREVENTION DISCUSSION	
7:30 - 10:00 p.m.	*Dinner in Cedar House*	
FRIDAY, OCTOBER 27		
7:30 - 8:30 a.m.	*Breakfast in Granhall*	
8:30 - 10:00	**Keynote Talks: What if - A comparative approach to the Apicomplexan protozoal organisms**	
8:30 - 9:15	Patricia Conrad	
9:15 - 10:00	Jereon Saeij	
10:00 - 10:15	*Morning Break in Mountain Room*	
10:15 - 12:00	**Open discussion, project proposals, wrap-up and adjournment**	**Nicola Pusterla**

Abstracts Overview

Goals and Overview

Nicola Pusterla and Stephen Reed

Second EPM Society Workshop

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- Jenny Evans, University of Kentucky
- Martin Furr, Oklahoma State University
- Daniel Howe, University of Kentucky
- Amy Johnson, University of Pennsylvania
- Robert MacKay, University of Florida
- Sharon Witonsky, Virginia Tech

Sponsors

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- Equus Standardbred Station, Inc.
- Gluck Equine Research Center
- Grayson Jockey Club Research Foundation
- IDEXX Laboratories
- Merck Animal Health
- PRN Pharmacal
- UCD, Davis, Center for Equine Health
- UCD, Davis, Pritchard Veterinary Medical Teaching Hospital

EPM Workshop - 2014

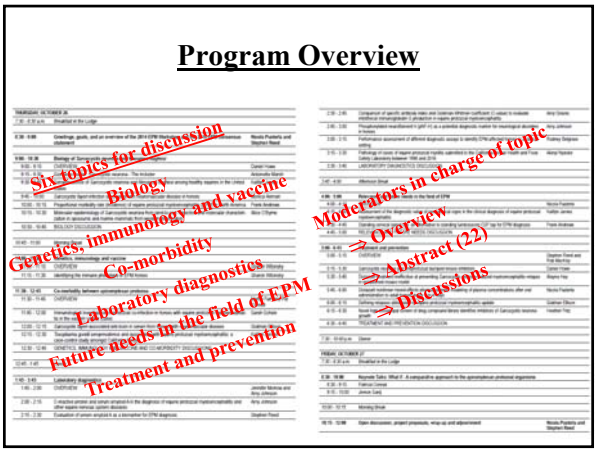
- 45 attendees (academia, private practice, research field, pharmaceutical industry, diagnostic field)
- Goal to better understand EPM, share information, identify unresolved areas and promote collaborative research
- Overviews, abstracts and discussions
  - Biology, genetics
  - Infectious model
  - Immunology, vaccine
  - Laboratory diagnostics
  - Treatment, prevention

EPM Workshop - 2014

- Considerable progress in biology, genome and life cycle of *S. neurona*, epidemiology, pathogenesis of EPM, diagnosis of both *S. neurona*/*N. hughesi* and treatment
- Identified fields in need of answers
  - Effect of parasite genotype on pathogenesis
  - Horse, intermediate versus accidental host
  - Role of immune response in protection and disease
  - Contribution of co-morbidity (co-infection)
  - Expand fundamental knowledge on *N. hughesi*

Updated EPM Consensus Statement

- First consensus statement focused on clinical diagnosis (2002)
- Updated statement (2016)
  - Solid foundation of parasite biology and disease pathogenesis
  - Epidemiology and risk factors
  - Emphasis on clinical diagnosis
    - Clinical signs compatible with EPM
    - Ruling out other neurological diseases
    - Immunodiagnostics on serum and CSF
  - Treatment recommendations
  - Prevention

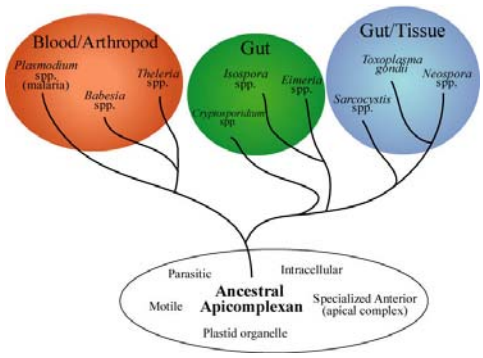

Program Overview
Six topics for discussion
Biology
Genetics, immunology and vaccine
Co-morbidity
Laboratory diagnostics
Future needs in the field of EPM
Treatment and prevention
Moderators in charge of topic
Overview
Abstract (22)
Discussions

Parasite Biology

Sarcocystis neurona and
Neospora hughesi

Daniel K. Howe
Department of Veterinary Science
University of Kentucky

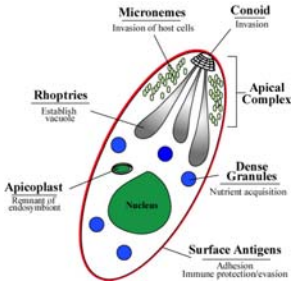
Phylum Apicomplexa



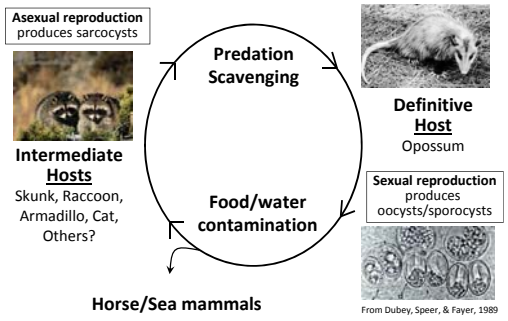
Ultrastructure of an Apicomplexan

- Eukaryotic organisms
- Possess a "toolkit" of specialized organelles and virulence factors
  - Mostly novel molecules
  - Candidate targets for:
    - Chemotherapeutics
    - Diagnosis
    - Vaccines

Cartoon apicomplexan cell (zoite)



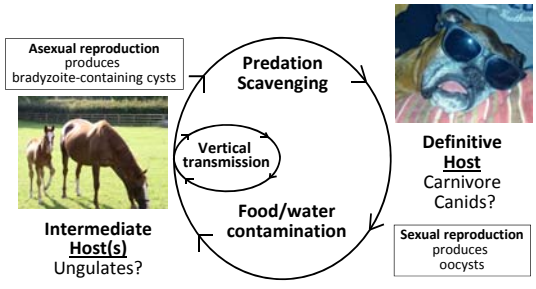
Sarcocystis neurona Life Cycle



Neospora hughesi Life Cycle

?

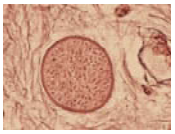
Neospora hughesi Life Cycle?



Tissue Cysts/Bradyzoites

- Quiescent stage
- Infection source for definitive host
- Cyst recrudescence
  - Neospora - Yes
  - Sarcocystis - No

Dubey, Speer, & Fayer, 1989



Dubey, Hemphill, Calero-Bernal & Schares, 2017

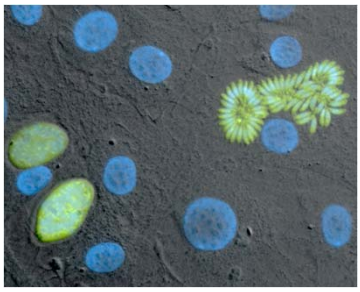
Oocysts/Sporocysts/Sporozoites

- Passed in feces of definitive host
- Environmentally resistant
- **Fundamental mode of *S. neurona* transmission to horses**

Dubey, Speer, & Fayer, 1989

Dubey, Hemphill, Calero-Bernal & Schares, 2017

Comments? Questions? Additions?



Sarcocystis neurona expressing YFP in monolayer of bovine turbinate cells (DAPI nuclear stain)

BIOLOGY OF *SARCOCYSTIS NEURONA AND NEOSPORA HUGHESI*

1
SIZE DOES MATTER: *SARCOCYSTIS NEURONA*-THE TRICKSTER

Antoinette E. Marsh, Michelle Carman, Daniel K. Howe, William J. Saville, Stephen M. Reed

College of Veterinary Medicine, Ohio State University, Columbus, OH; University of Kentucky Gluck Equine Research Center, Lexington, KY; Rood and Riddle Equine Hospital, Lexington, KY.

Sarcocystis neurona is an evasive parasite, taking at least 6 different life stage forms, infecting terrestrial animals such as horses and marine mammals and sea otters. Intensive studies on the *S. neurona* life cycle span over two decades. Long-term maintenance of the *in vivo* cycle of *S. neurona* in a research setting poses many challenges. These studies are expensive, time consuming and require maintaining animal colonies linked to breeding cycles and annual seasons. Thus, we wanted a system whereby we could manipulate the life cycle through both *in vitro* and *in vivo* studies, obtaining specific stages of the parasite, suspending the life-cycle at different points, and cryopreserving stage-specific parasites until needed. Our study aimed to determine if equine-derived *S. neurona* merozoites cultivated in vitro could be used to produce infective sarcocysts in the intermediate host. A raccoon-derived isolate, SN744, produced tissue sarcocysts in raccoons, including a large sarcocysts seen in the brain, following parasite inoculation with culture-derived merozoites. However, parallel experiments using an equine-derived isolate showed differences. We demonstrated that SN-MU1 (Missouri-derived *S. neurona* isolate from a horse with EPM) could form tissue sarcocysts in a raccoon although the sarcocysts were significantly smaller, less frequent, and challenging to detect. These results differ from earlier studies reporting that an EPM isolate failed to produce sarcocysts in either cats or raccoons following inoculation with culture-derived parasites. The retrospective analysis of tissues from earlier experimental inoculation studies were evaluated with the focus to detect small (<50 um) sarcocysts. We determined that an equine-derived isolate, SN-UCD1 did produce small cysts in an inoculated raccoon but not in two cats. These results are consistent with the sarcocysts reported in naturally infected sea otters. These results are the first to demonstrate the procedure to take two, geographically and antigenically distinct EPM isolates, cultivate the merozoites and schizonts *in vitro*, inoculate these stages into a laboratory animal and produce morphologically similar small sarcocysts distinguishable from the larger sarcocysts produced by the SN744 (SN37R) isolate. Our results also highlight the importance of immunohistochemistry staining for detecting the small sarcocysts that can be missed due to their size and lack of associated inflammation.

The authors acknowledge Duncan Alexander for the financial gift to support this work as well as NCI Cancer Center Support Grant P30 CA016058 for the shared Comparative Pathology resources at the Ohio State University.

2
SEROPREVALENCE OF *SARCOCYSTIS NEURONA* AND *NEOSPORA HUGHESI* AMONG HEALTHY EQUINES IN THE UNITED STATES

Kaitlyn James, Woutrina Smith, Patricia Conrad, Andrea Packham, Leopoldo Guerrero, Mitchell Ng, Nicola Pusterla

Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA; Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA.

Equine protozoal myeloencephalitis (EPM), an infectious neurologic disease of horses, is caused by two parasites, *S. neurona* and *N. hughesi*. The objective of this cross-sectional study was to describe the general seroprevalence of *S. neurona* and *N. hughesi* infection among healthy horses using an indirect fluorescent antibody test, as well as determine potential risk factors (geographic region, breed, use, gender, and age) for seroprevalence. Whole blood from 5,250 horses was collected across 18 states in October 2013, along with risk factor information. An indirect fluorescent antibody test was used to determine antibody titers to the two protozoal parasites, and mixed effects logistic regression models were created to determine prevalence odds ratios. The overall seroprevalence of *S. neurona* and *N. hughesi* in horses was 78% and 34%, respectively. Thirty one percent of horses were seropositive to both *S. neurona* and *N. hughesi* and 18% were seronegative to both parasites. Horses from the South, of Warmblood breed, and of older age were associated with seropositivity to *S. neurona*. There was no significant difference in *N. hughesi* seroprevalence across the country, but Warmblood breed and increasing age were associated with seropositive animals. Implications of these results are contemporary knowledge on the background

infection rates and geographic distribution of the two causative agents of EPM in horses. Increasing age as a risk factor for *Sarcocystis* and *Neospora* exposure is expected but the findings of breed significance and the differing geographical distributions for *S. neurona*, but not *N. hughesi*, deserve further investigation.

3
SARCOCYSTIS FAYERI INFECTION ASSOCIATED WITH NEUROMUSCULAR DISEASE IN HORSES

Monica Aleman, Karen Shapiro, Silvia Siso, John E. Madigan, Sam Crosby, Diane C. Williams, Daniel Rejmanek, Beatriz Aguilar, Patricia A. Conrad

School of Veterinary Medicine, University of California, Davis, CA.

Sarcocystis fayeri-induced toxicity causing muscle and intestinal sarcocystosis has been reported in humans consuming raw horse meat [1,2]. Clinical manifestations include intermittent or chronic myalgia, myositis, muscle wasting, arthralgia, fatigue, headache, bronchospasm, rashes, facial swelling, fever, cardiomyopathy, and glomerulonephritis [1]. Sarcocysts in skeletal muscle of equids has been commonly regarded as an incidental finding. However, there have been isolated case reports of muscle sarcocystosis and anecdotal descriptions of horses with unexplained gait deficits and neuromuscular disease of undetermined etiology responding to the treatment with antiprotozoal drugs [3]. Also, experimental infection of *S. fayeri* in ponies produced clinical manifestations of disease in one study [4]. Further, the observation of encysted parasites in horses' skeletal muscle with neuromuscular disease by one of the authors (MA), prompted the authors to investigate the prevalence and molecular characterization of *Sarcocystis spp.* infection in equids. For comparison of findings, healthy horses were used as controls. Our findings indicated that *Sarcocystis fayeri* infection was common in young mature equids with neuromuscular disease and could be associated with myopathic, neurogenic, and mixed (myopathic and neurogenic) processes [5]. The number of infected muscles and number of sarcocysts per muscle were significantly higher in diseased than in control horses. *Sarcocystis fayeri* was predominantly found in low oxidative highly glycolytic myofibers. This pathogen had a high glycolytic metabolism. Common clinical signs of disease included muscle atrophy, weakness with or without apparent muscle pain, gait deficits, and dysphagia in horses with involvement of the tongue and esophagus. Horses with myositis were lethargic, apparently painful, stiff, and reluctant to move. Similar to humans, sarcocystosis and cardiomyopathy can occur in horses. Similar clinical signs of progressive muscle wasting, weakness, and lethargy in horses with granulomas, eosinophilic and plasmacytic-lymphocytic myositis associated with *Sarcocystis spp.* have been reported by others [6,7]. Although our study did not establish causality but a possible association (8.9% of cases) with neuromuscular disease; the assumption of *Sarcocystis spp.* being an incidental finding in every case might be inaccurate. A more recent study by others found the presence of *S. fayeri* antitoxin in serum from horses with neurologic disease [8]. Further studies are needed to determine the role of *S. fayeri* infection in the development of neuromuscular disease in horses.

References

1. Fayer R, Esposito DH, Dubey JP. Human infections with Sarcocystis species. Clin Microbiol Rev 2015;28:295-311.
2. Kamata Y, Saito M, Irikura D, et al.A toxin isolated from *Sarcocystis fayeri* in raw horsemeat may be responsible for food poisoning. J Food Prot 2014;77:814-819
3. Traub-Dargatz JL, Schlipf JW Jr, Granstrom DE, et al. Multifocal myositis associated with *Sarcocystis sp* in a horse. J Am Vet Med Assoc 1994;205:1574-1576.
4. Fayer R, Dubey JP. Development of *Sarcocystis fayeri* in the equine. J Parasitol 1982;68:856-860.
5. Aleman M, Shapiro K, Siso S, et al. *Sarcocystis fayeri* in skeletal muscle of horses with neuromuscular disease. Neuromuscul Disord 2016;26:85-93.
6. Herd HR, Sula MM, Starkey LA, et al. *Sarcocystis fayeri*-induced granulomas and eosinophilic myositis in 2 related horses. Vet Pathol 2015;doi:10.1177/03009815584073:1-4.
7. Cawthorn RJ, Clark M, Hudson C, et al. Histological and ultrastructural appearance of severe *Sarcocystis fayeri* infection in a malnourished horse. J Vet Diagn Invest 1990;2:342-345.
8. Ellison SP, Li Austin. *Sarcocystis fayeri* associated anti-toxin in serum from horses with neuromuscular disease. Intern J Appl Res Vert Med 2016;14:152-158.

4

PROPORTIONAL MORBIDITY RATE (INCIDENCE) OF EQUINE PROTOZOAL MYELOENCEPHALITIS (EPM) IN NORTH AMERICA

Frank Andrews, Agricola Odoi, Sharon Witonsky, Carla Sommerdahl

Equine Health Studies Program, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA; University of Tennessee, College of Veterinary Medicine, Knoxville, TN; Virginia Maryland Regional College of Veterinary Medicine, Blacksburg, VA.

EPM is a serious neurologic disease in horses. The objectives of this study were to: (a) assess temporal changes in the proportion of cases reported to veterinary teaching hospitals in North America and (b) assess the perception of veterinary practitioners regarding incidence of EPM. Medical records were extracted from the Veterinary Medical Data Base (VMDB) from 1990-2015. Proportional morbidity rate (PMR) of EPM was computed and compared across breeds and years. An online survey of veterinary practitioners was conducted from January 10, 2016 to March 13, 2016 to assess equine practitioner perceptions regarding incidence of EPM. The PMR was 0.70% (1,823/263,862). There was a significant decrease in PMR observed from years 2009 to 2015 of the study. Standardbreds (1.59%), Walking Horses (1.37%), and Thoroughbreds (1.31%) had significantly ($P<0.05$) higher PMR compared to the PMR for all breeds. The majority (63%) of practitioners thought that the incidence of EPM had not changed (44%) or increased (19%) in the previous 2-4 years. Other than 1996-1998, PMR for EPM in horses did not change from 1990 through 2007. However, for the past 7 years, the number of EPM cases presented to veterinary teaching hospitals has significantly decreased, noted by a decrease in PMR. This was in contrast to the practitioners' perceptions where a majority of them thought cases were staying the same or increasing. Also, it appears that Standardbreds, Walking Horses, Thoroughbreds, and geldings are more likely to be diagnosed with EPM. The data suggest that practitioners are treating EPM in the field and fewer cases are being referred to veterinary teaching hospitals. However, the data should be interpreted with caution as reporting of EPM cases through the VMDB is dependent on the veterinary teaching hospitals.

5

MOLECULAR EPIDEMIOLOGY OF *SARCOCYSTIS NEURONA* FROM LAND-TO-SEA: DETECTION AND MOLECULAR CHARACTERIZATION IN OPOSSUMS AND MARINE MAMMALS FROM WESTERN WASHINGTON

Alice O'Byrne, Dyanna Lambourn, Daniel Rejmanek, Brittany Dalley, Katherine Haman, Elizabeth Vanwormer, Andrea Packham, Patricia Conrad, Karen Shapiro

Pathology, Microbiology and Immunology, University of California, Davis, CA; Washington Department of Fish and Wildlife, Lakewood, WA; UCD School of Veterinary Medicine, University College Dublin, Belfield, Dublin, Ireland.

Increasing reports of marine mammal deaths attributed to the parasite *Sarcocystis neurona* have been published in recent decades. Infected opossums, the only known definitive hosts, shed environmentally robust *S. neurona* sporocysts in their faeces [1]. Sporocysts can contaminate the marine environment via overland runoff [2], and their subsequent ingestion by marine mammals can lead to fatal encephalitis [2]. The aim of this study was to determine the prevalence of *S. neurona* in opossums from western Washington and to compare genetic markers between *S. neurona* in opossums and marine mammals. Thirty-two fresh road kill opossums were collected along western Washington, and 27 brain samples from marine mammals for which the cause of death was suspected to be protozoal encephalopathy were provided by the Washington Department of Fish and Wildlife. Following amplification of the ITS1 gene, three opossums (9.7% prevalence) and twelve marine mammals (40.7% prevalence) were confirmed to be positive for *S. neurona*. Positive cases were further molecularly characterized at two markers, sn7 and snSAG3. Genetic identity of *S. neurona* was demonstrated among one marine mammal and two opossums. These preliminary results support the hypothesis that sporocysts shed from opossums can contaminate the marine environment via overland run off, resulting in marine mammal infections. Investigations that identify specific temporal and spatial parameters associated with *S. neurona* infections in opossums may further assist targeted mediation strategies for reducing the burden of illness in susceptible hosts.

The authors would like to acknowledge funding from FVE/Merck and the STARS program at UC Davis for the opportunity to pursue this project.

References:

1. Dubey, J., Saville, W., Lindsay, D., Stich, R., Stanek, J., Speer, C., Rosenthal, B., Njoku, C., Kwok, O., Shen, S. and Reed, S. (2000). Completion of the Life Cycle of *Sarcocystis neurona*, Journal of Parasitology, 86(6), pp.1276-1280.
2. Rejmanek, D., Miller, M., Grigg, M., Crosbie, P. and Conrad, P. (2010). Molecular characterization of *Sarcocystis neurona* strains from opossums (Didelphis virginiana) and intermediate hosts from Central California. Veterinary Parasitology, 170(1-2), pp.20-29.

Updates on: Genetic bias, Immunology and Vaccines for EPM

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OCTOBER 26TH, 2017

Genetic bias associated with EPM

- Increased incidence of EPM due to *S. neurona (Boy et al. 1990).*
- Thoroughbreds
- Standardbreds
- Quarter horses
- No breed associations reported with *Neospora hughesi*.

[redacted]

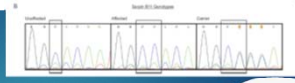
Genome wide association study

- Gaubatz MS thesis 2013 (Howe)
- Methodology: screen the whole equine genome for differences between 2 traits/phenotypes
- Single nucleotide polymorphisms (SNPs): mutations in gene, either help identify that another gene and associated function may be altered or less likely is an altered gene and associated function affecting specific phenotype.
- Case definition: UK VDL EPM horses examined histopathology to identify samples with *S. neurona* present (H&E and possibly Immunohistochemistry (IHC))
- 36 EPM horses
- No stated controls

GWAS (Gabatz et al., 2013)

- Samples: formalin fixed paraffin embedded tissue: cut 5 scrolls of 5um sample
- DNA extracted
- Analyzed samples using Equine SNP50 BeadChip (Illumina): 54, 602 SNPs distributed across all 31 autosomes, average probe spacing 43.23kb
- Average call rate used for quality control
- Samples from 24 horses sent for DNA extraction
- Issues with how long samples had been fixed in formalin affected ability to read SNPs on chip: 5 samples, call rate >88% so >88% SNPs read; those samples fixed <24 months
- No difference source of DNA (i.e., liver, spleen, tongue, etc).
- Results: inconclusive

Review of literature



- Finno et al. 2015: identification of frameshift variants in Connemarra ponies with hoof wall separation disease (HWSD)
- Genome wide association analysis: n=15 affected, n=24 controls
- Identified region on chromosome 8 of affected vs. controls
- Family of SERPINB genes in this region
- Next generation sequencing of 2 affected and 2 unaffected
- Identified 9,758 single nucleotide variants (SNVs) of which 363 segregated with HWSD cases vs. controls
- Of the 363, 16 were located in annotated genes or <700bp from ATG
- One additional SNV between SerpinB2 and Serpin B10
- Gene associated SNVs genotyped using larger set
- Sequencing identified 1230 small insertions and deletions (indels): 28 segregated with disease, 11 were within or close (<700bp) to genes.
- Of the 11 indels, 6 were intronic, 1 was coding and 4 were up or downstream; those were genotyped

Table 1. Variants segregating with HWSD phenotype.

Genomic Location	Variant Type	Effect	gDNA	cDNA	Protein
chr8:80111598	1 bp-insertion	Frameshift *SERPINB11*	g.80111598_80111599insC or: g8883_8884insC	c.504_505insC	p.Thr169Hisfs*3
chr8:80259666	SNV	Downstream; 1431bp from *SERPINB2* and upstream; 2596 bp from *SerpinB10*	g.80259666T>C	N/A	N/A
chr8:80319671	12 bp-insertion	Upstream; 691bp from *SerpinB8*	g.80319671_80319683insTGAAAAATAAAT	N/A	N/A
chr8:80319673	4 bp deletion	Upstream; 677bp from *SerpinB8*	g. 80319673del	N/A	N/A

Four variants were unique to the 23 affected Connemara ponies and heterozygous in 27 obligate carriers.

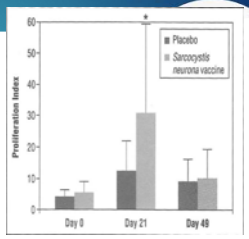
Gene	Expression	Std. Error	P(H1)	Result
B2M	1.000	-	-	-
Serpin B2	0.704	0.080 – 2.861	0.482	Not Different
Serpin B8	0.542	0.222 – 1.515	0.054	Not Different
Serpin B10	0.825	0.170 – 3.753	0.665	Not Different
Serpin B11	0.064	0.010 – 0.274	≤0.000	DOWN-Regulated

Genetics, Immunology and Vaccine

Sharon Witonsky

Vaccine development

- No currently commercial efficacious vaccines available
- Previous vaccine: meta-stim adjuvant, UCD-1 isolate (Ft. Dodge) (Marsh et al., 2004)
- Vaccine induced: seroconversion delayed hypersensitivity reaction
- No protection studies performed



Vaccine development: SnSAG1

- 5 horses vaccinated rSAG1
- 5 horses vaccinated adjuvant
- All horses challenged SAG1 expressing strain.

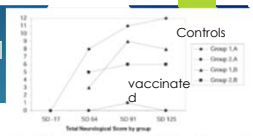
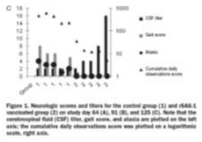


Table IV. Serum neutralization titers pre- and post-vaccination. Pre- and post-vaccine sera were evaluated for the ability to neutralize Sarcocystis neurona infections in vitro. The last dilution of serum showing no parasite growth in 50% of wells containing irradiated mouse fibroblasts was considered the $TCID_{50}$. The lowest dilution was 1:4 for all sera tested

Horse ID	Pre-vaccine $TCID_{50}$	Post-vaccine $TCID_{50}$
1	< 4	32
3	< 4	64
6	< 4	128
7	< 4	64
9	< 4	32



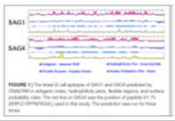
Ellison and Witonsky, 2009

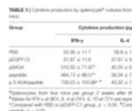
What is needed vaccine development

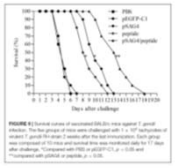
- Confirming predicted protective immune response: M1 DC1, CD4 Th1 CD8 IFN-gamma: Macrophage 1 , Dendritic cell1, T-helper-1 CD4 T-cytotoxic
- Identify immunodominant antigens
- Adjuvant?
- Development and testing of a vaccine

Progress on vaccine development for toxoplasmosis

- Vaccines based on antigens which stimulate humoral and CMI (SAG4, SAG5b, SAG5c, SAG4 vs vaccines based on virulence factors
- SAG4 based vaccine (Zhou and Wang, 2017)
- DNA based vaccine
- Challenge against RH strain in Balb/c mice
- Partial protection



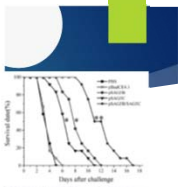


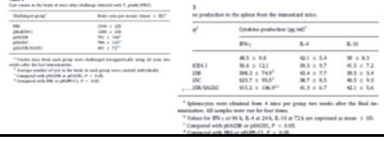


SAG5B and SAG5C combined vaccine protects mice against *Toxoplasma gondii* infection

Gang Lu[a], Jian Zhou[a,b], Aihua Zhou[a], Yali Han[a], Jingjing Guo[a], Pengxia Song[d], Huaiyu Zhou[a], Hua Cong[a], Ming Hou[a], Lin Wang[a,c,*], Shenyi He[a,c,**]

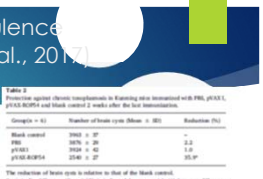
- 5 different subtypes of SAG, SAG5A-5E expressed in bradyzoites and tachyzoites
- Prediction of B and T-cell epitopes
- Recombinant DNA plasmids
- Vaccinate and challenge RH strain

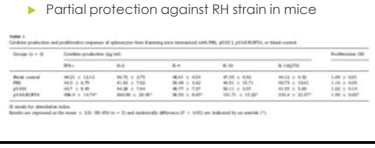


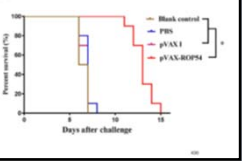


Candidate vaccines against virulence factors for toxoplasma (Yang et al., 2017)

- Virulence particles (ROP54) rhoptry effector protein, which affects the ability of toxoplasma to infect
- Disruption of ROP54 altered virulence in mice
- DNA based vaccine induced mixed Th1 and Th2 response
- Partial protection against RH strain in mice







Immune system in protection and disease

- No immune system, no horse!
- Predicted protective immune response

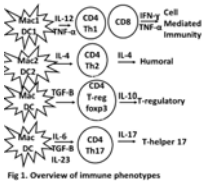


Fig 1. Overview of immune phenotypes

Host pathogen balance

Natural seroprevalance >50%

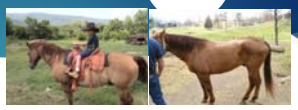
0.5-1% of horses get disease

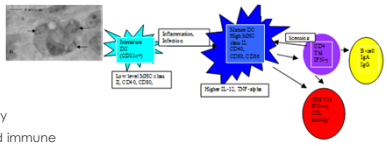
Factors

- Stress (shipping, showing, training, pregnancy)
- Season
- Location
- Age
- Other diseases

Role Cell Mediated Immunity

EPM horses have decreased immune responses



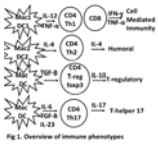


Evidence for protective immune response

- Mouse models: Infected B6 mice develop increased CD4 and CD8 memory response (Witonsky et al., 2003)
- B-cells are not needed for protection (uMT) (Witonsky et al.. 2008?)
- CD8 cells and IFN-gamma needed for protection (Witonsky et al., 203b, 2005)

Non-protective immune response in EPM: equine

- Decreased antigen specific responses in EPM horses (Tornquist et al. 2001).
- Increased IL-4 expression, decreased IFN-gamma expression, decreased SAG-1 proliferation (Spencer et al., 2004, 2005).
- Decreased proliferation to phorbol myristate acetate and ionomycin in naturally and experimentally infected horses (Yang et al., 2006; Witonsky et al., 2008).
- Decreased antigen specific responses, most likely due to decreased antigen presenting cell function (Lewis et al., 2014).
- Increased number CD3 T-cells present in lesions in CNS of EPM affected horses (Scott et al., 2003).



What information is needed:

- Confirming the predicted protective immune response in horses
- Identifying the immune response in horses that develop EPM

GENETICS, IMMUNOLOGY AND VACCINE

6
IDENTIFYING THE IMMUNE PHENOTYPE IN EPM HORSES

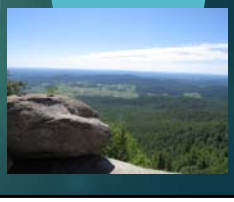
Alayna Hay, Caroline Leeth,Tanya LeRoith, Kevin Lahmers,Tom Cecere, David Lindsay, Frank Andrews, Fabio del Piero, Amy Johnson, Bettina Wagner, Steve Reed, Martin Furr, Nicola Pusterla, Rob MacKay, Tom Divers, Savannah Weatherford, **Sharon Witonsky**

College of Veterinary Medicine/Animal and Poultry Science, Virginia Tech, Blacksburg, VA; Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA; University of Pennsylvania, School of Veterinary Medicine, Kennett Square, PA; College of Veterinary Medicine, Cornell University, Ithaca, NY; Rood and Riddle Equine Hospital, Lexington, KY; Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK; School of Veterinary Medicine, University of California, Davis, CA; College of Veterinary Medicine, University of Florida, Gainesville, FL.

There are gaps in our understanding the mechanisms why only 0.5-1% of exposed horses develop Equine Protozoal Myeloencephalitis (EPM), as well as what the role of the causative agent, *Sarcocystis neurona* (*S. neurona*) in disease. Based on these gaps, our overall goal of this study is to: Identify the immune response within the central nervous system (CNS) of EPM affected horses, and its association with *S. neurona* infection. We hypothesized that: EPM affected horses do not develop a protective CD4+ Th1 CD8+ interferon gamma (IFN-γ) response, and *S. neurona* is associated with the development of histopathological changes. We are accomplishing these goals through the following aims: Aim 1: Determine the local immune response in the CNS of EPM affected horses, and Aim 2: Determine the frequency at which *S. neurona* is present in the CNS lesions of EPM affected horses. Our case definition for our study, is horses that have clinical signs consistent with EPM and are positive for *S. neurona* antibodies in the cerebrospinal fluid (CSF) are positive. Horses that do not have clinical signs of EPM, and have normal neurologic exams and test negative for antibodies in the CSF serve as control subjects. EPM positive horses are being divided into treated and untreated groups to determine the effect of treatment. The study is ongoing. Based on the data that we have gathered thus far, histopathologically, horses that are acutely affected appear more likely to have acute inflammation vs. horses with more recurrent signs have had degenerative changes with some evidence of previous inflammation. Immune phenotyping based on serum and CSF cytokines as well as immunohistochemistry (IHC) staining is being performed. *S. neurona* detection based on IHC staining and PCR is being performed. A summary of our results to date will be presented.

Co – morbidity among apicomplexan parasites

EPM SOCIETY MEETING
OCTOBER 25, 2017



Survival and growth of infectious protozoa in immunocompetent hosts

- Outrun the host response by fast replication or mutation
- Impair development of expression of immunity in the host (immune evasion or restriction)

The result:

- A "non-sterilizing" immunity which reduces parasite burden and limits pathological damage without wiping out the invader

Modulation of immune effects

- Cleavage of Fc – bound IgG by lysosomal enzymes of the parasite (Schistosoma)
- Elaboration of parasite endopeptides which suppress IL-1 expression by host cells
- Antigen switching and antigenic mimicry
- Suppression of antigen specific immune responses by parasite proteins (Trypanosoma and Toxoplasma)

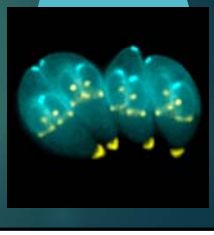
Protozoan Co-infections – *Toxoplasma gondii*

- *T gondii* induces a highly polarized Th1 response
  - Due to upregulation of IL-12
- Immune suppression induced is stage specific
  - Acute vs chronic toxoplasmosis inhibits resistance to Leishmania infection
  - Acute vs chronic toxoplasmosis reduces tissue damage in Leishmania infected mice
  - No effect upon Th-1 mediated response to Leishmania infection in Toxoplasma infected mice

Santiago, et al Infection Immunity 67 (9)

Protozoan co-infections ("polyparasitism")

- Common in humans
  - May be the "norm" rather than the exception
- In areas where protozoan infections are common
  - 10.4% of children harbor 2 protozoan infections
  - 57% harbor 3 infections
- Reflects
  - Exposure
  - Nutritional status
  - Health co-morbidities
  - Poor access to health care



Co-infections in cats

- Strong association between FeLV, FIV, and Leishmania infection
- Strong association between FeLV and Toxoplasma infection

Sobrinho et al, Veterinary Parasitology 187 (1-2)

FeLV and toxoplasma:

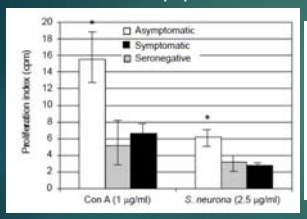
- 57% of FeLV (+) infected with toxoplasma
- 2% FeLV (–) infected with toxoplasma

Witt et al. JAVMA 1989

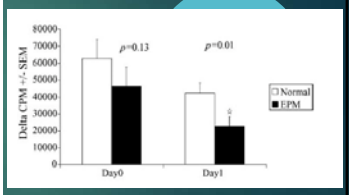
Co-morbidity Between Apicomplexan protozoa

Martin Furr and Patricia Conrad

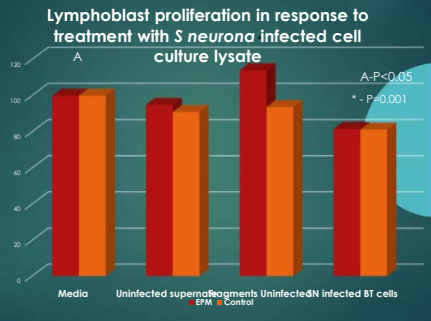
Sarcocystis –mediated immunosuppression



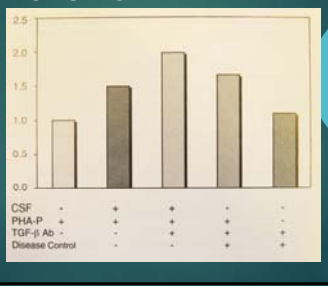
Tornquist, et al 2001



Yang et al 2006



Immunosuppresive microenvironement



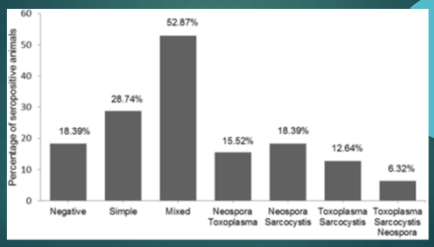
Furr and Pontzer, 2001

Sarcocystis and Neospora hughesi co-infections in 3123 horses

Serologic Status	Percent seropositive
Neospora hughesi only positive	38 (1.2%)
Sarcosystis neurona only positive	840 (26.8%)
Neospora and Sarcocystis both positive	25 (0.8%)
Neospora and Sarcocystis both negative	2220 (71%)

Pusterla, et al The Veterinary Journal 2016

Protozoal Co-infections in horses



Cazarato et al, 2016 Microbial Pathogenesis

Impact and relevance in equine disease?

- Is Sarcocystis infection a cause of immunosuppression allowing secondary infections with other organisms?
- To what degree does this immunosuppression limit treatment efficacy?
- Are other parasitic, bacterial, or viral co-morbidities influencing EPM susceptibility?
- Do infection with various strains of the *S neurona* or *N hughesi* influence disease susceptability or clearance of organism ?

CO-MORBIDITY BETWEEN APICOMPLEXAN PROTOZOA

7
IMMUNOLOGICAL INVESTIGATION OF PROTOZOAL CO-INFECTION IN HORSES WITH EQUINE PROTOZOAL MYELOENCEPHALITIS IN THE EASTERN UNITED STATES

Sarah Schale, Daniel Howe, Michelle Yeargan, Jennifer Morrow, Amy Graves, Amy L. Johnson

College of Veterinary Medicine, Oregon State University, Corvallis, OR; University of Kentucky Gluck Equine Research Center, Lexington, KY; Equine Diagnostic Solutions, LLC, Lexington, KY; New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, PA.

Sarcocystis neurona is the primary causative agent of equine protozoal myeloencephalitis (EPM), although disease due to *Neospora hughesi* also occurs. Polyparasitism is linked with increased disease severity in marine mammals with encephalitis. The primary aim of this study was to assess whether horses with EPM due to *S. neurona* also had evidence of exposure to *N. hughesi* or *T. gondii*. Inclusion criteria included neurologic disease, antemortem, and/or postmortem diagnosis of EPM or CVSM, and availability of serological results or archived samples for testing. Antemortem diagnosis of EPM was based on SnSAG2, 4/3 serum: CSF titer ratio ≤50 and exclusion of other diseases. Antemortem diagnosis of CVSM was based on positive myelographic results and exclusion of other diseases, in addition to SnSAG2, 4/3 titer ratio ≥100. One hundred one horses were included: 49 with EPM (48 due to *S. neurona* and 1 due to *N. hughesi*) and 52 with CVSM. Horses with EPM were more likely than horses with CVSM to have positive immunologic results for *S. neurona* on serum (95.9% vs. 76.9%), CSF (98.0% vs. 44.2%), and serum: CSF titer ratio (91.8% vs. 0%). However, positive results for *Neospora* and *Toxoplasma* were uncommon, with total seroprevalence rates <15% in the study population for both parasites. The proportions of EPM cases testing positive for *Neospora* and *Toxoplasma* were not different from the proportions of CVSM cases testing positive. These results do not indicate a substantial role for polyparasitism in EPM in the eastern US.

8
SARCOCYSTIS FAYERI ASSOCIATED ANTI-TOXIN IN SERUM FROM HORSES WITH NEUROMUSCULAR DISEASE

Siobhan Ellison and Austin Li

Pathogenes Inc., Reddick, FL; Fairfield, FL.

Sarcocystosis in horses can be due to *Sarcocystis neurona* or *S. fayeri* each with different clinical outcomes. It is generally believed that *S. fayeri* infection does not cause inflammation in equine tissues. The purpose of this study was to evaluate the seroprevalence of *S. fayeri* cyst-toxin (SFt) antibodies in horses with neuromuscular disease and associate neuromuscular disease with inflammation measured by serum C-reactive protein (CRP). Serum CRP was quantitated in 248 normal, untreated horses with a median value of 17 µg/ml (0-99 µg/ml). Thirty-five clinically normal, SAG 1 seronegative horses were vaccinated with SnSAG1 recombinant protein and seven horses were sham vaccinated with adjuvant on day 0 and day 21. One month post-vaccination SAG1-vaccinated horses seroconverted against SAG1, but not SFt, while sham vaccinated horses remained seronegative to both antigens. A female 9-year-old Warmblood showing signs of neuromuscular disease underwent muscle biopsy (Devaney CA), muscle cysts were present and she was diagnosed with clinical sarcocystosis. Treatment with ponazuril did not change clinical presentation and she continued to decline. Post-ponazuril she was seronegative for *S. neurona* and seropositive for SFt. Ponazuril was discontinued and treatment was initiated with decoquinate/levamisole with an improvement in gait. The mare resumed training. Decoquinate treatment was continued and the horse was monitored for changes in clinical signs (ataxia, weakness, muscle atrophy) quarterly. After daily therapy for 6 months the horse was seronegative for SFt and remained clinically normal. ELISA testing using *S. neurona*-specific and SFt as antigens were used to determine the seroprevalence of *Sarcocystis* antibodies in normal and diseased horses. Reactive SFt antibody was present in 24% of sera collected from 42 clinically normal horses. This study showed that antibodies against *Sarcocystis* antigens were found in sera from normal and diseased horses. Exposure detected by antibody to both *Sarcocystis* species in normal (87%) and diseased (74%) horses were more common than the presence of single species antibody. Single reactivity to SFt was detected in 61.5% of normal and 37% diseased animals. Single reactivity to *S. neurona* was detected in 61.5% of normal and 48% of diseased horses. *Sarcocystis neurona* seropositive sera was more often associated with neuromuscular disease when compared to SFt positive sera, however horses with antibodies against both antigens were more likely to show neuromuscular disease than those with single infections. When a cut-off for normal serum CRP concentration

was less than 16 µg/ml, an elevated CRP (72.6%) was detected in most horses that showed clinical signs. Significantly more horses with neuromuscular disease had an elevated CRP when compared to normal horses (P = 0.0135) using Fisher's exact test. This study suggests that *S. fayeri* cyst protein is associated with neuromuscular disease in some horses and may be associated with inflammation that is detected with serum CRP levels.

9

TOXOPLASMA GONDII SEROPREVALENCE AND ASSOCIATION WITH EQUINE PROTOZOAL MYELOENCEPHALITIS: A CASE-CONTROL STUDY AMONGST CALIFORNIA HORSES

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While toxoplasmosis is not commonly considered a clinical disease of equines, previous seroprevalence studies have produced differing background rates of *Toxoplasma gondii* infection in horses globally. The objective of this study was to evaluate the epidemiologic association between *T. gondii* seroprevalence and horses with equine protozoal myeloencephalitis (EPM) clinical signs. Using a case-control study design, 720 resident California horses with neurologic signs compatible with EPM were compared to healthy, non-neurologic horses for the presence of *T. gondii* antibodies (via indirect fluorescent antibody tests [IFAT]). *Toxoplasma gondii* seroprevalence among cases and controls was determined at standard serum cut-offs: 40, 80, 160, 320, and 640. At a *T. gondii* titer cut-off of 320, horses with clinical signs compatible with EPM had 3.55 times the odds of a seropositive test compared to horses without clinical signs (P-value < .01) when adjusted for covariates. When restricted to the fall season and at the same titer cut-off, an EPM suspect horse had even higher odds, 6.4 times the risk for testing seropositive to *T. gondii*, compared to non-neurologic horses. The association of high *T. gondii* titers and clinical signs compatible with EPM is potentially reflective of toxoplasmosis in equines. Serologic testing of CSF and isolation of *T. gondii* in EPM suspect cases should be considered for future studies on the relationship between *T. gondii* and EPM.

2017 EPM Workshop Laboratory Diagnostics

Tahoe City, CA

Moderators:
Jennifer Morrow
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2016 ACVIM Consensus Statement

- 3 recommendations for diagnosis of EPM
  1) Neurologic exam to confirm clinical signs
  2) Exclusion of other potential diseases
  3) Immunodiagnostic testing of serum and CSF
     - Confirm intrathecal antibody production

Current IgG tests

- No known changes since 2014
- *S. neurona*
  - IFAT
  - SAG2, 4/3 ELISA
  - SAG1, 5, 6 ELISA
- *N. hughesi*
  - IFAT
  - SAG1 ELISA

General differences between the current IgG tests*

- The *S. neurona* strain used for the test
- Test format
- Dilution factor of samples as tested
- Validation samples and gold standard samples used
- Validation
- Recommended samples for testing
- Result interpretation
- Sensitivity and specificity
- Quality control measures

*slide modified from 2014 EPM Workshop

Commercially available immunologic tests for antibodies against *S. neurona*
**modified from 2016 ACVIM consensus statement, Table 1*

Test	Laboratory	Interpretation	Reported performance			References
			Sample	Sensitivity (%)	Specificity (%)	
WB[1]	EDS UC Davis	• Band pattern read and interpreted visually (subjective) • Results usually reported as negative, weak positive, low positive, or positive	Serum CSF	89[2], 80[3], 89[4], 90[5] 89[2], 87[3], 83[5]	71[2], 38[3], 87[4], 42[5] 89[2], 44[3], 86[5]	1. Granstrom *et al*. 1993 2. Granstrom 1997 3. Daft *et al*. 2002 4. Duarte *et al*. 2003 5. Morrow (pers. comm. 2014)
mWB[6]	Michigan State	• Similar to standard WB (above)	Serum	100[6], 89[4]	98[6*], 69[4] (**n.b., negative cases not from North America*)	6. Rossano *et al*. 2000
IFAT[4]	UC Davis	• Serum positive at ≥1:80 has ≥55% probability* of EPM • Serum negative at ≤1:40 has ≤33% probability* of EPM • CSF positive at ≥1:5 has 92% probability* of EPM	Serum CSF Serum:CSF titer ratio	89[4], 83[7], 94[9], 59[10] 100[7], 92[9], 65[10] 65[10]	100[4], 97[7], 85[9], 71[10] 99[7], 90[9], 98[10] 98[10]	7. Duarte *et al*. 2004 8. Duarte *et al*. 2006 9. Johnson *et al*. 2010 10. Johnson *et al*. 2013
SAG2, 4/3 ELISA[11]	EDS	• Serum positive for exposure at ≥1:250 • CSF correlates well with EPM if ≥1:40 • Serum:CSF titer ratio very predictive of EPM if ≤100	Serum CSF Serum:CSF titer ratio	30-86 (depending on cutoff)[12], 71[10] 77-96 (depending on cutoff)[12], 88[10] 86 (cutoff ≤50) or 93 (cutoff ≤100)[12], 88[10]	37-88 (depending on cutoff)[12], 50[10] 58-96 (depending on cutoff)[12], 86[10] 96 (cutoff ≤50) or 83 (cutoff ≤100)[12], 100[10]	11. Yeargan and Howe 2011 12. Reed *et al*. 2013
SAG1, 5, 6 ELISA[13]	Pathogenes	• Serum positive at ≥1:8, indicating infection	Serum	N/A	N/A	13. Ellison & Lindsay 2012

Commercially available immunologic tests for antibodies against *N. hughesi*
**modified from 2016 ACVIM consensus statement, Table 3*

Test	Laboratory	Interpretation	Reported performance	References
IFAT	UC Davis	• Serum positive at ≥1:320; negative at <1:40 • CSF positive at ≥1:5	• Serum Se 100%, Sp 100% at cutoff of 1:640 • Serum Se 100%, Sp 71% at cutoff of 1:320 • Se and Sp estimates calculated using samples from experimentally infected horses, not EPM cases	Packham *et al*. 2002
ELISA	EDS	• Serum positive at ≥1:500 • CSF positive at ≥1:5 • Serum:CSF titer ratio provides most accurate EPM diagnosis	• Serum Se 94%, Sp 95% compared to WB detection antibodies (not EPM cases)	Hoane *et al*. 2005

Test comparisons, focusing on EPM caused by *S. neurona – part 1*

**modified from 2016 ACVIM consensus statement, Table 2*

Reference	Tests (and samples) compared	Sample origin	Results	Author conclusions
Duarte *et al*. 2003 *J Vet Diagn Invest*	• WB (serum) • mWB (serum) • IFAT (serum)	• Necropsy cases (9 positive, 39 negative)	• Similar Se (89%) for all 3 • Variable Sp (IFAT 100%, WB 87%, mWB 69%)	IFAT accuracy was better than WB tests.
Saville 2007 *ACVIM forum EPM SIG*	• WB (serum) • mWB (serum) • IFAT (serum) • SAG1 ELISA (serum)	• Experimental cases (1 *S. neurona* positive, 1 *S. fayeri* positive, 2 negative) • Clinical cases (3 positive, 10 negative) • Necropsy case (1 positive)	• Variable for each case; limited agreement between tests	WB and IFAT were most accurate, though IFAT was cross-reactive with *S. fayeri*. mWB tended to have false positive results while SAG1 ELISA tended to have false negative results.
Johnson *et al*. 2010 *J Vet Intern Med*	• IFAT (serum, CSF) • SAG1 ELISA (serum)	• Necropsy cases (9 positive, 17 negative) • Clinical cases (10 positive, 29 negative)	• Marked difference in Se (IFAT serum 94%, IFAT CSF 92%, SAG1 ELISA serum 13%) • Comparable Sp (IFAT serum 85%, IFAT CSF 90%, SAG1 ELISA serum 97%)	Low Se limited the usefulness of the SAG1 ELISA.

Test comparisons, focusing on EPM caused by *S. neurona – part 2*

**modified from 2016 ACVIM consensus statement, Table 2*

Reference	Tests (and samples) compared	Sample origin	Results	Author conclusions
Reed *et al*. 2010 *ACVIM forum*	• WB (CSF) • IFAT (serum) • SAG1 ELISA (serum) • SAG2, 4/3 ELISA (serum:CSF ratio)	• Necropsy cases (7 positive, 5 negative) • Clinical cases (6 positive, 2 negative)	• Variable Se (SAG2, 4/3 ELISA 90%, WB 90%, IFAT 70%, SAG1 ELISA 55%) • Variable Sp (SAG2, 4/3 ELISA 100%, WB 95%, SAG1 ELISA 90%, IFAT 85%)	SAG2, 4/3 ELISA serum:CSF ratio was the most accurate.
Renier *et al*. 2012 *ACVIM forum EPM SIG*	• IFAT (CSF) • SAG2, 4/3 ELISA (serum:CSF ratio)	• Necropsy cases (6 positive, 17 negative) *(n.b., 1 positive case due to N. hughesi not S. neurona)*	• IFAT Se (100%) higher than SAG2, 4/3 ELISA Se (83%) • SAG2, 4/3 ELISA Sp (100%) higher than IFAT Sp (82%)	IFAT advantages include testing for *N. hughesi* and use as serum stand-alone test.* *(n.b., SAG2, 4/3 ELISA serum:CSF ratio had higher overall accuracy.)* *These "advantages" are currently not considered accurate.
Johnson *et al*. 2013 *J Vet Intern Med*	• IFAT (serum, CSF, serum:CSF ratio) • SAG2, 4/3 ELISA (serum, CSF, serum:CSF ratio)	• Necropsy cases (11 positive, 28 negative) • Clinical cases (6 positive, 14 negative)	• SAG2, 4/3 ELISA serum:CSF ratio was most accurate (97%) • IFAT CSF and serum:CSF ratio also had high accuracy (88%)	Serum testing alone was least accurate; more accurate methods should be used. SAG2, 4/3 ELISA serum:CSF ratio was most accurate.

Summary test comparisons*

- Serum tests less accurate
  - Generally due to low specificity
  - SAG1 showed poor sensitivity
    - no longer commercially available
- Poor to fair test agreement
- SAG2, 4/3 ratio most accurate (3/6 studies)
  - Compared to WB, SAG1, IFAT
- No comparison studies for SAG1, 5, 6

*slide modified from 2014 EPM Workshop

EPM Workshop 2014 – Diagnostic testing

Questions for discussion part one (for existing Sn and Nh IgG tests):

- Diagnostic value of serum only
- When to recommend *N. hughesi* testing
- Sequential testing – usefulness for diagnosing an active infection or for evaluating effectiveness of treatment
- Effect of blood contamination of CSF on testing and how is blood contamination of CSFs evaluated for each test
- What is effect of previous treatment (within 1-4 months prior to workup) on testing

EPM Workshop 2014 – Diagnostic testing

Questions for discussion part two (other tests):

- Knowledge about biomarkers such as serum amyloid A, C reactive protein, heavy chain neurofilament, and anti-myelin protein P2
- Using the SAG1, 5, 6 peptide ELISA, what is the frequency of mixed infections
- Is there evidence for SAG6 strains infecting horses in nature
- Are there any other tests being developed and should there be

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LABORATORY DIAGNOSTICS

10
C-REACTIVE PROTEIN AND SERUM AMYLOID A IN THE DIAGNOSIS OF EQUINE PROTOZOAL MYELOENCEPHALITIS AND OTHER EQUINE NERVOUS SYSTEM DISEASES

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Accurate antemortem diagnosis of EPM can be challenging and requires evidence of intrathecal antibody production (e.g. SnSAG2, 4/3 serum: CSF titer ratio < 100). Some advocate the use of acute phase protein measurements in addition to serology, which alone results in substantial false positives. The purpose of this pilot study was to determine if C-reactive protein (CRP) and serum amyloid A (SAA) were elevated in cases of EPM compared to other neurological diseases. Serum and CSF CRP and SAA were measured for 25 cases of clinical equine neurologic disease: EPM (10), cervical vertebral stenotic myelopathy (CVSM) (10), neuroborreliosis (2), equine motor neuron disease (1), degenerative myelopathy (1), and leukoencephalomalacia (1). Nine of 10 EPM cases had a SnSAG2, 4/3 titer ratio < 25. The untested EPM case was confirmed postmortem, as were 4 other EPM cases. Serum CRP was above reference range in only 1 EPM case (14.4 mg/L; reference <0.1-10 mg/L). No EPM cases had elevated serum SAA. Cerebrospinal fluid CRP and SAA also failed to differentiate cases of EPM (CRP median 3.35 mg/L, range 0.19-13.43 mg/L; SAA median 0.1 mg/L, range <0.1-2.4 mg/L) from CVSM (CRP median 4.015 mg/L, range 0.16-9.62 mg/L; SAA median 0.62 mg/L, range <0.1-2.91 mg/L). No consistent relationships between SnSAG 2, 4/3 antibody levels and serum CRP or SAA were detected nor was there a relationship between the two acute phase proteins in cases of EPM. Results from this pilot study suggest that neither SAA nor CRP in serum or CSF aid diagnosis of EPM.

These findings have been reported as an oral abstract at the 2017 ACVIM forum. The authors would like to acknowledge Dr. Carolyn Cray and the Acute Phase Protein Laboratory at the University of Miami for assisting with this project.

11
EVALUATION OF SERUM AMYLOID A AS A BIOMARKER FOR EPM DIAGNOSIS

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Equine Protozoal Myeloencephalitis (EPM) is an important disease of horses. It is often considered challenging to diagnose due to association with an array of varying clinical symptoms. In ante-mortem cases, the condition is only considered accurately diagnosed through identification of intrathecal antibody production. Serum Amyloid A (SAA), a major acute phase protein in the horse, is an important early indicator of infectious and inflammatory disease with a marked elevation in systemic conditions. Its success as a biomarker of systemic conditions promotes it for consideration in compartmentalized disease such as EPM where it is less well characterized. The aim of this study was to evaluate the use of SAA in EPM by determining if an increase is observed in serological samples associated with EPM positive cases. A total of 101 serum samples were included in the study consisting of 49 EPM positive samples from 40 horses and 52 negative cases. EPM diagnoses were either known necropsy confirmed (T=7) or diagnosed through a serum:CSF SnSAG2, 4/3 titre ratio of < 100 (T=33). SAA concentrations were determined for all samples and the results were used to perform diagnostic value statistical analysis. Four different SAA cut-off values were arbitrarily selected to assess the diagnostic value of SAA in diagnosing EPM in horses. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated using 0, 50, 100, and 200 ug/ml as cut-off values. The area under the ROC curve was calculated as 0.504 indicating that SAA is a poor differentiator of EPM positive and negative samples. Kruskal Wallis Test attributed no statistical significance to differences in SAA concentrations between EPM positive and negative groups with P found to be 0.92. 92.3% specificity was observed when using 200 ug/ml SAA to diagnose EPM, however, this was paired with 6.1% sensitivity contributing to an overall poor accuracy of 37.2%. The study demonstrates that SAA concentration is not of significance when used to identify and diagnose EPM in a single time point sample.

12

COMPARISON OF SPECIFIC ANTIBODY INDEX AND GOLDMANN-WITMER COEFFICIENT (C-VALUE) TO EVALUATE INTRATHECAL IMMUNOGLOBULIN G PRODUCTION IN EQUINE PROTOZOAL MYELOENCEPHALITIS

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Interpretation of assays for detection of IgG against *Sarcocystis neurona* in cerebrospinal fluid (CSF) may be influenced by the presence of serum IgG. Normal passive transfer of serum antibodies occurs across the blood-brain-barrier at a proportionality which is altered when intrathecal IgGs are synthesized in the central nervous system (Furr, 2002). Since this proportionality can also be affected by iatrogenic blood contamination, it is important to distinguish between these possible causes. The use of the Immunoglobulin G (IgG) index calculation as an indicator of extravascular IgG synthesis originated from human medicine as a diagnostic aid in multiple sclerosis (Ganrot and Laurell, 1974; Link and Tibbling, 1977). Several studies have looked at the use of similar assessments for horses tested after experimental infection with *S. neurona* (Heskett and MacKay, 2008) or for clinical diagnosis of EPM (Furr, Howe and Yeargan, 2011). Both of these studies preceded the development, validation and diagnostic use of the *S. neurona* SAG 2, 4/3 ELISA assay (Reed et al., 2013). Using this assay, we have compared two ways to evaluate the origin of *S. neurona* IgG in CSF. The sample set consisted of 101 paired serum and CSF samples from 94 horses that went to necropsy (including 3 samples which were additional time-point collections from the same horse) and 4 horses with well defined clinical diagnoses such as EHV1 infection. By diagnosis, 28 horses had EPM, 29 horses had CVSM, 39 horses had other neurologic diagnoses and 5 were non-neurologic horses. *S. neurona* antibody titers were determined by the SAG 2,4/3 ELISA, albumin concentrations by spectroscopy, and IgG concentrations by RID. SAG 2,4/3 ELISA serum/CSF ratio (SNELISA ratio) was defined in this set as ≥100 not EPM and <100 EPM. Specific antibody index (SAI) was defined as ≤1.0 not EPM and >1.0 EPM. The C-value, previously defined at a 1.0 cut off value, was found to be improved using a 4.0 cut off value in this study and is defined in the calculations as ≤4.0 not EPM and >4.0 EPM. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using necropsy (T=94)/clinical (T=4) diagnosis as the gold standard. Results were analyzed as: SNELISA ratio alone, SAI alone, C-value alone, SAI and ratio, C-value and ratio. Specificities and PPVs improved with the combined indices over lone results. The sensitivity of the SNELISA ratio alone was 92.9%, specificity 90.4%, PPV 78.8%, and NPV 97.1%. Adding the SAI to the SNELISA ratio had no effect on sensitivity or NPV, but increased specificity to 94.3%, PPV to 86.7%. Adding the C-value to the SNELISA had no effect on the sensitivity or the NPV, but increased the specificity to 97.1% and the PPV to 92.9%. For a subset of 11 blood contaminated CSFs, the SNELISA ratio plus C-value was 100% for all 4 statistics.

13

PHOSPHORYLATED NEUROFILAMENT H (pNF-H) AS A POTENTIAL DIAGNOSTIC MARKER FOR NEUROLOGICAL DISORDERS IN HORSES

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Neurofilaments are structural proteins of the neuron that are particularly concentrated in axons and are released following neuronal and axonal degeneration; abnormal accumulations have been documented to occur in neurodegenerative diseases of humans. The current study investigated 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 were obtained from horses diagnosed with neurologic disorders, including equine protozoal myeloencephalitis (EPM) (n=38) and cervical vertebral stenotic myelopathy (CVSM) (n=23). Control serum and CSF samples were obtained from clinically healthy horses (n=57). 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 and CSF pNF-H levels in comparison to controls or CVSM cases (Table 1).

Table 1. The median and range concentration of pNF-H in different groups in different samples.

Group	Serum (ng/mL)	CSF (ng/mL)
Control	0.0 (0.0–2.0)[a] ($n = 57$)	0.663 (0.0–6.896)[a] ($n = 34$)
EPM	0.359 (0.0–10.870)[b] ($n = 38$)	7.286 (0.0–26.636)[b] ($n = 36$)
CVSM	0.229 (0.0–4.775)[c] ($n = 23$)	1.679 (0.0–15.857)[a] ($n = 22$)

[abc] Different superscripts within columns indicate significant difference at $p \leq 0.05$.

The correlation between CSF and serum pNF-H levels was poor in both the whole study population and among subgroups of horses. There was significant association between the likelihood of EPM and the concentrations of pNF-H in either the serum or CSF. This study demonstrated that pNF-H could be detected in serum and CSF samples from neurologic and control horses and indicated that pNF-H levels have the potential to provide objective information to aid in early diagnosis of horses afflicted with neurologic disorders.

Full article now available: Res Vet Sci. 2017 Jul 18;114:401-405.

14
PERFORMANCE ASSESSMENT OF DIFFERENT DIAGNOSTIC ASSAYS TO IDENTIFY EPM-AFFECTED HORSES IN A CLINICAL SETTING

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Current antemortem EPM diagnostic strategies determine serum, CSF, and serum: CSF antibody titers to differentiate acute or heightened infection from general exposure to *S. neurona*. To more effectively identify EPM-affected patients and better evaluate assay performance, we compared paired results from two different EPM assays (the IFAT and the SAG 2, 4/3 ELISA) within a subpopulation of patients at an equine clinical hospital. Sampled across four years, IFAT CSF samples, in addition to paired SAG serum and CSF, were submitted soon after collection (n=88 horses). For a subpopulation (n=18), antibody ratios for serum: CSF for both IFAT and SAG were calculated on paired samples and tested using the same aliquots. EPM positive samples were defined as: ≥1:160 serum and ≥1:5 CSF antibodies on IFAT; ≥1:250 serum and ≥1:2.5 CSF antibodies on SAG; ≤64 and <100 ratios on serum: CSF ratio for IFAT and SAG assays, respectively. Descriptive statistical analysis, including overall percent agreement (OPA), positive percent agreement (PPA), negative percent agreement (NPA), Cohen's kappa coefficient (k), and McNemar p test, was performed to compare assay components, as a perfect reference EPM test standard is nonexistent. When paired results for the SAG serum: CSF ratio were compared to the IFAT CSF, the OPA, PPA, and NPA were 87.50, 47.37, and 98.55, respectively, indicating that both tests can equally diagnostically exclude EPM ($p<0.02$; n=88). Additionally, the SAG ratio was interpreted as EPM less frequently than the IFAT CSF; moderate agreement between the assays (k=0.55, 95% CI 0.33-0.78) also points to this disparity in EPM identification. Similarly, comparison between the SAG to IFAT serum: CSF ratio results revealed an OPA, PPA, and NPA of 88.89, 85.71, and 90.91, respectively, with stronger agreement between the tests (k=0.77, 95% CI 0.46-1.00). These assay results did not demonstrate an identification bias toward either assay (n=18; p=0.48), suggesting the assays could be potentially substituted for each other, though a larger sample size should be considered in future analysis. Comparisons between IFAT or SAG serum versus the SAG or IFAT ratio, respectively, showed a poor agreement of results: OPA and k were 44.44 and -0.23 (95% CI -0.63-0.17), and 44.44 and 0.07 (95% CI, -0.07-0.22), respectively. Additionally, while ratio calculations for both assays require positive CSF samples, positive SAG CSF samples occasionally yielded negative ratios (NPA 60.76; k=0.24 (95% CI 0.10-0.38); n=88). Interestingly, the IFAT CSF perfectly correlated with the ratio (OPA 100; k=1.00; n=18). A lack of corresponding necropsy results on sampled horses, and a small subpopulation for direct ratio comparisons, unfortunately limits assessment of EPM assay accuracy (i.e. sensitivity and specificity), though past research has evaluated assay accuracy [1]. Clinicians relying solely on serum testing for a diagnosis may likely mistake infection for exposure given the high seroprevalence [2]. CSF testing alone seemed appropriate

for the IFAT assay, but did not seem sufficient on the SAG assay. In conclusion, clinicians selecting the IFAT assay could potentially only analyze CSF, while the SAG assay seems to perform best when using the serum: CSF ratio.

References
1. Johnson, A.L., Morrow, J.K., Sweeney, R.W., 2013. Brief Communication 596–599.
2. Witonsky, S., 2016. ACVIM Consensus Statement 491–502. doi:10.1111/jvim.13834

15

PATHOLOGY OF CASES OF EQUINE PROTOZOAL MYELITIS SUBMITTED TO THE CALIFORNIA ANIMAL HEALTH AND FOOD SAFETY LABORATORY BETWEEN 1990 AND 2016

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Equine protozoal myeloencephalitis (EPM) is a disease that affects mainly the central nervous system (CNS) of horses, and is produced by the apicomplexan parasite *Sarcocystis neurona*. EPM is prevalent in California and infection with *S. neurona* is regularly included in the list of differential diagnoses for horses with CNS disease submitted to the California Animal Health and Food Safety Laboratory system (CAHFS), for necropsy and diagnostic work up. We present here a retrospective study of cases of EPM submitted to CAHFS between 1990 and 2016. Diagnoses were based on characteristic histologic lesions of the CNS, with or without serology and immunohistochemistry (IHC) for *S. neurona*. During this period a total of 87 horses were diagnosed with EPM in four branches of CAHFS (Davis, Fresno, Tulare and San Bernardino). Of these, the diagnosis was based on histopathology alone in 45 cases, histopathology plus serology (9 cases), histopathology plus IHC (25 cases), or histopathology plus serology and IHC (8 cases). Of the 87 cases, 41 had encephalomyelitis, 24 had only myelitis, and 20 had only encephalitis; information on the location of the lesions in the remaining 2 cases was not available. These findings stress the importance of collection and examination of the brain and entire spinal cord, as lesions are frequently segmental and of variable distribution. While histopathology may be diagnostic in cases in which merozoites and/or schizonts are seen microscopically, other ancillary tests, including IHC and serology are needed in many other cases to confirm a diagnosis of this disease.

Relevance and Future Needs in the Field of EPM

Nicola Pusterla

Relevance and Future Needs

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From the Clinician's Perspective

- **Diagnostic field**
  - **Reliably validated and potentially improved diagnostics**
  - **Ways to support a diagnosis w/o performing a CSF tap**
- **Clinical diagnosis**
  - **Is a clinical diagnosis and treatment trial justifiable**
  - **Gait abnormality and EPM, is there a link**
- **Treatment**
  - **Can we expect new drugs and should we consider combination treatments (anti-protozoal + biologic response modifiers)**
  - **Cost analysis of treatment trial versus CSF collection/analysis**
- **Prevention**
  - **Drugs and indications to use them**

From Industry's Perspective

- **Development of new/novel drugs**
  - **FDA-approved drugs show 60-65% efficacy**
  - **Case definition and quantitative serodiagnostics could potentially improve the efficacy of approved drugs**
  - **Need to establish a cost-effective and reliable animal model**
- **Prevention**
  - **Development of a vaccine**
  - **Standardize prophylactic protocols and gather data (relapsing/recurrence rate, rate of sero-reversion)**
  - **Pharmacokinetic modeling of FDA-approved drugs to establish effective dose and administration frequency to prevent infection**
- **Post-licensing studies**

RELEVANCE AND FUTURE NEEDS IN THE FIELD OF EPM

16
ASSESSMENT OF THE DIAGNOSTIC VALUE OF NEUROLOGICAL SIGNS IN THE CLINICAL DIAGNOSIS OF EQUINE PROTOZOAL MYELOENCEPHALITIS

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While immunodiagnostics are an important aspect in supporting an EPM diagnosis, the presence of relevant neurologic deficits assessed via a comprehensive neurological examination may represent the first step at correctly diagnosing an EPM suspect horse. The objective of this study was to define the importance of selected clinical signs in an effort to further refine the EPM diagnostic interpretations. Using retrospective data collected from two referral equine hospitals, neurological signs of horses with suspected EPM based on a *S. neurona* and/or *N. hughesi* serum to cerebrospinal fluid (CSF) antibody ratio less than 100 (n=71) were compared with neurologic signs of horses with non-EPM horses with other neurologic diseases based on a *S. neurona* and/or *N. hughesi* serum to CSF antibody ratio equal to or above 100 (n=218). Logistic regression models were created to determine associations between clinical signs and EPM outcome for each hospital. One hospital demonstrated muscle weakness and asymmetrical presentation of clinical signs as significant factors predicting an EPM suspect outcome, while the other hospital found no association with any clinical signs and an EPM diagnosis. The hospital that found associations with EPM diagnosis and muscle weakness/asymmetrical presentation also had 17% of its population diagnosed with cervical stenotic myelopathy, compared to 3% at the other hospital. This study highlights the importance of determining neurological deficits through the neurological examination in diagnosing EPM associated with *S. neurona* and/or *N. hughesi* infection. Generally, horses in this study with suspect EPM were more likely to demonstrate muscle weakness and asymmetrical presentation, while horses with other neurologic conditions were more likely to demonstrate sensory deficits; however, these signs were dependent on the referral population of the hospital, as areas with younger horses of Thoroughbred breed demonstrated different clinical signs indicative of EPM compared to areas with older, more mixed breed equine populations.

17
STANDING CERVICAL SPINAL TAP: AN ALTERNATIVE TO STANDING LUMBOSACRAL CSF TAP FOR EPM DIAGNOSIS

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Performing a standing lumbosacral CSF tap (LS) is technically difficult and because of potential blood contamination many practitioners elect to diagnose equine protozoal myeloencephalitis (EPM) based on neurologic examination, positive serum titers and response to treatment. A standing cervical spinal CSF tap (CS) was recently introduced as an alternative to the LS to obtain CSF samples in horses with neurologic disease. The purpose of this study was to describe the procedure of obtaining CSF from the CS and compare red blood cell counts (RBC), total nucleated cell count (WBC) and total protein concentration (TP) in CSF obtained from CS or LS in horses presented for neurologic disease. CSF was obtained from LS (n=5) or CS (n=4) in standing horses that presented to the LSU VTH for neurologic disease. CSF was processed immediately after collection. RBC and WBC were counted manually using a hemocytometer and TP in was measured spectrophotemetrically using the pyrogallol red technique, with human total protein as the standard. The CS was safe and easy to perform and resulted in no adverse effects. However, one horse became recumbent after sedation and synovial fluid was obtained on the first CS attempt. The CS procedure was repeated in lateral recumbency and a clean sample was obtained. LS CSF RBC counts ranged from 16 to 24,500 cells/µL, nucleated cell counts ranged from 1-82 cells/µL and TP ranged from 32-130 mg/dl in the 5 horses. LS CSF in 3/5 horses had >10,000 RBC/µL and 2/5 had mild blood contamination (440 RBC/µL and 16 RBC/µL). CS CSF RBC counts ranged from 7-57,000 cells/µL, nucleated cell count ranged from 1-2,450 cells/µL and TP ranged from 40-340 mg/dl in the 4 horses. CS CSF in 3/4 horses had <42 RBC/µL. One horse had 57,000 RBC/µL, 2,450 nucleated cells/µL and a TP of 340 mg/dL and at necropsy there was severe suppurative meningitis that was considered bacterial in origin. All horses tested negative for *S. neurona*. In conclusion, although this was a small sample size, the CS procedure described here was performed on standing and sedated (detomidine [0.01 mg/kg, IV] and butorphanol [0.01 mg/kg, IV]) horses using a 3 inch 20ga spinal needle without adverse effects. The CSF obtained from the CS had less blood contamination than samples taken from the LS space in these horses. The CS method of CSF collection was less technical difficult that the LS collection procedure and resulted in less blood contamination in these horses.

Current Therapeutic & Preventative Approaches for EPM

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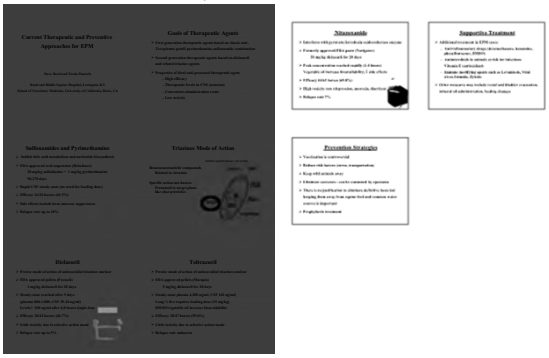
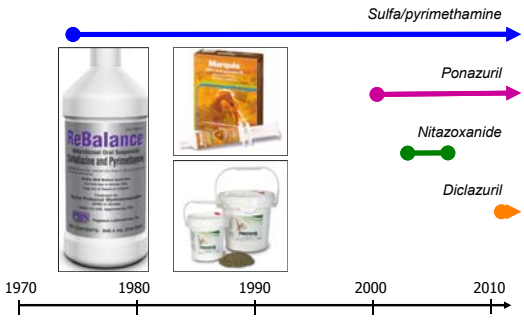
EPM tx and px, circa 2014



More than 40 years of treatment



FDA-Approved EPM therapies

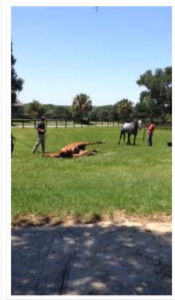
- Ponazuril, diclazuril, sulfadiazine/pyrimethamine
- ~60% showed improvement
  - ≥ 1-2 grades OR negative WB
  - Number cured?
- Rationale for treatment duration
- Horses enrolled on signs plus WB
  - Likely underestimate of tx efficacy
- No comparative data

Conventional Antiprotozoal Treatment

- Marquis (or Protazil)
  - Loading dose (3X Marquis)
  - 1 to 2 months (±corn oil, DMSO(?!))
- Sulfa/pyrimethamine
  - 3-6 months
- Combination treatment
  - Marquis *OR* Protazil 2 months, *PLUS*
  - Sulfa/Pyr 6 months
  - ~$3,500

Unofficial/Off-Label/Illegal

- Toltrazuril (Baycox)
  - Australia, Canada
  - Widespread use in young racehorses
- Compounded drugs
  - Toltrazuril ± pyrimethamine
  - Sulfadiazine/pyrimethamine
- Diclazuril
  - Na salt
  - Intravenous



Pending approval

- Decoquinate
  - Calf anticoccidial
  - Decoquinate/levamisole (Orogin® [Oroquin-10])
  - FDA approval in process
  - Deccox-M (1/3 cup daily)

Immunostimulants

- Levamisole (Levasole Injectable, Merck Intervet)
  - Dendritic cell activation, interferon γ, polarization toward cell-mediated immunity
  - Aminorex, rexamino
- Zylexis (Zoetis)
  - *Parapox ovis* (orf) – interferon γ, enhanced innate immunity
- Eqstim (IDEXX)
  - Killed *Propionibacterium acnes* – enhanced innate immunity
- 4Life Transfer Factor (4Life Research)
  - Specific cell-mediated factors from colostrum and egg yolk small MW

Treatment Issues

- Foundational data 15 to 20 y old, possibly underestimates
- Excessive reliance on anecdotal/unscientific ”evidence”
- Low percentage “cures”
- Lack of comparative data
- Treatment endpoint definitions
- Relapses
- Promiscuous use of EPM drugs in racehorses
- Progression of in vitro testing to clinical usage
- Dearth of data supporting use of BRM

Prevention strategies

- Only previous vaccine lacked proof of efficacy
  - Withdrawn
- Reduction of risk factors (stress, transportation)
- Reduction of exposure to/contamination by opossums
- No evidence for elimination of intermediate hosts
- Prophylactic treatment

Prevention Issues

- Application of modern vaccine technologies
  - Live organism (e.g., controlled exposure)
  - Live organism attenuated/inactivated
  - ”Sub-unit” defined antigen
  - Killed organism, enhanced adjuvants
- Treatment as prophylaxis

TREATMENT AND PREVENTION

18
SARCOCYSTIS NEURONA AND ANTIPROTOZOAL BUMPED KINASE INHIBITORS

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Disruption of essential protein kinase function has been explored for therapy of parasitic diseases. However, the difficulty of inhibiting parasite protein kinases to the exclusion of host homologues poses a practical challenge. A possible path around this difficulty is the use of bumped kinase inhibitors (BKIs) targeting calcium-dependent protein kinases that contain atypically small “gatekeeper” residues and are crucial for motility and proliferation of apicomplexan parasites. Interrogation of *Sarcocystis neurona* genome and transcriptome information revealed a calcium-dependent protein kinase 1 (CDPK1) homologue with the glycine gatekeeper residue found in other apicomplexans, thus implying that BKIs might be effective against *S. neurona* infection and equine protozoal myeloencephalitis (EPM). Recombinant SnCDPK1 was tested against four BKIs shown previously to inhibit *Toxoplasma gondii* TgCDPK1 and, hence, tachyzoite invasion and growth. SnCDPK1 activity was inhibited by low nanomolar concentrations of these BKIs and *S. neurona* growth was inhibited at 40-120 nM concentrations. Thermal shift assays confirmed these BKIs bind CDPK1 in *S. neurona* cell lysates. Treatment with BKIs before or after invasion suggested that these inhibitors interfere with *S. neurona* invasion of mammalian host cells at lower concentrations (0.5-2.5 μM) but additionally interfere with intracellular division at 2.5 μM. *In vivo* proof-of-concept experiments were performed in a murine model of *S. neurona* infection. The infected groups treated for 30 days with BKI-1553 (n=10 mice) had no signs of disease, while the control (untreated) group had severe clinical signs. Elevated antibody responses were found in all 10 of the control infected mice, but in only two of the treated infected mice. Parasites were found in brain tissues of all 10 of the control infected mice, but in only one of the treated mice. The BKIs used in these assays have been chemically optimized for potency, selectivity, and pharmacokinetic properties, and hence are good candidates for treatment of EPM.

19
DICLAZURIL TREATMENT INEFFECTIVE AT PREVENTING *SARCOCYSTIS NEURONA* INDUCED MYELOENCEPHALITIS RELAPSE IN ESTABLISHED MOUSE MODEL

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The debilitating and potentially fatal neurologic disease, Equine Protozoal Myoencephalitis (EPM), is one of the most common neurologic diseases seen in the equine population of the United States. Disease develops as a result of unintentional ingestion of the pathogenic parasite, *Sarcocystis neurona*. Treatment options consist of the anticoccidial drug diclazuril and numerous other antiprotozoal drugs. Weeks to months after cessation of antiprotozoal drug treatment and corresponding neurologic improvement, horses may present with clinical disease symptoms again. Little is known whether this reoccurrence of symptoms is from relapse or reinfection. We sought to understand if relapse was possible following appropriate treatment with diclazuril, a commonly used medication for the treatment of EPM. Using a mouse model of EPM, we subjected infected mice to treatment with or without diclazuril for 30 and 60 days. All untreated mice developed neurologic symptoms consistent with *S. neurona* infection within 30 days post infection. All diclazuril treated mice developed neurologic symptoms within 60 days of cessation of treatment. Cerebellum samples were examined for lesions characteristic of those associated with *S. neurona* infection and immunohistochemically for presences *S. neurona*. Sera immunoglobulin levels were analyzed and results suggest that treatment did not completely eliminate the effect potentially allowing migration of *S. neurona* to the CNS after cessation of treatment. In conclusion *Ifnγ* $^{-/-}$ mice treated appropriately with diclazuril only remained asymptomatic while on treatment.

These results suggest that treated horses which present with recurrent symptoms may have persistent infection and more effective treatment options should be explored.

20

DICLAZURIL NONLINEAR MIXED-EFFECTS PHARMACOKINETIC MODELING OF PLASMA CONCENTRATIONS AFTER ORAL ADMINISTRATION TO ADULT HORSES EVERY 3 TO 4 DAYS

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The purpose of this study was to determine if a low dose of diclazuril given every 3 to 4 days would achieve steady-state concentrations in plasma known to be inhibitory to *Sarcocystis neurona* and *Neospora caninum in vitro*. Six healthy adult horses received 0.5 mg/kg of 1.56% diclazuril pellets orally every 3 to 4 days for a total of 5 administrations. Blood was collected via venipuncture immediately before (trough concentrations) and 10 hours after (peak concentrations) each diclazuril administration. Plasma samples were analyzed by high-pressure liquid chromatography (HPLC). Steady-state pharmacokinetics was performed using non-linear mixed effects modeling (NLME) with the primary parameters as fixed effects and inter-individual variability as the random effect. The population-derived peak concentration (C_{MAX}) was 0.284 µg/mL (284 ng/mL) and the terminal half-life was 1.6 days, but with a large variation (CV 136%). Plasma concentrations increased until the 4th day, but did not accumulate thereafter, reaching a steady-state for the final 3 doses. In conclusion, the study results showed that diclazuril given at a low dose every 3 to 4 days provides plasma concentrations in excess to inhibitory in vitro concentrations for *S. neurona* and *N. caninum* that can be maintained at a steady-state for subsequent doses. Further, this protocol has in the opinion of the authors two main advantages, it improves compliance (twice weekly drug administration instead of daily drug administration) and reduces the amount of diclazuril administered (71% less drug administered when compared to daily drug administration). Future studies are needed to determine if the established dosing regimen will be effective at reducing infection rate with subsequent EPM development in high risk horse populations.

21

DEFINING RELAPSES ATTRIBUTED TO EQUINE PROTOZOAL MYELOENCEPHALITIS UPDATE

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Disease caused by *Sarcocystis* spp are associated with a variety of clinical signs including abortion, hemorrhages, hair loss, muscular disease, and neurological dysfunction. In horses, clinical disease is associated with *Sarcocystis fayeri* and *S. neurona*. The genesis of and clinical progression of disease caused by these protozoans in horses is not well defined but acute, chronic, and relapsing disease is recognized in horses undergoing treatment and recovery from sarcocystosis. In this study, we investigated the seroprevalence of species specific antibodies to *S. neurona* and a protein derived from *S. fayeri* sarcocysts (toxin) in 71 horses with a history of relapsing neuromuscular disease. Horses were grouped by presence of antibody and treated with decoquinate given at 0.5 mg/kg body weight to determine the response to antiprotozoal treatment with specific aspects of disease progression. Recurrence of disease was evaluated by gait score. This study indicated that there were three disease presentations associated with chronic sarcocystosis that had been attributed to clinical equine protozoal myeloencephalitis caused by *S. neurona* (EPM). Most relapsing remitting disease in this group of horses was associated with the presence of antibodies against *S. fayeri* protein (49 %) or anti-myelin protein (43 %), not *S. neurona* (8 %). Treatment was successful in the *S. neurona* group horses at 3 (100%), 6 (75%), and 9 (100%) months. Treatment was successful in the *S. fayeri* group horses (with or without *S. neurona* antibodies) in 39%, 26%, 75%, and 100% of the horses at 3, 6, 9, and 12 months, respectively. Repeat environmental exposure to *S. neurona*, rather than persistent but unapparent infections, was shown by increasing antibody titers to *S. neurona* in the clinically normal horses. Persistent subclinical disease was indicated by an elevated CRP concentration in clinically normal horses. Horses in the *S. fayeri* group that were treated for 6-months showed a decline in *S. fayeri* related antibody. Dysfunctional inflammatory immune responses stimulated by sarcocystosis may result in the development of clinical signs due to autoimmune polyneuritis shown by circulating anti-myelin protein antibodies that were found in some horses (n=29). Treatment with decoquinate was significantly more effective in treating sarcocystosis related disease as compared to autoimmune polyneuritis ($p > 0.5$) when success was determined by an absence of clinical signs of EPM in these horses at 3, 6, and 9 months.

22

NOVEL HIGH-THROUGHPUT SCREEN OF DRUG COMPOUND LIBRARY IDENTIFIES INHIBITORS OF *SARCOCYSTIS NEURONA* GROWTH

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The apicomplexan parasite *Sarcocystis neurona* is the primary etiologic agent of equine protozoal myeloencephalitis (EPM), the most significant infectious progressive neurologic disease in horses. Many horses the U.S. are at risk of developing EPM; serologic studies estimate that 50% of all horses in the U.S. have been exposed to *S. neurona* and treatments for EPM are at best 60-70% effective. Advancement of treatment for EPM requires new technology to identify novel compounds. To address this critical need, we developed and validated, then implemented the use of a novel high-throughput screen to test 725 FDA-approved chemical compounds from the NIH clinical collections library. Our screen identified 18 novel compounds with confirmed inhibitory activity against *S. neurona* growth, including compounds active in the nM concentration range. Many of the inhibitory compounds identified have well-defined mechanisms of action, making them useful tools to study parasite biology in addition to being potential therapeutic agents. In comparing the activity of inhibitory compounds identified by our screen to that of other screens against other apicomplexan parasites, we found that most of the compounds (15/18; 83%) have activity against one or more related apicomplexans. Interestingly, nearly half (44%; 8/18) of the inhibitory compounds were reported to have activity against dopamine receptors. These studies demonstrate the use of a robust new tool for discovering new chemotherapeutic agents for EPM and potentially provide new reagents to elucidate biologic pathways required for successful *S. neurona* infection.

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