2019 Annual Report
Prepared by:
Dr. Laurent Couëtil, DVM, PhD
Professor, Veterinary Clinical Sciences
Director, Equine Sports Medicine Center
Director, Equine Research Programs
# INDEX

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mission</td>
<td>1</td>
</tr>
<tr>
<td>Goals</td>
<td>1</td>
</tr>
<tr>
<td>Achievements of Equine Sports Medicine Center (ESMC)</td>
<td>1</td>
</tr>
<tr>
<td>Treadmill Evaluations</td>
<td>1</td>
</tr>
<tr>
<td>Continuing Education and Extension Service</td>
<td>1-5</td>
</tr>
<tr>
<td>Outreach</td>
<td>5-7</td>
</tr>
<tr>
<td>Research</td>
<td>7-9</td>
</tr>
<tr>
<td>Research Projects in Progress Supported with Pari-Mutual Funds</td>
<td>7</td>
</tr>
<tr>
<td>Research Projects Completed Supported with Pari-Mutual Funds</td>
<td>7-8</td>
</tr>
<tr>
<td>Competitive Equine Research Fellowship</td>
<td>8</td>
</tr>
<tr>
<td>Externally Funded Equine Research Projects Conducted in 2019</td>
<td>8-9</td>
</tr>
<tr>
<td>Publications Supported by the Equine Research Internal Funds</td>
<td>9-11</td>
</tr>
<tr>
<td>Referred Scientific Articles</td>
<td>9</td>
</tr>
<tr>
<td>Abstracts and Proceedings</td>
<td>10</td>
</tr>
<tr>
<td>Book Chapters</td>
<td>10</td>
</tr>
<tr>
<td>Referred Scientific Publications</td>
<td>11</td>
</tr>
<tr>
<td>Havemeyer Workshop</td>
<td>12</td>
</tr>
<tr>
<td>Research Advisory Board Membership 7/2018-6/2019</td>
<td>13</td>
</tr>
<tr>
<td>Research Advisory Board Membership 7/2019-6/2020</td>
<td>14</td>
</tr>
</tbody>
</table>

## Appendix A (Blue)

- **Equine Health Update** - Equine Sports Medicine Center Newsletter
  Vol. 21, Issue No. 1 – 2019

- **Equine Health Update** - Equine Sports Medicine Center Newsletter
  Vol. 21, Issue No. 2 – 2019

## Appendix B (Gold) ~ Research Projects In Progress Supported with Pari-Mutual Funds


- **Hooser S.** Detection of Black Walnut Wood in Equine Bedding by PCR.

- **Lescun T., Hermida J., Main RP., Little D., Weng H-Y.** Collagen Orientation and Tensile Strength of Equine Proximal Sesamoid Bones.

• Little D., Lescun T. Mechanosensitive Channels in Equine Musculoskeletal Soft Tissues.

**Appendix C (Green)** ~ Research Projects Completed Supported with Pari-Mutuel Funds

• Dos Santos AP., Taylor SD., Woolcock A., Christian JA., Ruple A. **Validation of a Novel Assay to Detect Intraerythrocytic Reactive Oxygen Species (ROS) by Flow Cytometry in Horses.**

• Hendrix K., Kritchevsky J. **Recovery of Salmonella Bacterial Isolates from Pooled Equine Fecal Samples.**

• Lescun T., Breur G., Nauman E., Chandrasekar S., Adams S., Jones Y., Main R. **Finite Element Modeling and Implant Nanosurfacing to Enhance Equine Fracture Treatment.**

• Taylor S., Cooper B., Grady S., Lescun T., Moore G., Davern A., Brunner T. **Plasma Drug Concentrations of Ketorolac Tromethamine, Phenylbutazone and Flunixin Meglumine in Horses Following Single-dose Intravenous Administration.**

• Taylor S., Grady S., Lescun T., Moore G., Davern A. **Analgesic Efficacy and Safety of Ketorolac, Phenylbutazone and Flunixin in a Model of Foot Lameness in Horses.**

**Appendix D (Purple)**

~ Refereed Scientific Articles


~ Abstracts and Proceedings


• Tinkler SH., Penny Lecca I., Weil A., Couëtil LL. Cardiopulmonary Parameters in Field Anesthetized Working Equids at High Altitude in the Peruvian Andes. World Equine Veterinary Association Congress, Verona Italy. October 2019.

Appendix E (Gray) ~ Refereed Scientific Publications


**Appendix F (Tan) ~ Havemeyer Workshop on Equine Asthma – Proceedings**

- Dr. Couetil chaired the organizing committee for the Dorothy R. Havemeyer Foundation Workshop: Equine Asthma; Current Understanding and Future Directions that was held in Custer, SD in May 2019. The workshop was supported in part by PVM.
MISSION
To provide first class veterinary diagnostic and investigative support to the horse industry in Indiana and to educate owners, trainers, and veterinarians.

GOALS:
The goals of the ESMC are to pioneer leading-edge research in the area of equine sports medicine, to provide training to future equine veterinarians and veterinary technicians, to offer continuing education to Indiana veterinarians and horsemen, and to diagnose and treat causes of decreased performance in horses.

ACHIEVEMENTS OF EQUINE SPORTS MEDICINE CENTER (ESMC)

Treadmill Evaluations:
Treadmill diagnostic work-ups are an important activity at the ESMC. Twenty client-owned horses were evaluated on the treadmill in 2019. This brings the total number of horses evaluated since the opening of the ESMC in April 1996 to 523. Treadmill demonstrations at the ESMC continue to be a major attraction for local, national and international visitors to the Purdue campus. In the past year 8 treadmill demonstrations were given to groups or dignitaries who visited the Purdue campus.

Continuing Education and Extension Service:
• Continuing Education presentations:
  • Couetil L.
    International
    • Health effects of equine asthma. The Dorothy R. Havemeyer Foundation Workshop on Equine Asthma, Custer, SD. May 2019.

    National
    • The American Association of Equine Practitioners Convention 360° on Upper and Lower Respiratory Tract Disorders, College Station, TX, 2019.
      ▪ Diagnosis and management of equine inflammatory airway disease (IAD)
      ▪ Diagnosis and treatment of equine recurrent airway obstruction (RAO)
      ▪ How to use inhalant therapy for management of equine respiratory disorders
      ▪ Update on exercise-induced pulmonary hemorrhage (EIPH)
      ▪ Workshop on the use of inhalant therapy in horses: Metered dose inhalants, nebulizers, and other devices
Regional and State


- Purdue Horseman’s Forum, Purdue College of Veterinary Medicine, IN, February 9th, 2019.
  - Update on PVM research
  - Equine Sports Medicine: high-speed treadmill demonstrations

- Davern A.

Regional and State
- Advanced imaging – standing CT cases. Purdue Horseman’s Forum, West Lafayette, IN, February 9th, 2019.

- Farr A.

Regional and State
- Purdue Horseman’s Forum, Purdue College of Veterinary Medicine, IN, February 9th, 2019.
  - THIS! IS! JEOPARDY! Test Your Equine Knowledge.

- Gedeus T.

Regional and State
- Centaur Education Meeting, The Centaur Equine Specialty Hospital, IN, March 20th, 2019.
  - Practical session: CT of individual heads and collation of examination results

- Haanen G.

Regional and State

- Hogan D.

Regional and State
- Cardiology. Purdue Horseman’s Forum, West Lafayette, IN, February 9th, 2019.

- Kozol J.

Regional and State
• Lescun T.
  National
  • Veterinary Orthopedic Society Conference, Breckenridge, CO, February 2019.
    ▫ Drilling; not so fast
    ▫ Nanotechnology and osseointegration

Regional and State
• Hawaii Veterinary Medical Association Annual Meeting, Honolulu, HI, November, 2019. Invited speaker.
  ▫ Nerve Blocks: Do I Trust Them?
  ▫ Foot Lameness
  ▫ Laminitis: Managing Chronic Cases
  ▫ Current Approaches to Synovial Sepsis


• Segarra F.
  Regional and State
  • Purdue Horseman’s Forum, Purdue College of Veterinary Medicine, IN, February 9th, 2019.
    ▫ Managing osteoarthritis

• Skelton J.
  Regional and State
  • Purdue Horseman’s Forum, Purdue College of Veterinary Medicine, IN, February 9th, 2019.
    ▫ THIS! IS! JEOPARDY! Test Your Equine Knowledge
    ▫ Hoof care

• Taylor S.
  International
  • International Veterinary Emergency and Critical Care Annual Conference, National Harbor, MD, 2019.
    ▫ Metabolic resuscitation in equine sepsis
    ▫ Equine piroplasmosis
    ▫ Equine protozoal myeloencephalitis: an update
    ▫ Unique large animal neurologic cases

National

• Tinkler S.
  International
    ▫ Total of 2 lecture hours and 2 lab hours
  • Equitarian Initiative Workshop, Puerto Jimenez Costa Rica, January 2019.
    ▫ Diverse veterinary models for equid health care delivery in Africa
- Welfare impacts of the donkey skin trade on donkeys and owners
- Multiagency cooperative model for improved working equid and community health: Peru model
- Applied research on working equids
  - Equitarian Initiative, Mollepata and Cusipata Peru, January 2019
    - Created and presented in Spanish to equid owners: 2 hour lecture, 2 hour lab - given 2 times
    - Medicina preventiva - clase para paramedicos veterinaries
  - Equitarian Initiative Ecuador scoping trip for future working equid projects, Quito, Ecuador, April 2019
  - Equitarian Initiative Volunteer Veterinarian, Puerto Jimenez, Costa Rica 2019

National

Regional and State
- What is the Equitarian Initiative/Working Equids – lecture. SCAAEP Purdue, October 2019.

- Townsend W.
  Regional and State

- Waxman, S.
  Regional and State

Committee service
International
- Couetil L.:
  Dorothy Havemeyer Workshop on Equine Asthma – Chair of the organizing committee

- Lim CK.:
  Member of the European College of Veterinary Diagnostic Imaging Credentials Committee (2017-2019)

- Tinkler S.:
  Vice President, Equitarian Initiative (Board of Directors 2016-present)

- Townsend W.:
  Research Committee Member, International Equine Ophthalmology Consortium. 2013-present
National
- Couetil L.:
  American College of Veterinary Internal Medicine
  - Board of Regents Nomination Committee
  - Large Animal Internal Medicine Nomination Committee

- Kritchevsky J.:
  American Humane Society Scientific Advisory Committee for American Humane Farm Program
  Equine Endocrinology Working Group

- Lescun T.:
  American Association of Equine Practitioners, Avenues Task Force, 2012-present
  American College of Veterinary Surgeons Foundation, Board of Trustees (2018-present)
  European College of Veterinary Surgeons, Examination committee, 2019 (Invited ACVS external observer)

- Townsend W.:
  Genetics Committee, American College of Veterinary Ophthalmologists, 2012-present

State
- Lescun T.:
  Purdue Liaison to the Board of Directors, Indiana Association of Equine Practitioners 2016-present

Outreach:

• Purdue’s Equine Sports Medicine Center Web site dedicated to informing horse owners about equine-related activities at Purdue University has undergone a major update this year. The address of the site is: [https://vet.purdue.edu/esmc/index.php](https://vet.purdue.edu/esmc/index.php)

• Outreach activities
  
  o **Purdue Horseman’s Forum**, Purdue Veterinary Medicine, West Lafayette, IN, February 9th, 2019.
    ▪ Continuing Education annual meeting for horse owners and veterinarians
    ▪ Approximately 200 registrations, 12 presentations, treadmill demonstrations, acupuncture demonstration, loading in a trailer demonstration and tour of Large Animal Hospital.
  
  o **Centaur Equine Specialty Hospital Continuing Education Meeting**, March 20th, 2019.
    ▪ Continuing Education annual meeting for horse owners and veterinarians
    ▪ Theme: “Diseases of the Equine Teeth and Sinuses”
• Lay Publications:
  o The Equine Sports Medicine Center continued publication of its newsletter called “Equine Health Update” established as a source of information for Indiana’s horse industry. Dr. Stacy Tinkler is the editor for the newsletter since January 2012. Two issues were released in 2019 and articles are accessible from our Web site. The newsletters are included in Appendix A (Blue).

  o Clinton H, Taylor SD. Neonatal Isoerythrolysis: Why Blood Typing can be Important. Purdue University College of Veterinary Medicine Equine Health Update. 2019.

  o Couetil L.: 
      http://www.insideindianabusiness.com/story/39883804/purdue-asthma-test-helps-heal-racehorses
    o Couetil L. Cough Causes Concern. Answer to a Reader’s Question. EQUUS, Spring 2019.
      https://www.veterinarypracticenews.com/purdue-professor-to-study-equine-asthma-treatment/
      https://www.smithsonianmag.com/science-nature/kentucky-derby-even-mild-cases-asthma-can-slow-down-elite-racehorses-180972102/
    o Ed Kane interviewed Dr. Couetil: Hay is for Horses, But What if it Leads to Asthma? DVM360. December 2019. 
      http://veterinarymedicine.dvm360.com/hay-horses-what-if-it-leads-asthma


Tinkler SH.: 
- Edited the following articles:
  - Gillian Hasslinger (PVM’20): Equine Melanomas: Not your Ordinary Lumps and Bumps.
  - Levi Smith (PVM’20): A Villain in your Backyard: Anaplasmosis and Tick Prevention

Tinkler SH, Yankoviak E. “I’ll Bet You a Dime Your Horse Doesn’t Have Lyme!” Equine Health Update for Horse Owners and Veterinarians, Vol. 21, Issue No. 1, July 2019.


Research:

Research activities from investigators of the Equine Sports Medicine Center are summarized below. The names of members of the ESMC are underlined.

Research projects in progress supported with Pari-Mutual Funds: 
Progress reports for the following projects are included in Appendix B (Gold).

- Hooser S. Detection of Black Walnut Wood in Equine Bedding by PCR.

Research projects completed supported with Pari-Mutual Funds: 
Complete reports for the following projects are included in Appendix C (Green).

- Dos Santos AP., Taylor SD., Woolcock A., Christian JA., Ruple A. Validation of a Novel Assay to Detect Intraerythrocytic Reactive Oxygen Species (ROS) by Flow Cytometry in Horses.
- Hendrix K., Kritchevsky J. Recovery of Salmonella Bacterial Isolates from Pooled Equine Fecal Samples.
Lescun T., Breur G., Nauman E., Chandrasekar S., Adams S., Jones Y., Main R. **Finite Element Modeling and Implant Nanosurfacing to Enhance Equine Fracture Treatment.**

Taylor S., Cooper B., Grady S., Lescun T., Moore G., Davern A., Brunner T. **Plasma Drug Concentrations of Ketorolac Tromethamine, Phenylbutazone and Flunixin Meglumine in Horses Following Single-dose Intravenous Administration.**

Taylor S., Grady S., Lescun T., Moore G., Davern A. **Analgesic Efficacy and Safety of Ketorolac, Phenylbutazone and Flunixin in a Model of Foot Lameness in Horses.**

**Competitive Equine Research Fellowship supported with Pari-Mutual Funds:**
The PVM Equine Research Fellowship is for the recruitment of outstanding M.S. or Ph.D. track students to conduct applied/clinical research in the area of equine medicine at Purdue University to address issues of importance to the health and performance of Indiana racehorses and other equine athletes. The fellowship provides one year (M.S.) or two years (Ph.D.) of stipend support from the PVM Equine Internal Fund and additional years of funding support for degree completion will come from the graduate program of the respective department.

Jesus Hermida, DVM, second-year PhD student in VCS – Faculty advisors: Drs. Tim Lescun & Russell Main: *Application was approved by the ERAB and the Equine Research Fellowship was granted for the 2018-2019 academic year and renewed for a second year based on satisfactory progress during the previous year.*

**Externally funded equine research projects conducted in 2019:**


Couetil L.L., Olave C., Ivester K. Role of Dietary Pro-Resolving Lipid Mediators in Equine Asthma. Boehringer Ingelheim Vetmedica. $15,000.


Taylor SD. Mesenchymal Stem Cells to Treat Equine Sepsis. AgSEED Basic Research. $49,572.


Tinkler S., Van Wormer L., Varnum A. Veterinary Student Training in Working Equid Medicine and Capacity Building in Remote Communities in the Peruvian Andes. SPANA (Society for the Protection of Animals Abroad) Outreach Program. £5,000

Witonsky S., Taylor SD. Identifying the Role for IL-17α in EPM-Affected Horses. Virginia Polytechnic Institute and State University.

*Publications supported by the Equine Research Internal Funds: * Appendix D [Purple]

The names of members of the ESMC are underlined.

**Refereed Scientific Articles:**


Abstracts and Proceedings:


Book Chapters:

- Splint bone fractures and removal
- Fractures of the head


Taylor SD., Section Editor for the Laboratory Tests Section of Blackwell’s 5-Minute Veterinary Consult: Equine. 3rd ed. Published, December 2019.


Refereed Scientific Publications: [Appendix E (Gray)]


Dr. Couetil chaired the organizing committee for the Dorothy R. Havemeyer Foundation Workshop: Equine Asthma; Current Understanding and Future Directions that was help in Custer, SD in May 2019. The workshop was supported in part by PVM.
<table>
<thead>
<tr>
<th>Doctor’s Name</th>
<th>Year Serving</th>
<th>Address</th>
<th>Phone</th>
<th>E-mail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Marianne Ash</td>
<td>1</td>
<td>Indiana Horse Council Rep. Indiana BOAH</td>
<td>317-544-2411</td>
<td><a href="mailto:MASH@BOAH.IN.GOV">MASH@BOAH.IN.GOV</a></td>
</tr>
<tr>
<td>Dr. Brian Biggers</td>
<td>1</td>
<td>Indiana Association of Equine Practitioners (IAEP)</td>
<td>574-210-8450</td>
<td><a href="mailto:bgbiggers@comcast.net">bgbiggers@comcast.net</a></td>
</tr>
<tr>
<td>Dr. Colleen Brady</td>
<td>1</td>
<td>ANSC – AGAD 227</td>
<td>765-494-8433</td>
<td><a href="mailto:bradyc@purdue.edu">bradyc@purdue.edu</a></td>
</tr>
<tr>
<td>Dr. Laurent Couetil</td>
<td>Chair</td>
<td>VCS-Lynn G408</td>
<td>765-494-6808</td>
<td><a href="mailto:couetill@purdue.edu">couetill@purdue.edu</a></td>
</tr>
<tr>
<td>Dr. Kari Ekenstedt</td>
<td>2</td>
<td>BMS</td>
<td></td>
<td><a href="mailto:kje0003@purdue.edu">kje0003@purdue.edu</a></td>
</tr>
<tr>
<td>Dr. Jan Hawkins</td>
<td>1</td>
<td>VCS-Lynn G416B</td>
<td>765-494-8563</td>
<td><a href="mailto:hawkinsj@purdue.edu">hawkinsj@purdue.edu</a></td>
</tr>
<tr>
<td>Mrs. Jeri Kieninger</td>
<td>2</td>
<td>7990N 475E, Rochester, IN 46975</td>
<td>574-223-8518</td>
<td><a href="mailto:jack-jeri@rtcol.com">jack-jeri@rtcol.com</a></td>
</tr>
<tr>
<td>Dr. Bruce Murphy</td>
<td>2</td>
<td>Indiana Thoroughbred Owners &amp; Breeders Assoc.</td>
<td>765-366-0287</td>
<td><a href="mailto:Racehorse4523@yahoo.com">Racehorse4523@yahoo.com</a></td>
</tr>
<tr>
<td>Dr. Hsin-Yi Weng</td>
<td>3</td>
<td>CPB – VPTH 127</td>
<td>765-494-0445</td>
<td><a href="mailto:Weng9@purdue.edu">Weng9@purdue.edu</a></td>
</tr>
<tr>
<td>Doctor’s Name</td>
<td>Year Serving</td>
<td>Address</td>
<td>Phone</td>
<td>E-mail</td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------------</td>
<td>----------------------------------------------</td>
<td>----------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Dr. Marianne Ash</td>
<td>1</td>
<td>Indiana Horse Council Rep. Indiana BOAH</td>
<td>317-544-2411</td>
<td><a href="mailto:MASH@BOAH.IN.GOV">MASH@BOAH.IN.GOV</a></td>
</tr>
<tr>
<td>Dr. Brian Biggers</td>
<td>1</td>
<td>Indiana Association of Equine Practitioners (IAEP)</td>
<td>574-210-8450</td>
<td><a href="mailto:bgbiggers@comcast.net">bgbiggers@comcast.net</a></td>
</tr>
<tr>
<td>Dr. Colleen Brady</td>
<td>1</td>
<td>ANSC – AGAD 227</td>
<td>765-494-8433</td>
<td><a href="mailto:bradyc@purdue.edu">bradyc@purdue.edu</a></td>
</tr>
<tr>
<td>Dr. Laurent Couetil</td>
<td>Chair</td>
<td>VCS-Lynn G408</td>
<td>765-494-6808</td>
<td><a href="mailto:couetill@purdue.edu">couetill@purdue.edu</a></td>
</tr>
<tr>
<td>Dr. Kari Ekenstedt</td>
<td>2</td>
<td>BMS</td>
<td></td>
<td><a href="mailto:kje0003@purdue.edu">kje0003@purdue.edu</a></td>
</tr>
<tr>
<td>Dr. Jan Hawkins</td>
<td>1</td>
<td>VCS-Lynn G416B</td>
<td>765-494-8563</td>
<td><a href="mailto:hawkinsj@purdue.edu">hawkinsj@purdue.edu</a></td>
</tr>
<tr>
<td>Dr. Steve Hooser</td>
<td>1</td>
<td>CPB</td>
<td>765-494-6931</td>
<td><a href="mailto:shooer1@purdue.edu">shooer1@purdue.edu</a></td>
</tr>
<tr>
<td>Mrs. Jeri Kieninger</td>
<td>2</td>
<td>7990N 475E, Rochester, IN 46975</td>
<td>574-223-8518</td>
<td><a href="mailto:jack-jeri@rtcol.com">jack-jeri@rtcol.com</a></td>
</tr>
<tr>
<td>Dr. Bruce Murphy</td>
<td>2</td>
<td>Indiana Thoroughbred Owners &amp; Breeders Assoc.</td>
<td>765-366-0287</td>
<td><a href="mailto:Racehorse4523@yahoo.com">Racehorse4523@yahoo.com</a></td>
</tr>
</tbody>
</table>
APPENDIX A

• *Equine Health Update* - Equine Sports Medicine Center Newsletter Vol. 21, Issue No. 1 – 2019

• *Equine Health Update* - Equine Sports Medicine Center Newsletter Vol. 21, Issue No. 2 – 2019
A Horse Owner's Guide to Botulism

By Alyssa Biscoglio, DVM Student (Class of 2020)
Edited by Stacy H. Tinkler, DVM, MPH, Dipl. ACVIM-LA

What is botulism?
Botulism is a paralytic disease affecting equines world-wide, and is caused by neurotoxins produced by the bacterium Clostridium botulinum. This bacterium is typically present in soil, decaying plant matter and animal carcasses. C. botulinum is a spore forming bacteria that thrives in anaerobic (where there is little to no oxygen) and basic (pH levels are > 6) environments. There are several types of C. botulinum toxins and they are classified as types A through G. Clinical botulism in horses has been attributed to C. botulinum types A, B, C, and D. Most cases of equine botulism in the United States occur in Kentucky and the Mid-Atlantic states, with types B and C most commonly reported. Type A toxin is reported more frequently in the Western United States in sporadic outbreaks, and Type B is the most commonly reported serotype in the whole US (>85% of cases).

How does a horse contract botulism?
Horses acquire C. botulinum toxin via three main routes: ingestion of spores (toxicoinfectious), wound infections, and through ingestion of preformed toxins in decaying plant matter, improperly preserved haylage or feed contaminated with decaying animal carcasses. The toxicoinfectious route commonly occurs in foals who ingest C. botulinum spores, and is sometimes referred to as Shaker Foal Syndrome due to the observed muscle tremors caused by the neurotoxin. The ingested spores become active bacteria in the animal’s gut and begin producing toxins. Toxicoinfectious botulism is most common in foals 1 to 3 months of age, although it has been observed in foals as young as 7 days of age. Wounds may become infected with C. botulinum if not kept clean and dry. Deep puncture wounds may create anaerobic environments that foster bacterial growth and subsequent toxin formation.

How does botulism affect my horse?
Botulinum toxin causes paralysis because it blocks the transmission of electrical impulses from nerves to muscles via binding to the junction where these impulses are transferred. Once the communication between nerves and muscles is lost, muscles become weak and/or paralyzed. Once the toxin is bound to these junctions there is no way to remove it. Therefore, the only way for an animal to heal is for new junctions to form. Regeneration of these junctions may take around 7 to 10 days but return to full strength may take an affected animal up to 1 month. During earlier stages of disease horses typically demonstrate weakness with an intolerance to exercise, a short stiff gait, and inability to swallow. Horses may appear sleepy, their third eyelid may protrude, and their tongues may hang out of their mouths or be slow to retract. Horses will often drool and “play with their food/water” due to their inability to swallow. Foals exhibit similar signs and are typically noted dribbling milk from their mouth and nostrils. Colic may also be observed in the early stages of disease due to decreased gastrointestinal motility. Weakness observed in horses may progress to muscle tremors and increased time spent laying down. In the final stages of disease, horses may become unable to rise as full paralysis sets in. Ultimately, the muscles that allow horses to breathe become paralyzed, resulting in death.

(continued on page 2)
How is botulism diagnosed and treated?
Definitive diagnosis of botulism is challenging. In the majority of cases, diagnosis of botulism is made using the horse's history and clinical signs. Other neurologic diseases such as rabies, sleeping sickness, EPM, or viral encephalitis must also be ruled out. Toxin gene detection by using a PCR test after culture of C. botulinum from GI contents or feed is now the preferred test for diagnosis. Treatment of equine botulism may be very costly due to the intensive care required. Treatment also comes with many risks, as even well managed down horses may suffer complications such as colic, pressure sores, and pneumonia. The treatment for botulism consists of early administration of anti-toxin as well as supportive care. The anti-toxin works by binding circulating toxin, and preventing toxin binding to the nerve cell surface receptor. Horses that are treated with anti-toxin have a much higher survival rate than those that are not. Supportive care includes feeding and oral fluids through a nasogastric tube or intravenous nutrition as well as IV fluids in animals that are unable to swallow. Additionally, horses may need to wear a muzzle to prevent intake and aspiration of shavings used as bedding. The stall should be heavily bedded if the horse is down for long stretches of time. Horses unable to rise may need additional padding and to be rotated every few hours to prevent development of pressure sores. Some horses may need a urinary catheter placed, and horses unable to blink will need artificial tears. Severe cases may require a ventilator if the horse is unable to breathe on its own. Horses mildly affected by botulism have a fair prognosis with proper medical management. Horses unable to rise have a very poor prognosis and foals have a good prognosis if the disease is caught early on and anti-toxin is administered. Horses typically require 10 to 14 days to gradually recover. A longer recovery may be required if large amounts of toxin are ingested.

How can botulism be prevented?
Botulism may be prevented through proper vaccination, wound care, and administration of quality feed. Vaccination is only effective for Type B toxin, which is responsible for the majority of horse related botulism cases. Vaccination is recommended in endemic areas, or animals at greater risk for exposure, such as those on cured forages or round bales. Pregnant mares in endemic areas should be vaccinated and given a booster 4-6 weeks before foaling. Foals from unvaccinated mares can be transiently and passively protected using antitoxin or vaccinated with the type B toxoid but it is not labeled for foals.

All feed should be inspected for foreign material, decaying plant material, poisonous plant material, and decaying animal carcasses before it is fed. Avoid feeding round bales as they may contain decaying plant or animal material that may go un-noticed. Square hay bales are best as they may be inspected as they are fed. Keeping wounds clean and dry may help prevent infection. Puncture wounds, castration incision sites, and foal navels that have not been dipped may be at greater risk of C. botulinum infection.

References:
Above are two different presentations of melanomas.
https://www.sporthorsevets.com/equine-melanoma/
https://thehorse.com/16830/canine-melanoma-vaccine-testing-in-horses-underway/

I graduated from Purdue’s Veterinary Technology program in 1990. I started out my career doing overnight patient care in the Large Animal Hospital (just when tracking for the senior students was getting started). I spent 6 years alternating with another technician between third shift doing patient care and day shift, helping with the surgery section. I worked in Clinical Pathology for about 4 years before leaving for a research position in West Lafayette. I was away from Purdue for about 15 years. During this time, I spent several years working for the USDA with livestock behavior research to help improving livestock health and well-being for better production.

In 2014, I found the opportunity to return to the Purdue Large Animal Hospital, again doing patient care on second shift. I love the work that I get to do with the patients, students and clients, but I especially love the hours as I am not a morning person at all! I have 5 horses that range from 12-35 years old and a pack of “tiny dogs” (2 Jack Russell’s and 3 rescued Chihuahuas) that keep me well-entertained. I’m a member of the Red Hats and Purple Chaps and I participate in many parades with our ladies in Indiana, Louisville and Chicago.

Hello! My name is Brandi Maxie and I am an RVT who joined the Large Animal team at Purdue in July 2018. I was born and raised in central Ohio where I earned my RVT from Columbus State Community College in 2002. I worked for 5 years at The Ohio State University Veterinary Teaching Hospital in the Equine surgery, medicine and ICU departments. I spent the next 12 years at OSU Large Animal Services (Ambulatory) where I enjoyed an incredible variety in my duties that challenged me and helped me grow.

The collegiality and support here at Purdue has smoothed the transition. I am expanding my skills and am challenged every day in the best way. The way that the technicians are utilized here is very impressive and they are truly passionate about their patient care, career path, and student development.

In my spare time, I enjoy showing, lure coursing, and doing therapy work with my 5 Rhodesian Ridgebacks. I also enjoy hiking nature trails with the dogs, traveling, trail-riding my horse and cooking.
Equine granulocytic anaplasmosis (EGA), often referred to as Anaplasmosis, is a disease caused by an infection from bacteria called *Anaplasma phagocytophilum*. EGA has also been previously referred to as equine ehrlichiosis or equine granulocytic ehrlichiosis. Anaplasma bacteria live in the gut of *Ixodes* ticks, similar to the bacteria that causes Lyme disease, and is transmitted to horses through a tick bite. The bacteria then enter the circulation, and once the bacteria enter the bloodstream, they invade the white blood cells that normally protect the body from these types of infections. After invading the white blood cells in the blood, the *Anaplasma* organism alters white blood cell function, and the horse loses the capability of fighting off other infections. The organism also affects the bone marrow production of red blood cells, white blood cells and platelets. The mechanism for the red cell and platelet changes is not completely understood, but an immune-mediated cause has been proposed. In addition, the presence of the bacteria is thought to induce profound inflammatory reactions causing damage to organs like the spleen, liver, lungs, and leaky blood vessels leading to edema.

**Clinical Signs** – Studies state that it takes less than 14 days after the tick bite for the animal to begin showing signs of *Anaplasma* infection. The course of the disease depends on the age of the horse and how long the infection produces illness. Horses over 4-years-old tend to develop a fever, stop eating, take on a yellowish color, have small pin-point areas of bleeding on their gums (Figure 1), have swelling in their lower limbs, are often lethargic, and may seem wobbly or not want to move at all. Younger horses do not tend to develop as severe clinical signs, and horses less than 1-year-old may not show many signs at all. Because the body is unable to protect itself from other infections, animals infected with *Anaplasma* may also develop signs associated with secondary infections or may injure themselves from being wobbly and uncoordinated. These clinical signs may point your veterinarian to rule out other diseases like liver disease, equine infectious anemia (EIA), immune-mediated destruction of red cells and platelets, and other viral diseases.

**Diagnostics** – Anaplasmosis can be diagnostically challenging and several different blood tests may be helpful but not all are definitive. A stained blood smear examined microscopically may show multiple bluish-gray inclusions of the immature stages of *Anaplasma* in the white blood cells (Figure 2), but there is only a short-window of time that the *Anaplasma* may be maturing in those cells (typically days 3-5 post-infection), and the presence of the inclusions cannot be relied upon for diagnosis. A PCR test has been shown to correctly and accurately identify the presence of *Anaplasma phagocytophilum* DNA in samples that may not have sufficient amounts of the organisms in the blood cells to identify under the microscope. A complete blood cell count can be done to identify decreased numbers of blood cells, and, while helpful or suggestive of infection, is not a definitive diagnostic test. Lastly, paired antibody titers collected several weeks apart can also be used but would not aid in a timely diagnosis.

**Treatment and Prognosis** – Luckily, Anaplasmosis can be treated and has an excellent prognosis. An antibiotic, oxytetracycline, can be administered through an IV catheter for a few days for effective treatment, or a combination of IV and oral antibiotics, oxytetracycline and doxycycline, also have been shown to work well. The addition of anti-inflammatories like flunixin meglumine (Banamine) help in making horses feel better while recovering from the infection and reduce any systemic inflammation that may be occurring. Typically, there will be clinical improvement within 12-24 hours. If the horse goes untreated, the disease should improve on its own within 2-3 weeks; however, the weight loss, limb swelling, and wobbliness could lead to complications. In a hospital setting, other treatments like fluids through an IV catheter could help the horse recover.
I’ll bet you a dime, your horse doesn’t have Lyme!

Co-authored by Stacy H. Tinkler, DVM, MPH, Dipl. ACVIM-LA and Erin Yanoviak, DVM (Class of 2018)

**Lyme disease** is a tick-borne disease caused by an overactive immune response to the bacterium *Borrelia burgdorferi* (Figure 1). Lyme disease affects multiple organ systems, and in humans commonly infects the skin and joints. In the United States, black-legged ticks (*Ixodes scapularis*) infected with *B. burgdorferi* can transmit this bacterium to animals and humans. Even though greater than 50% of *Ixodes* ticks in prevalent areas such as the northeastern states and Wisconsin are infected with *B. burgdorferi*, only 2% of humans bitten by these ticks are likely to develop Lyme disease. Indiana is an emerging risk area for Lyme disease because it is located between Lyme disease endemic areas (Figure 2). This disease not only affects humans, but also our furry companions including dogs and horses; however, true *B. burgdorferi* infection with resulting Lyme disease in horses is not common.

Most humans are infected by the immature form of the tick known as the nymph. Nymphs are difficult to see because they are less than 2mm in size. Nymphs and tick larvae typically feed on small to medium sized animals, whereas adult ticks typically feed on larger herbivores such as deer, sheep, cows, and horses (Figure 3). The Lyme disease bacteria lives in the tick’s gut and is transferred to the animal during blood meals. Once the tick attaches to a host, the Lyme disease bacteria travels from the gut to the tick’s salivary glands. This bacteria is then introduced into the host through the tick’s saliva when bitten. *Borrelia burgdorferi* has 3 outer surface proteins, OspA, OspC and OspF, and these surface proteins help the bacteria hide from the immune system. For example, by displaying OspA, *B. burgdorferi* is able to remain in the gut of the tick, and by displaying OspC, it can adhere to the tick’s salivary glands. The tick must be attached for at least 36-48 hours for the host to be infected.

**MYTH:** My horse is lame and has a swollen joint so it has Lyme disease.

**FACT:** There is much that we do not know about Lyme disease in horses. Symptoms are variable but some documented syndromes attributed to Lyme disease in horses include: neurologic disease (equine neuroborreliosis), eye disease (uveitis), and skin disease (cutaneous pseudolymphoma). Unlike human Lyme disease, excess fluid around the joints has been minimal in most Lyme-suspect horses. Infection with other tick-borne organisms such as *Anaplasma phagocytophilum* is a much more common cause of tick-borne disease in horses.

**MYTH:** You must always treat when horses have a positive blood test for Lyme disease. **FACT:** Diagnosing Lyme disease in humans as well as horses is a challenge, and there are no definitive antemortem (in the live animal) tests in the horse. Serum tests for antibodies or for the *B. burgdorferi* outer surface proteins Osp A, C and F document exposure to *B. burgdorferi*, meaning that a tick with *Borrelia* bit the animal being tested and that it was exposed to or infected with *B. burgdorferi*, not that it is necessarily suffering from Lyme disease. Many clinically normal horses living in areas where Lyme is present often have detectable antibody levels to *B. burgdorferi*, and the disease is often over-diagnosed. Ruling out other more common diseases that might explain a horse’s symptoms should be done first, and is most important.

**FACT:** Treatment of horses not showing signs of disease but with a positive blood test will result in the unnecessary treatment of many horses resulting in extra expense, increased risk of possible adverse reactions, and inappropriate use of antibiotics. Horses with symptoms, and in which all the other more common causes of eye, neurologic and skin disease have been ruled out should be the only animals treated with antibiotics. Treatment for *Borrelia* includes the tetracycline antibiotics, such as oxytetracycline, doxycycline, and minocycline. In comparison to treatment of Lyme disease in humans, treatment of the disease in horses is complicated by the difficulty in confirming the diagnosis, poor bioavailability of oral antibiotics commonly used for treatment, and the longer duration of infection in horses prior to beginning treatment. The appropriate duration of antibiotic treatment is still unknown in horses but should be based on clinical response to therapy and, to a lesser degree, a decrease in serum antibody level. Treatment with steroids is not recommended unless the horse has acute and severe eye or...
Lyme (continued from page 5)

neurologic disease as their use has been associated with harmful outcomes in some cases. More research is needed in this area.

**MYTH:** The prognosis for recovery for all Lyme disease in horses is good.

**FACT:** The prognosis for horses treated for neuroborreliosis is reportedly poor, with only a single case of successful treatment reported in the literature. The prognosis for horses with Lyme-induced uveitis is also poor for recovery of vision. Antibiotic treatment resulted in an excellent outcome in one horse case with Lyme pseudolymphoma in the literature. Differences in prognosis, poorer in horses versus humans with confirmed Lyme syndromes, are likely related to duration of infection before treatment, and species differences in bioavailability of the antibiotic treatments.

**MYTH:** There is no way to prevent Lyme disease and treatment is the best way to combat the disease.

**FACT:** The best way to prevent equine Lyme disease is to prevent tick exposure and prolonged tick attachment. Late summer, fall, and early winter are the most common times for adult ticks to attach so during these times removing ticks should be a priority.

**How to remove a tick (Figure 4)**

1. Use a fine-tipped tweezer to grasp the tick as close to the skin surface as possible
2. Pull upward with steady, even pressure
3. Clean the bite area with soap or rubbing alcohol

References:

**Take Home Points**

Lyme disease in horses remains a challenge to diagnose because testing for Borrelia is difficult and there is a large variation in clinical signs when this disease does occur in horses. It is possible for a horse with vague clinical signs of Lyme disease to test positive and to not actually have the disease. More common causes of such clinical signs must be ruled out before making a diagnosis of Lyme disease. Since Indiana is an emerging risk area for Lyme disease, it is important to keep this disease in the back of your mind but understand that true cases of disease are still rare, and diagnosis and treatment are complicated and controversial in horses. An ounce of prevention is still worth a pound of cure, and this is especially true as it relates to equine Lyme disease. The most important way to prevent Lyme disease is to prevent exposure to ticks as best you can.


**Anaplasmosis (continued from page 4)**

eter, standing leg wraps, and stall confinement help improve the recovery time for those with Anaplasmosis. Unfortunately, there is still the chance of relapse of infection within 30 days, even in treated animals.

**Tick Prevention** – Utilizing tick repellents and preventative treatments are the best way to prevent equine granulocytic anaplasmosis, as well as other tick-borne diseases like Lyme disease. Some ways to help prevent your horse from being bitten by ticks is to keep areas where your horses are pastured dry, regularly disturbed, and well-exposed to the sun. Other preventative management measures include mowing pastures, clearing debris and leaves from the pastures, and if possible, deterring and keeping deer away. Topical products are available for horses that help prevent tick exposure. Products with permethrin have been well-associated with high success of repelling ticks. Products are available as wipe-on, pour-on, spot-on, and spray-on application that all seem to have similar effectiveness when applied appropriately. It is important to remember that dirt, sweat, and water can shorten the length of effectiveness of the tick repellents. It is important to consult your veterinarian about the best product for your specific horses’ needs.

References:
https://extension.umn.edu/horse-health/tick-diseases-horses
If you have spent any time in a racing or performance horse stable, chances are you have seen a tub or two of thyroid supplement medication. A large number of performance horses receive thyroid medication daily. You might ask, “Is hypothyroidism really that common in the horse world?” The answer to that question is no. Next you might ask, “So what’s the deal with all the horses on thyroid supplement?” The answer to that is, “It’s complicated.” There are a large number of reasons why horses are placed on thyroid medication—some good and some not-so-good.

Thyroid hormones are important for maintaining resting metabolic rate and energy metabolism. Like people, the thyroid gland in a healthy horse produces all the thyroid hormone the body needs. If the thyroid gland doesn’t make enough hormone, hypothyroidism occurs.

Unlike people, true hypothyroidism is extremely rare in adult horses. When it does occur, clinical signs include cold intolerance, dry eye, and a roughened hair coat. Your veterinarian can diagnose hypothyroidism by performing a thyroid gland stimulation test. Just measuring blood thyroid hormone concentrations is not sufficient to determine whether or not a horse is hypothyroid. This is because there are a huge number of factors that can lower resting blood thyroid hormone levels for a short period of time, but the thyroid gland is fine and will respond to stimulation when more thyroid hormone is needed.

At times, a horse’s thyroid gland is normal, but an increased dose of thyroid hormone supplement is prescribed in order to increase a horse’s metabolic rate. This is done when a horse is suffering from Equine Metabolic Syndrome and has higher than normal blood insulin concentrations even after when placed on a low-carbohydrate diet. The signs of Equine Metabolic Syndrome include having a high body condition score, increased fat long the top line and a creasy neck, and, most importantly, laminitis. Adding thyroid supplement to the treatment plan of a horse with Equine Metabolic Syndrome promotes fat utilization and weight loss. Talk to your veterinarian if you are worried about Equine Metabolic Syndrome in your horse. You can discuss whether including thyroid hormone treatment is a good choice.

Finally, some place horses on thyroid supplement if they feel the horse is backing off feed, losing energy, or showing any of a host of non-specific issues. In these cases, thyroid hormone is used as a non-specific “pick-me-up.” There are two problems with taking this approach. First, a serious medical problem might be covered up or missed. Any horse that loses weight or becomes depressed needs to be evaluated. Secondly, giving hormone takes away the need for horse’s own thyroid gland to make hormone, and the gland will shut down. It will not be able to respond normally to the stimuli that would usually cause more hormone secretion.

Thyroid hormone can be a life-saving therapy, helping to prevent or manage laminitis in horses with metabolic disease. But it should never be given “just because.” It causes changes to every cell in the body; it should only be given if you understand exactly why the horse needs to receive it under the guidance of your veterinarian.

**Melanomas (continued from page 2)**

**Treatment options**

Treatment options for metastatic melanomas are limited, but equine melanomas remains an ever-growing field of research. Surgical removal is curative in some cases (melanocytic nevi, dermal melanosomas), but ineffective for others (dermal melanomatosis) due to the high prevalence of internal spread, invasiveness, and difficult surgical correction. In addition, many tumors previously removed may return, and may even increase in size and number. Chemo- therapeutic agents, such as cisplatin, may be effective for smaller tumors (<3 cm) when implanted directly, but have had inconsistent results in treatment of larger, metastatic tumors or those already treated with other therapies. Electrochemotherapy, or delivery of electrical pulses, has been used as a supplemental therapeutic option to help absorption of cisplatin into the melanoma tissue, but is less effective than other treatment options. Cimetidine, a histamine receptor antagonist, was previously considered an effective therapy, but is less electrical pulses, has been used as a supplemental therapeutic option 4-cisplatin, may be effective for smaller tumors (<3 cm) when implanted directly, but have had inconsistent results in treatment of larger, metastatic tumors or those already treated with other therapies. Electrochemotherapy, or delivery of electrical pulses, has been used as a supplemental therapeutic option to help absorption of cisplatin into the melanoma tissue, but is less effective than other treatment options. Cimetidine, a histamine receptor antagonist, was previously considered an effective therapy, but it should never be given "just because." It causes changes to every cell in the body; it should only be given if you understand exactly why the horse needs to receive it under the guidance of your veterinarian.

**New treatments on the horizon**

In recent years, a new therapy option has spread to the equine community following treatment of canine melanomas with vaccine therapy. A DNA vaccine called Oncept™ has been circulating in research studies and shows promise for use in treating equine melanomas. A recent study involved the administration of Oncept™ to horses using the same vaccination protocol as used in dogs (4-biweekly injections, followed by a 6 month booster). Results are promising for use of this vaccine in horses, but it is still undergoing clinical trials at this time before becoming an officially approved treatment. Another new and upcoming option is a plasmid DNA vaccine called ImmuneFX that contains a gene from the bacteria Streptococcus pyogenes. The vaccine is injected into tumors, and the tumors then express the gene which causes a strong anti-tumor response in the body and effectively reduces tumor burden by 40-50%. Lastly, keep an eye out for betulinic acid for melanoma chemotherapy. It’s derived from birch bark and is reported to have strong anti-inflammatory properties! Although only minimal research has been done in this area so far, these three options hold promise for treating equine melanomas.

**References:**

The Equine Sports Medicine Center

Purdue’s Equine Sports Medicine Center is dedicated to the education and support of Indiana horsemen and veterinarians through the study of the equine athlete. The Center offers comprehensive evaluations designed to diagnose and treat the causes of poor performance, to provide performance and fitness assessments, and to improve the rehabilitation of athletic horses. Other integral goals of the Center are to pioneer leading-edge research in the area of equine sports medicine, to provide the highest level of training to future equine veterinarians, and to offer quality continuing education to Indiana veterinarians and horsemen. For more information visit our website:

www.vet.purdue.edu/esmc/
Navicular Disease

By Brent Unruh, DVM Student (Class of 2020)
Edited by Tim Lescun, BVSc, MS, PhD, Dipl. ACVS

What is navicular disease?
The navicular bone is a small bone present on the backside of the foot between the short pastern bone and the coffin bone. In a healthy horse, the navicular bone functions to equally distribute mechanical forces between the coffin (pedal) bone, short pastern bone and the deep digital flexor tendon (DDFT). Therefore, navicular disease is the result of degenerative changes occurring within the navicular bone or with the soft tissue structures that make up the navicular apparatus. The navicular apparatus is comprised of the distal sesamoid impar ligament, the navicular suspensory ligament, the navicular bursa and the deep digital flexor tendon (Figure 1). This disease is a common cause of lameness in horses 4 to 15 years of age, encompassing roughly 30% of all lameness cases. Some will refer to navicular disease as a syndrome because the inciting cause is unknown and it typically does not affect just one structure. The source of pain is multifactorial ranging from the bone itself to components of the navicular apparatus, or a combination of both. There are two main mechanisms that are believed to result in navicular disease: vascular compromise to the foot and biomechanical abnormalities. The most accepted mechanism is that biomechanical abnormalities of the foot alter the normal forces present on the navicular apparatus leading to tissue degeneration. Alterations in the biomechanical forces can be due to poor conformation of the foot and pastern, hoof imbalances, improper shoeing or trimming, excessive weight bearing and exercise on hard surfaces.

How do we diagnose it?
Diagnosing navicular disease can be achieved using the clinical presentation, a thorough lameness exam and imaging modalities. Horses suffering from navicular disease typically present with a progressive, bilateral forelimb lameness resulting in decreased performance, stiffness, short strides and an unwillingness to make short turns. On lameness exam, these horses may exhibit sensitivity over the frog of the foot when hoof testers are applied. Specific lameness tests and nerve blocks can be performed to further localize the origin of pain to the heel region. However, the anatomy of the equine foot is complex, so diagnosis of navicular disease must include imaging such as radiographs.

(continued on page 2)
Navicular Disease (continued from cover)

Advanced imaging, which includes ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI), is highly encouraged. MRI is the gold standard as it allows a more accurate assessment of bone and soft tissue structures of the foot (Figure 2).

How do we treat it?
There is not a gold standard treatment for navicular disease, as it is not curable. Instead, the treatment centers around managing the level of pain present through multiple, different techniques, such as medical or surgical therapy.

Medical Management
Medical management of navicular disease encompasses three major methods: rest and controlled exercise, corrective trimming and shoeing, and systemic and local medications. Although rest is important, it is recommended that stall rest be utilized for 3 weeks followed by 3 weeks of controlled exercise, such as walking. This period typically follows corrective shoeing allowing the horse to adjust to wearing the shoes and inflammation in the navicular apparatus to subside. The method of corrective trimming and shoeing of horses with navicular disease is crucial for the management of pain. It is imperative that the veterinarian and farrier utilize the available imaging modalities to choose the most appropriate type of shoes to restore balance to the foot and reduce biomechanical forces on the navicular apparatus. Common shoe types utilized in navicular patients include, but are not limited to, regular shoes with heel elevation and egg-bar shoes (Figure 3 & 4). Shoes with heel elevation decrease the tension placed on the DDFT to allow better weight distribution and reduce the pressure applied to the navicular apparatus. The main disadvantage to this shoe type is that it slows heel growth, making patient selection an important factor. Egg-bar shoes are not as effective as heel elevation shoes, but are useful in patients with underrun heels because they help redistribute weight across the sole of the foot. Systemic medications should be utilized in addition to other management techniques (continued on page 4)
Neonatal Isoerythrolysis (NI) – Why Blood Typing Can Be Important

By Hannah Clinton, DVM Student (Class of 2020) – Edited by Sandra D. Taylor, DVM, PhD, Dipl. ACVIM-LA

Just like a person may be “A positive” or “O negative” with respect to blood type, horses have varying blood types as well. There are many different red blood cell antigens (factors) that determine a horse’s blood type, but the Aa and the Qa antigens have the potential to cause the biggest reactions. While a horse's blood type is important when it comes to blood transfusions, it is also important in the realm of reproduction.

If a mare does not have the Aa antigen, but the stallion does, the foal is at risk for developing a hemolytic blood disorder known as neonatal isoerythrolysis, or NI. NI is very rare and only occurs 1-2% of the time, but it is life-threatening when it happens. For NI to occur, the foal would have inherited the sire's dominant Aa positive blood type and, during gestation, the mare will be exposed to the foal’s blood, which is recognized by the mare’s body as a foreign substance. The mare will then develop antibodies towards the foal’s blood. A second way that a mare can be exposed to a foal’s blood is during foaling, but since antibodies take time to develop, the first foal from the same mare/sire pairing is unaffected in this scenario. However, when a second foal with the same genetics is born, the mare already has anti-Aa antibodies circulating in her blood. When the foal suckles colostrum in the first 24 hours, it absorbs the dam’s antibodies from the gut into the blood, which subsequently leads to destruction (lysis) of the foal’s red blood cells, causing hemolytic anemia.

A foal with NI will seem healthy at the time of foaling, but will become lethargic and depressed within the first 2-4 days of life. The foal’s mucous membranes and sclera (whites of the eyes) will appear icteric (yellow/jaundiced) due to pigment accumulation from the red blood cell destruction (Figures 1 and 2). How rapidly the disease progresses depends on the red blood cell antigen (Aa or Qa) and the severity of the red blood cell destruction. Seeking treatment as quickly as possible is necessary to save the foal’s life.

Most veterinarians will diagnose this disease based on the history of a multiparous mare with a foal that develops clinical signs (jaundice, weakness, depression) within a few days after birth. Bloodwork may be submitted to determine the degree of anemia and to determine if other concurrent diseases are present. If the anemia is mild, minimally handling the foal and allowing it to rest is often sufficient for recovery within a few days. However, if the PCV (red blood cell volume) is below 12% (normal: 25-30%) or if the foal is depressed and lacks a suckle reflex, a blood transfusion is appropriate.

(continued on page 4)
Navicular Disease (continued from page 2)

and include non-steroidal anti-inflammatory drugs (NSAIDs) and bisphosphonates. Bisphosphonates, such as tiludronate disodium and clodronate disodium, are utilized in navicular patients with radiographic changes within the navicular bone. These drugs work to inhibit bone resorption, potentially slowing the progression of the disease. These drugs should be used carefully as they affect all bones, not just the navicular bone. Local medications are implemented by injection directly into the navicular bursa. However, injection into the bursa is difficult so injections can be placed into the coffin joint, which allows diffusion into the navicular bursa. Medications injected directly into the bursa or coffin joint decrease inflammation and support joint health. These medications include corticosteroids, polysulfated glycosaminoglycans (PSGAGs), biologics (PRP or Irap) and hyaluronic acid.

Surgical Treatment

Surgical intervention for treatment of navicular disease includes navicular bursoscopy and palmar digital neurectomy. Navicular bursoscopy is a minimally invasive procedure allowing improved visualization of lesions in the navicular bursa and the DDFT (Figure 5). These lesions are then able to be debrided with the goal of alleviating pain from the disease. This technique can also be utilized to further diagnose the extent and presence of lesions that are not detected during other diagnostic tests. Palmar digital neurectomy is the process of the cutting the palmar digital nerve on both sides of the foot, as it is the nerve responsible for sensing pain in the foot. This is a salvage procedure and is performed when all other management modalities have failed. This procedure is contraindicated in patients with navicular disease involving the DDFT because it can increase the risk of rupturing this tendon. Other complications of this procedure include surgical site infections, nerve regrowth, and painful neuroma formation.

Neonatal Isoerythrolysis (continued from page 3)

The mare can serve as a donor if her blood is “washed” to remove antibodies, or a gelding can be used as a donor. Typically, 1-2 liters of washed red blood cells are transfused, but calculations can be performed to determine the exact amount needed for a particular foal. During treatment for NI, the foal can (and should) be allowed to nurse the mare. This is safe because after 18-24 hours, the foal’s intestines are no longer able to absorb colostral antibodies, and thecolostrum (which contains these antibodies) has already been consumed.

If a mare has previously had foals that developed NI or blood typing reveals that she is at risk, assessing a potential sire’s blood type is essential for preventing this disease. If an incompatible mare and stallion are bred, the foal should be prevented from suckling at birth and provided with an alternate source of colostrum. The foal’s antibody levels should be monitored at 18-24 hours to ensure adequate colostrum/antibody intake, and then can be allowed to return to suckling the mare thereafter.

In summary, NI is rare but life-threatening. Understanding the disease and how to prevent it can save your foal’s life. Please contact your veterinarian or the Purdue University Large Animal Medicine department if you are interested in blood typing your mare and stallion, or if you have questions. Happy foaling!

References:

Figure 3. Regular shoe with heel elevation. (https://lpinning.com/originals/bb/40/c7/bb40c775909230e148439f585cdef50e.jpg)

Figure 4. An egg-bar shoe (https://www.willowbrookequinefarriers.co.uk/)

Figure 5. Image during a navicular bursoscopy showing a lesion on the navicular bone. (https://www.semanticscholar.org/paper/Navicular-bursoscopy-in-the-horse%203A-a-comparative-Haupt-Carton/3836831fe54306ae4622d602a966704e194cf5d83/figure/6)
For years, Vitamin C (ascorbic acid) has been recognized for its ability to help us fight off the common cold and shorten the time that we’re out of commission! You may have taken supplements like Emergen-C® and Airborne® to boost your immune system during flu season, since Vitamin C is a powerful antioxidant and can be “used up” during illness.⁴ Since humans cannot make their own Vitamin C, severe depletion can lead to “scurvy.”

Horses are different from people in that they can make their own Vitamin C, but their Vitamin C stores can also become depleted during illness. This is especially true of severe illness, such as sepsis. Sepsis is defined as systemic (“whole-body”) inflammation secondary to infection, and is common in neonatal foals that don’t ingest adequate amounts of immunoglobulins in the dam’s colostrum (Figure 1), and in adult horses with severe pneumonia or enterocolitis. Septicemia is a term that specifically refers to infection within the bloodstream. Giving appropriate antibiotics is usually sufficient for eliminating infection in septic horses, but it is currently very difficult to treat the systemic inflammation that has gone haywire and become more exuberant than necessary. This inflammation is associated with oxidative stress (excessive free radical production throughout the body) and release of inflammatory mediators that can lead to decreased oxygen delivery to tissues and subsequent organ failure. An intensive care approach to treatment typically includes antibiotics, intravenous (IV) fluid support, blood pressure support, and IV nutrition. In adult horses, keeping the feet cold is important to help prevent development of laminitis (“founder”). Even with intensive treatment, the survival rate of septic horses is only 60-70%.²³

Vitamin C is well-known for its antioxidant effects, but its lesser-known qualities, including its anti-inflammatory and anti-bacterial properties, are just as important. Vitamin C has been shown to improve the efficiency with which white blood cells eliminate pathogens, and it also increases production of other antioxidants, including Vitamin E (α-tocopherol). In people, a transporter protein in the gastrointestinal tract limits the amount of Vitamin C that can be absorbed into the blood stream to approximately 500 mg per dose.⁴ This means that if high doses are needed, Vitamin C must be given intramuscularly (IM) or IV. It is unknown whether or not there is a similar limit to intestinal absorption of Vitamin C in horses, but IM or IV administration are reasonable options.

A recent study led by Dr. Sandra Taylor and Dr. Mindy Anderson at Purdue University’s College of Veterinary Medicine showed that when Vitamin C was given IV to septic horses, the white blood cells were protected from destruction by bacteria. Furthermore, the sepsis itself decreased Vitamin C levels in the blood of most horses prior to Vitamin C administration. A separate study conducted by the same researchers in collaboration with the University of Georgia’s College of Veterinary Medicine found that the higher the IV dose of Vitamin C given to healthy horses, the better the antioxidant capacity within the blood. These studies have paved the way for a future multi-center clinical trial in septic horses that present to the Purdue University Veterinary Teaching Hospital, other university veterinary teaching hospitals, and equine private practices to compare the outcome of horses treated with Vitamin C compared to those not treated with Vitamin C. We expect that Vitamin C administration will improve outcome, and that it might become an inexpensive standard-of-care treatment option for septic horses. Step-wise studies of this nature are critical for implementation of new treatment options for any disease, and researchers at Purdue University are committed to leading such studies with the end-goal of improving the health and welfare of horses worldwide.

It is likely that if Vitamin C is found to be beneficial in treating septic horses, high doses will be required and therefore will need to be given IM or IV. Alas, feeding a bunch of oranges likely won’t cut it! Stay tuned for results and recommendations!

Figure 1.
Neonatal foal with sepsis undergoing intensive treatment.

References:
Moon or Immune Blindness –
An Update on Equine Recurrent Uveitis

By Jessica White, DVM Student (Class of 2020) – Edited by Wendy Townsend, DVM, Dipl. ACVO

Equine recurrent uveitis (ERU), better known to some as “moon blindness,” has been documented as far back as 4th century AD when scholars suspected this fluctuant condition of the equine eye was due to changes in the moon. ERU is considered an immune-mediated disease that results in recurrence after a primary uveitis caused by infectious agents, environmental factors, and/or genetics. As the most common cause of blindness in horses, ERU is seen across many breeds and ages, but is most common in breeds such as Appaloosas, draft breeds, Knabstruppers, Icelandics, and some warmbloods. Two methods of classifying ERU exist: stage of disease and disease presentation. Stage of disease is broken into active (aka acute), quiescent, or end-stage. A horse with active ERU will have visible discomfort and inflammation of the eye as well as intra-ocular inflammation compared to a horse with quiescent ERU that will visibly appear comfortable with no evidence of current intraocular inflammation. End stage ERU is characterized by changes from chronic inflammation such as phthisis bulbi (a shrunken, nonfunctional eye) and vision loss, with or without cataracts, lens luxation, retinal detachment, or abnormal pupil structure. The three presentations of ERU are classic recurrent, insidious, and primary posterior. Classic ERU consists of an active episode, followed by periods of quiescent then recurrent active ERU. The insidious ERU horse will appear comfortable in the affected eye but has persistent low-level inflammation in the affected eye and is most common in Appaloosas, Knabstruppers, and draft breeds. Posterior ERU is less common than the other two but originates from inflammation of structures in the back of the eye rather than the front.

Several key signs that should make you suspicious of ocular disease are squinting, tearing, miosis (a small constricted pupil), redness or swelling of the tissues around the eye, and changes in the color, opacity, and surface of the cornea. Call your vet immediately if you have eye concerns as these can progress very quickly and may need immediate intervention.

Upon examination, your veterinarian will conduct a physical and complete ophthalmologic exam to assess what changes they see in the eye, determine what the potential causes are, and what further diagnostic tests and treatments they would like to recommend. Further diagnostics will often include a fluorescein stain of the cornea to check for ulcers, applying a topical anesthetic to the cornea to facilitate examination of the area around the eye, and tonometry to measure intraocular pressure. Depending on your region and index of suspicion by your veterinarian, Leptospirosis testing may be indicated for the bacteria _L. pomona_ which is a documented cause of ERU in some cases. If Leptospirosis is strongly suspected as the cause of active uveitis, the systemic antibiotic doxycycline will also be included in the treatment plan.

Once your veterinarian rules out other conditions, long-term treatment will be required to preserve vision and keep your horse comfortable. However, the prognosis for vision long-term is poor. The mainstays of medical treatment for ERU include mydriatics (pupillary dilators) and anti-inflammatories. Mydriatics, like atropine, are topically applied 1-3 times/daily to treat ocular spasms and pain from intraocular inflammation. Anti-inflammatories consist of topical and systemic steroids and/NSAIDs to help decrease inflammation, control pain, and preserve vision.

Intravitreal injections of gentamicin (IVG) have been evaluated and deemed effective in treating ERU that is refractory to core treatments. In IVG, the antibiotic gentamicin is injected into the gelatinous tissue in the eye called the vitreous and has been shown to have low complication rates and <15% recurrence rates.

Current surgical treatment options consist of cyclosporine implants or a procedure called a pars plana vitrectomy (PPV). Cyclosporine implants are sustained-release devices discharging consistent levels of this immunosuppressive drug to provide long-term control of inflammation and very low recurrence rates. The best candidates for cyclosporine implants are horses with well-controlled ERU but are looking for a more long-term treatment option than the current mainstay topical and systemic medications. In PPV the vitreous is removed while the horse is under general anesthesia. Candidates for PPV are ERU cases with recurrent posterior segment inflammation but whose disease is well-controlled on medications so as not to have difficulty post-operatively.

Stable management is key to reducing recurrence of these episodes of inflammation as well as good, consistent health maintenance. Environmental considerations to decrease ERU include improving rodent control to decrease leptospira exposure. Minimizing eye trauma is also crucial by giving horses a good quality fly mask, removing pasture weeds or low branches, avoiding using hay nets, and softening objects in their environment, i.e. tape over bucket handles. Proper maintenance of the ERU horse includes keeping up with good hoof and dental care, as well as optimized nutrition and deworming plans. Additionally, in horses with ERU it is best to

(continued on page 7)
split up vaccines, giving individual rather than multivalent vaccines and to spread them out over at least two different appointments one week or more apart. Depending on the horse, some veterinarians may also recommend Banamine be given 24 hours prior to vaccination to help decrease ocular inflammation post-vaccination. In the case of high Leptospirosis-risk areas, there is one equine Leptospirosis vaccine created to prevent the systemic dissemination of *L. pomona* which may help reduce the risk of ERU in high risk horses. However, this vaccine is not USDA-licensed for the prevention of ERU and the ability to prevent ERU has not been demonstrated in any trials at this point. Horses with ERU must NOT be given the vaccination as horses with ERU have developed horrible inflammation after receiving the leptospira vaccine.

References:
The Equine Sports Medicine Center

Purdue’s Equine Sports Medicine Center is dedicated to the education and support of Indiana horsemen and veterinarians through the study of the equine athlete. The Center offers comprehensive evaluations designed to diagnose and treat the causes of poor performance, to provide performance and fitness assessments, and to improve the rehabilitation of athletic horses. Other integral goals of the Center are to pioneer leading-edge research in the area of equine sports medicine, to provide the highest level of training to future equine veterinarians, and to offer quality continuing education to Indiana veterinarians and horsemen. For more information visit our website:

www.vet.purdue.edu/esmc/
APPENDIX B

Research Projects in Progress Supported with Pari-Mutual Funds:

- Figueiredo M., Lescun T., Gimble J. Enhancing the Repair Potential of Equine-Derived MSC for Treating Post-traumatic Osteoarthritis
- Hooser S. Detection of Black Walnut Wood in Equine Bedding by PCR
- Lescun T., Hermida J., Main RP., Little D., Weng H-Y. Collagen Orientation and Tensile Strength of Equine Proximal Sesamoid Bones
- Lim CK., Pemberton S., Heng HG., Kritchevsky J., Jones-Hall Y. Ultrasonographic Morphology of the Gastrointestinal Tract of Healthy Horses: In Vivo, Ex Vivo and Histological Comparison
- Little D., Lescun T. Mechanosensitive Channels in Equine Musculoskeletal Soft Tissues
Title: Enhancing the repair potential of equine-derived MSC for treating post-traumatic osteoarthritis (PTOA)

Investigators: Marxa Figueiredo (PI, BMS), Tim Lescun (Co-I, VCS), Jeff Gimble (LaCell, Inc.)

Date: 12/13/2019

Preliminary Progress Report

The purpose of this project has been to optimize the chondrogenic and anti-inflammatory potential of equine mesenchymal stem/stromal cells (eqMSC) by using a novel Laminin Receptor (LAMR1)-targeted small molecule, compound C3, for preventing post-traumatic osteoarthritis (PTOA) progression. Our preliminary data shows promise for C3 in promoting chondrogenesis of equine ASC and BM-MSC and reducing inflammatory response in equine synoviocytes. However, the efficiency of C3 still must be optimized to the biology of equine MSC, in order to improve and maximize cartilage-specific and anti-inflammatory responses. Based on our preliminary data and recent literature on equine MSC biology, we proposed to significantly optimize C3 efficacy and to test the hypothesis that equine MSC can be primed by compound C3 for enhanced chondrogenic and anti-inflammatory activity for preventing PTOA progression, and current progress in each Aim is summarized.

Aim 1. To examine whether the chondrogenesis and anti-inflammatory efficacy of compound C3 can be optimized for equine MSC in co-culture systems. Towards this Aim, we are approaching the equine synoviocytes first, and a new MS student recruited to the project (Huff) has developed new culture strategies and stimulation protocols to augment the inflammatory gene expression observed in eqSYN. With the new eqTNFa stimulation, we discovered that this stimulus far exceeds the previous stimulus we were utilizing to achieve pro-inflammatory gene expression in these cells. This is a more promising and robust system in which to test the efficacy of our compound C3 and an excellent basis for proceeding to more complex cell-cell interactions in coculture systems.

Aim 2. To examine whether the chondrogenesis and anti-inflammatory efficacy of compound C3 can be optimized for equine MSC pre-treated with Poly I:C. We have explored the effect of PolyI:C in reducing inflammatory gene expression in ASC and are working on the experimental conditions to augment the effect on priming these cells with this TLR ligand.

Other activities and immediate goals.

We have presented several local posters and talks on this project: A. VanSickle research day: 1 talk by Dr Figueiredo, 1 poster (2nd place competition, by Danielle Keating, DVM 3rd year); B. VCS Research Talks, Dr. Figueiredo; C. PVM research day, 1 poster by Danielle Keating; D. PULSe program Spring meeting, 1 poster by Annika Robinson-Hudspeth). E, Krista Huff (BMS MS student), BMS692 seminar Fall 2019; F. Krista Huff, Submitted abstract to present poster at Experimental Biology 2020 (San Diego, CA) for the American Society of Biochemistry and Molecular Biology.
Another grad student (Cosette Rivera) that contributed to this research in the ASC polarization preliminary data won 1st place *Omicron Graduate Student Research Award* (PVM research day 2019). We would like to also disseminate the results soon in an orthopedic conference, and submit grants for pilot equine clinical trials following this project’s completion to the Grayson Jockey Club Research and the American Quarter Horse Foundations. Current plans are to prepare a manuscript for submission to a peer-reviewed journal in collaboration with the co-investigators (Lescun and Gimble) within a year. We applied for 3 grants relating to this project, i.e. an Equine Fellowship for a PhD student (not successful), an AgSEED 2020 (results in March), and a PVM proposal (to be reviewed). We continue to utilize the equine cell and collaborative resources developed in the former pilot project, and hope that these related projects can enhance our chances to leverage this research into extramural funding.
Lay Article

Abstract. The relevance of this project to the IN horse industry is to develop drugs for treating or preventing post-traumatic osteoarthritis (PTOA) progression in equine athletes. These drugs already have some promising effects in cultures using cells isolated from equine athletes, such as mesenchymal stem cells from bone marrow and adipose tissue (fat) and synoviocytes. We propose to develop culture models that can mimic a ‘joint in a dish’ in order to better understand the therapeutic effect of our drugs on cartilage and in reducing inflammation. We also propose to examine whether we can enhance the ability of stem cells to repair joints using a molecule called Poly I:C, recently suggested as a way to ‘license’ stem cells towards enhanced joint repair. With these ‘joint in a dish’ and ‘licensing to repair’ approaches, we propose we can optimize stem cell therapy with our drugs for equine athletes suffering or at high risk for developing PTOA.

The long-term goals of the overall program. Within the past 2 years, our lab has become increasingly interested in MSC for anti-inflammatory and joint repair purposes, successfully pursuing a pilot project to bring our research approaches into equine MSC biology. Our goal is to incorporate our equine data into an extramurally funded application within the next 2 years, in continued collaboration with Drs. Lescun and Gimble. Target foundations may include the Grayson-Jockey Club Research, the American Quarter Horse, and/or the Morris Animal Foundations, which would facilitate translation of this work into the clinic.
Detection of Black Walnut Wood in Equine Bedding by PCR

Progress Report

12 December 2019

**Summary:** The goal of this project, to develop a PCR-based test to identify black walnut wood in bedding used for horses, with the aim to make the test available to ADDL clients, is nearly complete.

*Hypothesis:* Black walnut (*Juglans nigra*) wood can be identified in wood products used for equine bedding material ranging from sawdust to wood shavings using the polymerase chain reaction (PCR).

*Specific Aim 1,* to design and validate a method of sample preparation for black walnut wood detection using PCR, including determinations of the sensitivity and specificity of the method, has been completed (see attached abstract presented by SBH at the annual meeting of the American Association of Veterinary Laboratory Diagnosticians, October 27, 2019).

*Specific Aim 2,* to adapt the method (SA1) for the ADDL Molecular Diagnostics testing platform and perform in-house validation, has been largely completed. The method from SA1 has been adapted to the ADDL testing platform. Validation using stored samples from historical field cases in which horses were affected with clinical signs indicating exposure to black walnut wood, is underway.

*Specific Aim 3* is to make the test available to ADDL clients and demonstrate the method on samples from field cases. Further validation using samples from contemporary field cases submitted to the ADDL will be carried out as they arrive and are analyzed.

Work to be Completed: Following validation in SA2, if successful, the test will be made available to ADDL clients. We anticipate positive identification of black walnut by PCR in the historical samples that we have available. If, for some reason, such as the length of time of storage, historical samples test negative by PCR, we will complete initial validation of the PCR test using black walnut wood spiked into pine wood. Further test validation will be performed as samples arrive from field cases. Until a sufficient number of samples from field cases have been tested, a disclaimer will be added to final case reports stating that the test is still in the validation process. We anticipate being able to offer the test in the first half of 2020.
Detection of Black Walnut Wood in Equine Bedding by PCR

Stephen Hooser$^{1,2}$, Keith Woeste$^3$, Rebecca P. Wilkes$^{1,2}$, Hilary Richards$^1$, Angela Chan$^1$, Christina Wilson-Frank$^{1,2}$

$^1$Indiana Animal Disease Diagnostic Laboratory, West Lafayette, IN, $^2$Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, IN, $^3$Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center (HTIRC), Purdue University, West Lafayette, IN

American black walnut (Juglans nigra) is a common hardwood tree. Exposure to black walnut wood in bedding can cause severe laminitis and ventral edema in horses. Currently, when bedding from suspected cases of black walnut toxicity is submitted for diagnostic testing, the sample is visually and microscopically inspected for the presence of black walnut wood. Unfortunately, if the bedding is composed of sawdust, or if the bedding is heavily soiled, it is extremely difficult to determine if black walnut is present. Individuals who have the necessary training and experience in wood anatomy are few, difficult to find, and expensive to hire. The goal of this study is to develop a polymerase chain reaction (PCR)-based test to identify black walnut wood in bedding used for horses.

Pure samples of wood shavings or sawdust from black walnut and other trees, such as pine, spruce, cherry, ash, etc., were obtained from the Purdue University Wood Products Laboratory. The samples were individually ground using a freezer mill and extracted using a modified Qiagen DNEASY Plant DNA isolation kit, or were extracted using a MagMAX Core Nucleic Acid Purification Kit. PCR primers were designed to amplify three sequences specific to black walnut and often used as DNA barcodes, i.e., ITS1 – ITS4, the matK gene, and the trnT-trnF intergenic spacer. Amplified DNA products were verified to have come from black walnut wood by reaction with restriction enzymes and evaluation of the resulting DNA fragments, or by direct sequencing of the amplicons. Preliminary results revealed that ITS1 - ITS4 was the most reliable template, and that primer nesting (nested PCR) was able to produce visible amplicons in positive controls containing low amounts of black walnut DNA. Assay sensitivity was determined by performing PCR analysis on decreasing concentrations of DNA from pure samples of black walnut. Assay specificity was determined by performing PCR on pure samples of black walnut or other individual woods, or by mixing samples of varying concentrations of black walnut with combinations of those other woods.

These studies indicate that PCR can be used as a specific and sensitive assay to positively identify black walnut wood in bedding used for horses.

This work was supported by the State of Indiana, and Purdue University College of Veterinary Medicine research account funded by the Total Wagers Tax.
PROGRESS REPORT for COMPETITIVE EQUINE RESEARCH FUNDS, 2019

December 10, 2019

TITLE OF GRANT: Collagen orientation and tensile strength of equine proximal sesamoid bones

PI: Timothy B. Lescun; Co-Investigators: Jesus Hermida, Russell Main, Dianne Little, Hsin-Yi Weng

This ex-vivo experiment was designed to investigate the relationship between regional collagen fiber orientation (CFO), tensile mechanical properties and mineralized microstructural features of proximal sesamoid bone (PSB), in both horses who suffered a fracture of this bone and non-fracture control horses from samples collected over 5 years in a single racetrack in the state of Indiana.

The specific aims are:

1. To determine whether longitudinal CFO in the PSB is lower in horses with fracture of this bone compared to non-fracture control horses.
2. To compare the regional tensile mechanical properties, mineralized microstructural features and water content of the contralateral PSB from horses with a PSB fracture to non-fracture control horses.

Progress report:

Forelimb PSBs from twenty horses, ten euthanized due to PSB fracture (fracture horses) and ten euthanized for reasons other than a musculoskeletal injury (control horses) from horses obtained from the Indiana Horse Racing Commission necropsy program at the Indiana Animal Disease Diagnostic Laboratory at Purdue University were identified for this study. Figure 1.

Figure 1. Scout CT scan of the group of PSBs from 10 horses euthanized as a result of fracture.
Radiographs and MRI (3 Tesla – Purdue MRI facility) of all forelimb PSBs were performed in all 20 horses. Dissection from the soft tissue and microCT analysis (140 um and 90 um resolution) was performed. Once microCT was performed, further imaging using an ultrashort echo time (UTE) sequence with a 7 Tesla MRI was completed to evaluate bones more specifically for water content. Prior to destructive testing, the PSBs were imaged with a clinical CT 64 slice scanner GE with a 0.675 slice thickness to correlate microCT findings with clinically available CT imaging.

Protocols for histology and mechanical testing have been created with PSBs different from the samples of the study. Currently, we are developing the protocols for software analysis of the μCT, and 3T MRI, and 7T MRI. For the rest of the current year, the bones will be prepared for histology and mechanical testing.

Beginning in January, 2020, histology analysis and mechanical testing will begin, followed by analysis of CT and MRI results, histology and mechanical testing results, data analysis and interpretation, and final reporting. Currently, this project is on track to have the primary analysis work completed by April, 2020. We plan to use the data for both publication and as preliminary results for external funding opportunities for further analysis of PSB fracture in the horse.

Respectfully submitted,

Timothy B. Lescun, BVSc, MS, PhD, Diplomate ACVS
2018 Progress Report for Competitive Equine Research Grants Program

Title: Ultrasonographic morphology of the gastrointestinal tract of healthy horses: in vivo, ex vivo and histological comparison

PI: Dr. Chee Kin Lim

PACUC protocol: 1701001536

Step 1: In vivo sample collection:

14 horses have been examined via ultrasonography from 9/11/2018 – 10/12/2018

<table>
<thead>
<tr>
<th>Horse Name</th>
<th>Body No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max Attack</td>
<td>393 142</td>
</tr>
<tr>
<td>Alkabar</td>
<td>393 113</td>
</tr>
<tr>
<td>Mick</td>
<td>393 216</td>
</tr>
<tr>
<td>Freckles</td>
<td>393 329</td>
</tr>
<tr>
<td>Jones</td>
<td>389 740</td>
</tr>
<tr>
<td>Louie</td>
<td>390 433</td>
</tr>
<tr>
<td>Mocha</td>
<td>392 964</td>
</tr>
<tr>
<td>Rangoon Belle</td>
<td>392 962</td>
</tr>
<tr>
<td>Sister Fiona</td>
<td>392 963</td>
</tr>
<tr>
<td>Romantic Reason</td>
<td>392 974</td>
</tr>
<tr>
<td>Shania’s Code</td>
<td>389 457</td>
</tr>
<tr>
<td>Smarty’s Packin</td>
<td>392 736</td>
</tr>
<tr>
<td>Warrior</td>
<td>393 409</td>
</tr>
<tr>
<td>Carly</td>
<td>393 403</td>
</tr>
</tbody>
</table>

Status: Step 1 COMPLETED

Step 2: Ex vivo sample collection and ultrasonography

14 equine gastrointestinal samples have been collected and examined via ultrasonography from 9/11/2018 – 10/12/2018

Status: Step 2 COMPLETED

Step 3: Histopathology slides completed

All histology samples have been prepared, H&E slides prepared, and scanned into Aperio for review.

Status: Step 3 COMPLETED

Step 4: Review of ultrasonography images

To be performed between December 2018 and January 2019 by Dr. Lim and Dr. Heng.

Status: Step 4 TO BE COMPLETED
Step 5: Review of histopathology slides
To be performed between December 2018 and January 2019 by Dr. Jones-Hall
Status: Step 5 TO BE COMPLETED

Step 6: Review of data/draft manuscript
Deadline: End of January 2019
Status: Step 6 TO BE COMPLETED

Step 7: 1st draft of manuscript with data analysis complete
Deadline: End of February 2019
Status: Step 7 TO BE COMPLETED

Prepared by:
Chee Kin Lim DVM, BVSc (Hons), MMedVet (Diagnostic Imaging),
Fellow MCVS, Diplomate ECVDI
Clinical Assistant Professor of Diagnostic Imaging
Department of Veterinary Clinical Sciences
Purdue University
College of Veterinary Medicine
625 Harrison Street
Lynn Hall
West Lafayette, IN 47907
Phone: (765) 494-0116
Fax: (765) 496-1108
**Interim Progress Report: Competitive Equine Research Funds (2018)**

**Mechanosensitive Channels in Equine Musculoskeletal Soft Tissues**

**PI:** Dianne Little

**Date of Award:** 4/13/2018

**Hypothesis:** Transient receptor potential (TRP) channels and Piezo channel expression is upregulated in the palmar or plantar joint capsule of the fetlock joint with increasing severity of osteoarthritis, and in the flexor tendons and suspensory ligament with increasing evidence of suspensory desmitis or tendinopathy.

**Specific Aim 1:** Characterize the expression of mechanosensitive calcium TRP and Piezo channels across various sites in the equine fetlock joint and distal limb tendons from cadaveric donors of in different degrees of fetlock, tendon and ligament health.

**Specific Aim 2:** Characterize the functional role of mechanosensitive calcium channels in tendon and joint capsule extracellular matrix synthesis, matrix organization and contractility under simulated loading conditions.

**Personnel:**
Dr. Kara Negrini (PVM DVM’18), a candidate for the degree of Master’s of Basic Medical Sciences degree is working on this as her main project towards her thesis requirement, effective May 2018. Her salary is being funded through Dr. Little’s start-up funding from the Department of Basic Medical Sciences, and she is a teaching assistant for the Anatomy courses in the professional DVM program. Thus, she is dedicating approximately 0.2% FTE to this project, providing greater degrees of effort and expertise to this project than originally proposed.

**Progress Towards Research Goals:**
We are making substantial progress on the research goals, and on the underlying scientific premise, both of which will position us well for clinical relevance, benefit to the horse racing population of Indiana, future funding support and for completion of the proposed studies.

**Specific Aim 1:** To date, an initial n=11 grossly normal samples from superficial and deep digital flexor tendons, suspensory mid-body and dorsal and palmar joint capsule from research horses euthanized as part of unrelated studies have been obtained. In addition, n=3 samples from these horses had osteoarthritis in one or more joints, or with superficial digital flexor tendinopathies in one or more limbs, allowing for intra-individual
evaluation of normal and diseased tissues. It was not possible to obtain samples from the Indiana Horse Racing Commission Post-Mortem Program, but adequate tissues have been obtained without this resource. Based on our results from our parallel human subjects work (currently in review at the Orthopaedic Journal of Sports Medicine), demonstrating the importance of: 1) accurately characterizing immune cell populations associated with individual TRP channel expression in fibrosis, most notably associated with sub-phenotypes of resolving pro-fibrotic macrophage populations, and 2) accurately characterizing the different fibroblast sub-types present at different disease stages, we incorporated this into our study design for Aim 1. Therefore, we designed an antibody battery to effectively characterize the expression of various TRP and Piezo channels associated with specific cell types in these equine samples. This will generate more useful data with respect to clinical relevance, publication and future funding than the originally proposed focus only on channel expression would have allowed. The upgrades to the CellSens software on the Olympus microscope are finally complete, and protocols for the quantitative evaluation of immunoreactivity in histological sections are now developed.

Refinement to previously proposed approach: The range of antibodies available and likely to work in these equine tissues was far greater for immunohistochemistry of frozen sections, than for formalin fixed paraffin embedded sections. Therefore, tendon sections were frozen for cryosectioning in optimal cutting temperature (OCT) media and sections are currently being cut with the Leica Cryostat housed in the PI’s laboratory. While cryosection of equine tendon is challenging, the CryoJane system affixed to the cryostat is critical for the success of this endeavor. This approach has the additional benefit of saving fees associated with the Histology core, so these funds were used for the purchase of additional antibodies to allow for necessary immune cell and fibroblast sub-phenotype characterization.

Specific Aim 2: For the microphotopatterning component of this work, we have completed the necessary protocol modifications, trouble-shooting, programming, and equipment upgrades in Bindley compared to our previous work on different and substantially better multiphoton microscopy equipment we used at Duke University. This will allow for completion of the microphotopatterning components of Specific Aim 2 in a timely manner. Additionally, we have now validated markers of collagen alignment and of tendon and tendon fibrosis using the microphotopatterns proposed in Specific Aim 2.

Other than these critical refinements to our microphotopatterning protocols, the timeline of Specific Aim 2 has been delayed somewhat, for scientific and budgetary reasons:

First, based on our human data the expression of various TRP channels is tightly coordinated with specific macrophage sub-phenotypes, and with specific fibroblast sub-populations. This expression changes across the disease spectrum, at least in joint
capsule in osteoarthritis (normal, mild end-stage). We need to understand what these populations are, and how they change in normal and diseased equine joint capsule, tendon, and ligament before pursuing functional studies on incompletely characterized cell populations. Once we know the defined cell populations involved, we will be able to sort for these specific cell populations using flow cytometry and know what cell types are represented in our functional assays. This approach will lead to much more robust data and greater likelihood of both clinically relevant information and additional successful funding applications as we move forward.

Second, funding was received at 77% of the original request. This >20% cut in budget has meant that we cannot afford to lease the equipment for cyclic loading and imaging of calcium flux for 15 months, as originally proposed. This lease will now need to be condensed into a 4-6-month period of time once histology results from Specific Aim 1 are known. As a downstream complication of this, in order to avoid expiration or decline in efficacy of expensive purchased calcium channel agonists and antagonists, all work involving isolated cells (microphotopatterning, collagen gel contraction assays, and the cyclic loading work) will now be performed simultaneously once the equipment is leased using an additional set of cells isolated from specific tissues to generate balanced datasets. However, the benefit to these budgetary constraints is that these changes in logistics will allow us to sort for the specific cell populations we know to be involved, to ultimately produce more robust data, and to produce a more accurate timeline of cell/TRP/Piezo channel involvement at different points in the disease process.

**Summary:** Within the constraints identified above, we are close to completion of Specific Aim 1, and once results of this aim are known, are poised for successful completion of Specific Aim 2. Support from this funding application was acknowledged in presentations at the David Van Sickle Musculoskeletal Days in Fall 2018. We have grant submissions for human subjects in review at the Orthopaedic Research and Education Foundation; this combined human-equine clinical approach will strengthen not only the options for improved treatment of horses with joint capsule fibrosis and suspensory desmopathy/flexor tendinopathy but will increase the likelihood of successful federal funding applications to continue this work beyond that which is achievable with the current award.
APPENDIX C

Research Projects Completed Supported with Pari-Mutual Funds:

• Dos Santos AP., Taylor SD., Woolcock A., Christian JA., Ruple A. Validation of a Novel Assay to Detect Intraerythrocytic Reactive Oxygen Species (ROS) by Flow Cytometry in Horses

• Hendrix K., Kritchevsky J. Recovery of Salmonella Bacterial Isolates from Pooled Equine Fecal Samples

• Lescun T., Breur G., Nauman E., Chandrasekar S., Adams S., Jones Y., Main R. Finite Element Modeling and Implant Nanosurfacing to Enhance Equine Fracture Treatment

• Taylor S., Cooper B., Grady S., Lescun T., Moore G., Davern A., Brunner T. Plasma Drug Concentrations of Ketorolac Tromethamine, Phenylbutazone and Flunixin Meglumine in Horses Following Single-dose Intravenous Administration

• Taylor S., Grady S., Lescun T., Moore G., Davern A. Analgesic Efficacy and Safety of Ketorolac, Phenylbutazone and Flunixin in a Model of Foot Lameness in Horses
Validation of a novel assay to detect intraerythrocytic reactive oxygen species (ROS) in horses

Summary: Reactive oxygen species (ROS), also known as free radicals, are a family of unstable reactive molecules derived from normal cell metabolism. When in excess, ROS are implicated with cell injury, aging, and cell death. Animals have developed antioxidant defense mechanisms to prevent free radical formation. When ROS generation exceeds the antioxidant capacity of the cell, oxidative stress occurs. Oxidative stress happens in horses after exercise, and it is most likely required for physiological adaptation to exercise. However, correlation between production of ROS and race performance has not been evaluated to date.

As proposed, we have validated the use of DCFH-DA in equine RBC by flow cytometry. The assay has demonstrated high specificity, stability, and precision to measure ROS in equine RBC (detailed result report attached). The measurement is fast, cheap, easy to perform, and has adequate specificity, precision, and stability, representing a great improvement in comparison with the current available methods.

Short-term goals: We would like to determine the sensitivity of this assay in horses before and after intense exercise, and in pathological conditions. In the near future, we want to evaluate if horses with good performance will produce less ROS or if they have a more robust antioxidative system in comparison to animals with poor performance, as well as to evaluate the influence of antioxidant supplementation in racehorses.

We have prepared one poster for the Phi Zeta Research day and for the ASVCP annual meeting. A manuscript describing this assay is currently being written. We hope that the results of this research will help the Indiana Horse Industry by providing a new tool to improve detection of free radicals in horse blood and help determine whether or not ROS production significantly affect performance.

Below is a detailed report of our results:
1. Development of the proposed methodology and results

Thirty-one healthy horses, as determined by physical examination and absence of significant abnormalities from laboratory data (complete blood count and chemistry panel), were included in this study (Tables 1 and 2). Whole blood EDTA samples were collected from jugular venipuncture and analyzed within two hours of blood collection. All samples were divided into:

- Unstimulated vehicle control
- Stimulated vehicle control
- Unstimulated DCFH-DA
- Stimulated DCFH-DA

| Table 1: Mean, standard deviation, and range of each analyte evaluated in the complete blood count from the 31 horses used in the pilot study. |
|---|---|---|---|---|
| Analyte | Unit | Mean | Standard deviation | Range | Reference interval |
| Total serum protein | g/dL | 6.6 | 0.50 | 5.7-7.5 | 5.7-8.1 |
| Red blood cells | x 10^9/L | 7.83 | 0.84 | 6.31-9.72 | 6.00-12.00 |
| Hematocrit | % | 39.4 | 3.25 | 34.4-46.3 | 35.0-50.0 |
| Hemoglobin | g/dL | 13.8 | 1.17 | 11.9-16.1 | 11.0-19.0 |
| Mean corpuscular volume | fL | 50.5 | 3.16 | 45.4-57.3 | 35.0-55.0 |
| Mean corpuscular hemoglobin concentration | g/dL | 34.9 | 0.71 | 33.1-36.0 | 31.0-37.0 |
| Red blood cell distribution width | % | 18.7 | 0.88 | 16.6-20.5 | 15.6-20.3 |
| White blood cells | x 10^6/L | 7.2 | 1.21 | 4.5-9.4 | 6.0-12.0 |
| Segmented neutrophils | x 10^6/L | 4.2 | 1.05 | 2.8-7.4 | 3.0-7.0 |
| Lymphocytes | x 10^6/L | 2.6 | 0.78 | 1.2-3.9 | 1.5-5.5 |
| Monocytes | x 10^6/L | 0.23 | 0.12 | 0.06-0.50 | 0.05-0.80 |
| Eosinophils | x 10^6/L | 0.14 | 0.13 | 0.06 | 0.00-0.40 |
| Basophils | x 10^6/L | 0.04 | 0.06 | 0-0.23 | 0.00-0.20 |
| Platelets | x 10^9/L | 136 | 32.03 | 86-202 | 100-600 |
| Mean platelet volume | fL | 6.5 | 0.70 | 5.2-8.3 | 5.5-10.1 |

Stimulation was obtained with 2 mM hydrogen peroxide (H₂O₂, Sigma Aldrich, St. Louis, MO, USA). Unstimulated samples were obtained by the addition of the same volume of phosphate saline buffer (PBS). DCFH-DA (Sigma Aldrich) was diluted to 50 mM stock solution with dimethyl sulfoxide (DMSO, American Bioanalytical, Natick, MA, USA). Ten microliters of DMSO (vehicle control) or DCFH-DA at 500 µM were added to 5 mL round bottom tubes (brand). The blood samples were centrifuged 3,000 x g for 5 min at 4°C (Sorvall Legend X1R, Thermo Scientific, Whaltam, MA, USA). Plasma and buffy coat were removed with a Pasteur pipette. Ten microliters of red blood cells were diluted in 5 mL of PBS supplemented with 1% bovine albumin (PBSA, Calbiochem, Darmstadt, Germany). One hundred microliters of the red blood cell (RBC) solution were added to the respective tubes. The cells were incubated at 37°C for 20 min.
Table 2: Mean, standard deviation, and range of each analyte evaluated in the chemistry panel from the 31 horses used in the pilot study.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Range</th>
<th>Reference interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>mg/dL</td>
<td>86</td>
<td>9.15</td>
<td>60 - 103</td>
<td>73 - 124</td>
</tr>
<tr>
<td>Blood urea nitrogen</td>
<td>mg/dL</td>
<td>16</td>
<td>2.50</td>
<td>11 - 22</td>
<td>8 - 27</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mg/dL</td>
<td>1.2</td>
<td>0.16</td>
<td>0.9 - 1.5</td>
<td>0.6 - 1.8</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mg/dL</td>
<td>2.9</td>
<td>0.58</td>
<td>2 - 5.1</td>
<td>2.0 - 5.7</td>
</tr>
<tr>
<td>Calcium</td>
<td>mg/dL</td>
<td>12.1</td>
<td>0.43</td>
<td>11.2 - 12.8</td>
<td>10.7 - 13.4</td>
</tr>
<tr>
<td>Magnesium</td>
<td>mg/dL</td>
<td>1.9</td>
<td>0.19</td>
<td>1.5 - 2.3</td>
<td>1.6 - 2.7</td>
</tr>
<tr>
<td>Sodium</td>
<td>mmol/L</td>
<td>135</td>
<td>2.07</td>
<td>131 - 140</td>
<td>132 - 144</td>
</tr>
<tr>
<td>Potassium</td>
<td>mmol/L</td>
<td>4.2</td>
<td>0.75</td>
<td>2.5 - 6.3</td>
<td>2.7 - 4.8</td>
</tr>
<tr>
<td>Chloride</td>
<td>mmol/L</td>
<td>99</td>
<td>3.02</td>
<td>94 - 105</td>
<td>94 - 103</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>mmol/L</td>
<td>31</td>
<td>2.58</td>
<td>25 - 35</td>
<td>23 - 31</td>
</tr>
<tr>
<td>Anion gap</td>
<td>mmol/L</td>
<td>9</td>
<td>2.04</td>
<td>3.6 - 14.5</td>
<td>12 - 20</td>
</tr>
<tr>
<td>Total serum protein</td>
<td>g/dL</td>
<td>6.4</td>
<td>0.45</td>
<td>5.5 - 7.3</td>
<td>4.7 - 7.5</td>
</tr>
<tr>
<td>Albumin</td>
<td>g/dL</td>
<td>3.0</td>
<td>0.23</td>
<td>2.5 - 3.5</td>
<td>2.5 - 3.8</td>
</tr>
<tr>
<td>Globulin</td>
<td>g/dL</td>
<td>3.3</td>
<td>0.34</td>
<td>2.8 - 4.0</td>
<td>2.3 - 3.8</td>
</tr>
<tr>
<td>Albumin-Globulin ratio</td>
<td>-</td>
<td>0.9</td>
<td>0.10</td>
<td>0.7 - 1.1</td>
<td>0.7 - 1.3</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>IU/L</td>
<td>334</td>
<td>108.87</td>
<td>210 - 823</td>
<td>206 - 810</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>IU/L</td>
<td>22</td>
<td>6.86</td>
<td>12 - 41</td>
<td>3 - 24</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>IU/L</td>
<td>131</td>
<td>34.68</td>
<td>84 - 227</td>
<td>109 - 331</td>
</tr>
<tr>
<td>Gamma-glutamyl transferase</td>
<td>IU/L</td>
<td>31</td>
<td>8.61</td>
<td>17 - 56</td>
<td>12 - 46</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>mg/dL</td>
<td>1.4</td>
<td>0.88</td>
<td>0.6 - 5.4</td>
<td>0.1 - 2.6</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>IU/L</td>
<td>320</td>
<td>427.27</td>
<td>81 - 2497</td>
<td>88 - 453</td>
</tr>
</tbody>
</table>

After incubation, 10 µL PBS were added to the unstimulated cells and 10 µL 20mM hydrogen peroxide solution were added to the stimulated cells. After 20 min incubation at room temperature, the samples were quenched with 300 µL 1% PBSA and immediately analyzed by flow cytometry (BD Accuri™ C6 flow cytometer, Becton, Dickinson and Company, Franklin Lakes, NJ). ROS-dependent fluorescence intensity were detected by green fluorescence with an excitation wavelength of 488 nm (FL1 channel) with gating around RBCs only (gates were determined in specificity level one assay). At least 50,000 events were collected in the RBC gate. Data was exported from BD Accuri C6 Software as FCS files and analyzed with FlowJo 10.5.3 (FlowJo, Ashland, OR, USA) and Excel 2007 (Microsoft Office, Redmond, WA, USA). Median fluorescence intensity (MFI) and percentage of positive cells were obtained and statistical analysis was performed using GraphPad Prism 8.0.2 (GraphPad, San Diego, CA, USA).
1.1 Specificity assays

1.1.1 Specificity level one

Because DCFH-DA is not cell-specific, the fluorescence specificity to erythrocytes was determined based on correct gating and appropriate removal of “contaminant” cells, in this case, leukocytes and platelets. For this purpose, three EDTA whole blood samples were collected from three horses. Platelet-rich plasma (PRP) was obtained after the blood was allowed to settle for 20 minutes at room temperature. The plasma was transferred to another tube and centrifuged at 300 x g for 10 minutes at room temperature, and the supernatant corresponded to the PRP. Leukocytes were obtained after the buffy coat was removed from a second EDTA sample and transferred to 10 volumes of a RBC lysis solution (0.15 mM NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA, pH 7.4). After incubation on constant shaking for 10 minutes at room temperature, the sample was centrifuged at 3,000 x g for 5 minutes at room temperature and the pellet was resuspended again in 10 volumes of RBC lysis solution. The incubation and centrifugation was repeated and the supernatant was resuspended in 5 mL PBSA 1%. One third tube was used to obtain a RBC solution as described in the previous section. Samples from each horse were ran in duplicates and divided as described above (unstimulated vs. stimulated, vehicle vs. DCFH-DA). The samples were used to determine correct gating. The gates were saved as a template and used to analyze all remaining samples in this study.

In the PRP samples, the platelets were small events in a logarithmic scale and they were observed forming a narrowed cloud of events in the corresponding logarithmic forward (FSC-A) and side scatter plots (SSC-A). In the leukocyte samples, the leukocytes were the largest events. It is possible to observe an overlap within the RBC cloud and a large amount of either platelets or small fragments generated during lysis procedure. When the events are plotted in a linear FSC-A vs. SSC-A, the characteristic clouds of granulocytes and lymphocytes can be easily identified (image not shown). At last, the RBC samples provided one single cloud of events larger than the platelets, with a mild overlap with the leukocytes (presumably with lymphocytes, the smallest white blood cells present in blood). Despite the overlap, the separation technique yielded very low numbers of leukocytes (contamination with leukocytes was assessed by microscopic evaluation of direct smears, data not shown) and the high number of events collected per run (at least 50,000 events) ensured specificity (Figure 1).
**Figure 1:** Logarithmic forward (FSC-A) and side scatter plots (SSC-A) showing the gating strategy in one representative sample. (A) Depiction of all gates. (B) Platelet-rich plasma sample, containing mainly platelets (within blue gate represented in figure A). (C) Buffy coat sample, containing leukocytes (within gray gate represented in figure A) and most likely remaining platelets. (D) RBC solution sample, containing mainly red blood cells and small numbers of remaining platelets and leukocytes.

1.1.2 Specificity level two

To ensure that the cellular fluorescence reflected ROS generation, a series of triplicate samples from three different horses were incubated with 5 mM of sodium azide (NaN₃, Sigma Aldrich) for 10 minutes after incubation with vehicle or DCFH-DA. Sodium azide inhibits CAT, the enzyme responsible for the transformation of H₂O₂ to H₂O and O₂. After incubation, the erythrocytes were stimulated with H₂O₂ (as described above) or remained unstimulated. It was expected that the presence of sodium azide would cause a marked increase in the median fluorescence intensity (MFI) and in the percentage of cells positive for 2′,7′-dichlorofluorescein (DFC).

After Shapiro-Wilk normality test, the percentage of positive cells data was analyzed by Wilcoxon test and the MFI was analyzed with paired t test. The percentage of cells positive for green fluorescence in both unstimulated and stimulated samples was not statistically different with pre- incubation with sodium azide (Table 1). However, MFI were significantly increased in both unstimulated and stimulated samples after incubation with sodium azide (p = 0.0044 and 0.0456, respectively).
Table 3: Percentage of cells [median (range)] positive for 2′-7′-dichlorofluorescein (DFC) in a flow cytometric evaluation of erythrocytes pre-incubated with or without 5 mM of sodium azide and stimulated with 2 mM of hydrogen peroxide or left unstimulated. The median fluorescence intensity is also present (mean ± standard deviation). Different letters in the same column indicate statistical difference (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated</th>
<th>Stimulated</th>
<th>Unstimulated</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without sodium azide</strong></td>
<td>0.22 (0.15 - 0.33)</td>
<td>58.6 (53.1 - 66.2)</td>
<td>1.523 ± 0.023</td>
<td>2.896 ± 0.127</td>
</tr>
<tr>
<td><strong>With sodium azide</strong></td>
<td>0.14 (0.13 - 0.37)</td>
<td>81.4 (59.6 - 81.5)</td>
<td>1.585 ± 0.026</td>
<td>3.035 ± 0.130</td>
</tr>
</tbody>
</table>

1.1.3 Specificity level three

Level three determined the assay specificity in a dose-dependent manner. Samples from three different horses were incubated with 0 (vehicle only), 1, 5, 10, 50 or 100 µM DCFH-DA. Secondly, samples were incubated with increasing concentrations of H2O2 from 0 (PBS) to 6 mM with 2 mM increments. The percentage of positive cells and MFI were analyzed. Repeated measures two-way ANOVA with Geisser-Greenhouse correction and Tukey’s test post-hoc were used. Nonlinear regression using a robust fit method of normalized data was used to compare increasing concentration of DCFH-DA in the group with 6 mM hydrogen peroxide only.

The DCFH-DA concentration effect is statistical significant for both positivity (p = 0.0009, Table 4) and MFI (p < 0.0001, Table 5), but not for the concentration of peroxide. Based on these findings, we opted to adopt the smaller concentration of peroxide and 50 µM in the further experiments. On regression analysis, it was possible to observe that the fluorescent signal was not linear to the concentration of DCFH (Figure 2).

Table 4: Percentage of cells (mean ± standard deviation) positive for 2′-7′-dichlorofluorescein (DFC) in a flow cytometric evaluation of erythrocytes incubated with increasing concentrations of hydrogen peroxide (H2O2, columns, 0, 2, 4, and 6 mM) and increasing concentrations of 2′-7′- dichlorodihydrofluorescein diacetate (DCFH-DA, rows, 0, 1, 5, 10, 50, and 100 µM). Non applicable (N/A) indicates a combination of peroxide and DCFH-DA concentrations that was not performed. Different letters in the same column indicate statistical difference (p < 0.05). Values indicated with an asterisk (*) were not evaluated in multiple comparison.

<table>
<thead>
<tr>
<th>DCFH-DA</th>
<th>H2O2</th>
<th>0 mM</th>
<th>2 mM</th>
<th>4 mM</th>
<th>6 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>0.1 ± 0.001*</td>
<td>N/A</td>
<td>N/A</td>
<td>0.9 ± 0.066*</td>
<td></td>
</tr>
<tr>
<td>1 µM</td>
<td>N/A</td>
<td>9.3 ± 1.888a</td>
<td>7.3 ± 2.177a</td>
<td>5.7 ± 1.544a</td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>N/A</td>
<td>29.4 ± 3.832b</td>
<td>27.4 ± 4.888a</td>
<td>24.1 ± 2.686b</td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>N/A</td>
<td>37.4 ± 4.336c</td>
<td>38.1 ± 6.358b</td>
<td>38.2 ± 4.922b</td>
<td></td>
</tr>
<tr>
<td>50 µM</td>
<td>N/A</td>
<td>71.3 ± 4.692b,c</td>
<td>73.8 ± 2.217c</td>
<td>75.0 ± 2.751c</td>
<td></td>
</tr>
<tr>
<td>100 µM</td>
<td>0.4 ± 0.212*</td>
<td>88.0 ± 3.792d</td>
<td>86.5 ± 4.573c</td>
<td>91.6 ± 2.157d</td>
<td></td>
</tr>
</tbody>
</table>
Table 5: Median fluorescence intensity (MFI) of cells (mean ± standard deviation) positive for 2′-7′-dichlorofluorescein (DFC) in a flow cytometric evaluation of erythrocytes incubated with increasing concentrations of hydrogen peroxide (H₂O₂, columns, 0, 2, 4, and 6 mM) and increasing concentrations of 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA, rows, 0, 1, 5, 10, 50, and 100 µM). Non applicable (N/A) indicates a combination of peroxide and DCFH-DA concentrations that was not performed. Different letters in the same column indicate statistical difference (p < 0.05). Values indicated with an asterisk (*) were not evaluated in multiple comparison.

<table>
<thead>
<tr>
<th></th>
<th>DCFH-DA 0 µM</th>
<th>DCFH-DA 1 µM</th>
<th>DCFH-DA 5 µM</th>
<th>DCFH-DA 10 µM</th>
<th>DCFH-DA 50 µM</th>
<th>DCFH-DA 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mM</td>
<td>1.926 ± 0.008*</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>2.264 ± 0.017*</td>
<td></td>
</tr>
<tr>
<td>2 mM</td>
<td>N/A</td>
<td>2.385 ± 0.011a</td>
<td>2.353 ± 0.042a</td>
<td>2.336 ± 0.015a</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>4 mM</td>
<td>N/A</td>
<td>2.527 ± 0.016b</td>
<td>2.509 ± 0.024ab</td>
<td>2.482 ± 0.007b</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>6 mM</td>
<td>N/A</td>
<td>2.584 ± 0.018c</td>
<td>2.589 ± 0.039b</td>
<td>2.587 ± 0.030b</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>N/A</td>
<td>2.947 ± 0.074bc</td>
<td>2.986 ± 0.025c</td>
<td>3.004 ± 0.029c</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>50 mM</td>
<td>N/A</td>
<td>3.296 ± 0.133bc</td>
<td>3.253 ± 0.084c</td>
<td>3.415 ± 0.074d</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>100 mM</td>
<td>1.991 ± 0.012*</td>
<td>3.296 ± 0.133bc</td>
<td>3.253 ± 0.084c</td>
<td>3.415 ± 0.074d</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2: Nonlinear robust fit curves of normalized percentage cells positive for 2′-7′-dichlorofluorescein (left blue graph) and average median fluorescence intensity (MFI, right red graph) with incremental concentration of 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA; 0, 1, 5, 10, 50, 100 µM) stimulated with 6 mM hydrogen peroxide evaluated by flow cytometry. The curves are similar but generated different best-fit values.
1.2 Precision assay

Precision were determined based on the intra- and inter-assay repeatability. Stimulated and unstimulated samples and samples with and without DCFH-DA (as described above) from three different horses were run in triplicate for intra-assay precision, and five different analytical runs were performed for inter-assay precision. Acceptable coefficient of variation (CV) was ≤20% for both assays according to O’Hara et al. (2011).

The logarithmic mean of the median fluorescence intensity and the standard deviation were used to calculate the coefficient of variation. The formula used is represented below, where \( \ln(10) \) is the natural log of 10 and \( \sigma \) is the standard deviation. Data are present in tables 6 and 7. Acceptable coefficient of variation (CV) was ≤20% for both intra- and inter-assay according to O’Hara et al. (2011).

\[
CV(\%) = \frac{100\%}{\sqrt{10^{\ln(10)}\sigma^2}} - 1
\]

**Table 6**: The mean intra-assay coefficient of variation (CV) of the median fluorescence intensity from three horses in each of the five runs and in both conditions (unstimulated and stimulated cells) are present in the table, as well as the mean CV per condition.

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
</tr>
<tr>
<td>CV (%) per run</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>CV (%) per condition</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7**: The mean and standard deviation of the inter-assay median fluorescence intensity (MFI) for each horse, the mean inter-assay coefficient of variation (CV) of each horse, and the mean inter-assay CV per condition (unstimulated and stimulated cells) are present below.

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Horse 1</td>
<td>Horse 2</td>
</tr>
<tr>
<td>Mean MFI</td>
<td>1.954</td>
<td>1.971</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.016</td>
<td>0.067</td>
</tr>
<tr>
<td>CV (%)</td>
<td>3.7</td>
<td>4.6</td>
</tr>
<tr>
<td>Mean CV (%)</td>
<td>4.8</td>
<td></td>
</tr>
</tbody>
</table>
1.3 Stability assay

The capability of the samples to retain the initial measurement over time was determined by measuring fluorescence at 3, 6, 24, 36 and 48 hours post-collection of three different horses. The samples were kept refrigerated (4°C). Acceptable coefficient of variation (CV) was ≤20% in comparison to baseline according to O’Hara et al. (2011), using the same formula described above. Data are present in tables 8.

Table 8: Mean of the logarithmic median fluorescence intensity (MFI) is present below, as well as the standard deviation (SD) of unstimulated and stimulated cells at baseline, and 3, 6, 24, 36, and 48 hours after blood collection, and the coefficients of variation (CV) of each time in comparison to baseline.

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated</th>
<th></th>
<th></th>
<th>Stimulated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean MFI</td>
<td>SD</td>
<td>CV (%)</td>
<td>Mean MFI</td>
<td>SD</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.937</td>
<td>0.006</td>
<td>N/A</td>
<td>2.855</td>
<td>0.047</td>
</tr>
<tr>
<td>3 h</td>
<td>1.945</td>
<td>0.003</td>
<td>1.3</td>
<td>2.921</td>
<td>0.066</td>
</tr>
<tr>
<td>6 h</td>
<td>1.940</td>
<td>0.011</td>
<td>0.6</td>
<td>2.949</td>
<td>0.051</td>
</tr>
<tr>
<td>24 h</td>
<td>1.952</td>
<td>0.014</td>
<td>2.5</td>
<td>2.927</td>
<td>0.123</td>
</tr>
<tr>
<td>36 h</td>
<td>1.957</td>
<td>0.025</td>
<td>3.3</td>
<td>3.030</td>
<td>0.112</td>
</tr>
<tr>
<td>48 h</td>
<td>1.971</td>
<td>0.006</td>
<td>5.6</td>
<td>3.014</td>
<td>0.047</td>
</tr>
</tbody>
</table>

1.4 All horses

The assay was performed in all thirty-one horses to establish a pattern of response. The scatter data and median logarithmic MFI is present in the figure below.

**Figure 3:** Logarithmic MFI in unstimulated and stimulated samples are plotted in the graph. The horizontal bars represent the medians. Difference is statistically significant (Wilcoxon signed-rank test, p < 0.0001).
2. **Conclusions**

The assay is specific for erythrocytes based on gating and correct cell separation and precise, with maximum intra-and inter-assay CV of 13% and 18%, respectively. Stability was excellent for the unstimulated samples; however, the stimulated samples were stable only up to 24 hours after collection, presumably when cells deplete their intracellular antioxidant enzymes. The use of DCFH-DA to detect equine intraerythrocytic ROS with flow cytometry is a promising technique with multiple applications to study oxidative stress in horses.
ERAB Progress Report

“Recovery of Salmonella bacterial isolates from pooled equine fecal samples”

Dr. Kenitra Hendrix, Dr. Jose Goni and Dr. Janice Kritchevsky

Summary:

Current protocols for equine Salmonella culture include testing a series of five samples, usually collected at 24-hour intervals. The purpose of this study is to evaluate the sensitivity of culturing pools of five fecal samples for Salmonella culture. Testing pooled samples would offer the benefit of decreased cost of diagnostic testing.

Complete:

1. Obtain and propagate a pure culture of Salmonella Group E to serve as the reference bacteria. - complete

Salmonella E1 (ATCC 9270) is maintained in the ADDL Bacteriology section and utilized in subsequent specific aims.

2. Spike equine fecal samples with either $10^2$, $10^3$, $10^4$, or $10^5$ CFU Salmonella.
3. Pool 1 spiked and 4 non-spiked fecal samples collected over a 5-day time period into a single container.
4. Perform standard culture to recover the spiked Salmonella species from well-mixed composite fecal samples.

Two iterations of aims 2-4 were performed.

Phase 1 (Table 1): A series of five Salmonella samples from the feces donor horse were cultured and were negative for Salmonella. Feces in 20-gram aliquots from this Salmonella negative horse was initially spiked with either $10^2$, $10^3$, $10^4$, or $10^5$ CFU Salmonella. Ten grams were cultured for salmonella to show spiking was successful. The other ten grams were pooled with 40g of salmonella-negative feces, replicating a pool of one positive field samples and four negative field samples. Following both spiking and pooling, feces was homogenized for 1 minute at 230rpm. All spiking was successful based on positive culture results. All pools were positive, with the exception of the pool including the samples spiked with $10^3$ CFU. This raised concerns regarding the pooling technique itself, since the pool with less organism was culture positive. All pooled samples were cultured again after 8 days at 4°C, to simulate a field case in which only the first sample was positive for Salmonella. All pools were positive except for the one including the sample spiked with $10^2$ CFU.
Recovery of Salmonella bacterial isolates from pooled equine fecal samples

Table 1:

<table>
<thead>
<tr>
<th></th>
<th>First Culture</th>
<th>Second Culture (8d in fridge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20g feces spiked with (10^2) CFU</td>
<td>Salmonella</td>
</tr>
<tr>
<td>AP</td>
<td>10g A pooled with 40g negative</td>
<td>Salmonella</td>
</tr>
<tr>
<td>B</td>
<td>20g feces spiked with (10^3) CFU</td>
<td>Salmonella</td>
</tr>
<tr>
<td>BP</td>
<td>10g B pooled with 40g negative</td>
<td>Salmonella</td>
</tr>
<tr>
<td>C</td>
<td>20g feces spiked with (10^4) CFU</td>
<td>Salmonella</td>
</tr>
<tr>
<td>CP</td>
<td>10g C pooled with 40g negative</td>
<td>Salmonella</td>
</tr>
<tr>
<td>D</td>
<td>20g feces spiked with (10^5) CFU</td>
<td>Salmonella</td>
</tr>
<tr>
<td>DP</td>
<td>10g D pooled with 40g negative</td>
<td>Salmonella</td>
</tr>
</tbody>
</table>

Phase 2 (Table 2): In response to the unexpected results in phase 1, variations of homogenization protocols including the duration of homogenization and adding nutrient broth were tested. Samples were spiked with \(10^2\) CFU only, and then pooled as described in Phase 1. Triplicates of each set of conditions were tested, and all culture results were positive. This indicated that variables of the homogenization protocol did not affect the final culture results, and that \(10^2\) CFU in 50g of feces was consistently detected by culture.

Table 2:

<table>
<thead>
<tr>
<th>Control</th>
<th>Non-spiked feces</th>
<th>First Culture</th>
<th>Second Culture (8d in fridge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>1 minute; no broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>1b</td>
<td>1 minute; no broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>1c</td>
<td>1 minute; no broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>5a</td>
<td>5 minutes; no broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>5b</td>
<td>5 minutes; no broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>5c</td>
<td>5 minutes; no broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>B1a</td>
<td>1 minute; 20mL broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>B1b</td>
<td>1 minute; 20mL broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>B1c</td>
<td>1 minute; 20mL broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>B5a</td>
<td>5 minutes; 20mL broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>B5b</td>
<td>5 minutes; 20mL broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>B5c</td>
<td>5 minutes; 20mL broth</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>
In-progress:

A. Additional spiking study to determine the best pooling protocol:
   - 12 10-gram samples will be spiked with $10^2$ CFU/mL *Salmonella* (ATCC 9270)
   - Each spiked sample will be pooled with 40g of *Salmonella*-negative samples, and homogenized according to Table 2 above.
   - Each 50g spiked pool will be divided into 5 10-gram samples, and each will be cultured for *Salmonella* according to ADDL protocol.

B. For one calendar year, each time a series of 5 equine fecal samples is cultured for *Salmonella*, duplicate samples will be saved in the VTH and submitted as a single pooled sample for culture.
   - The fecal pool will be homogenized per the protocol selected in A.
   - 10g of the pool will be cultured for Salmonella according to the ADDL protocol.
Finite Element Analysis of Six Transcortical Pin Parameters and Their Effect on Bone–Pin Interface Stresses in the Equine Third Metacarpal Bone

Timothy B. Lescun1  Stephen B. Adams1  Russell P. Main2  Eric A. Nauman3  Gert J. Breur1

1 Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana, United States
2 Department of Basic Medical Sciences, College of Veterinary Medicine and Weldon School of Biomedical Engineering, College of Engineering, Purdue University, West Lafayette, Indiana, United States
3 Weldon School of Biomedical Engineering, College of Engineering, Purdue University, West Lafayette, Indiana, United States

Vet Comp Orthop Traumatol

Abstract

Objective The objectives of this study were to validate a finite element model of the equine distal limb transfixation cast and to determine the effect of six transcortical pin parameters on bone–pin interface (BPI) stresses in the third metacarpal bone.

Study Design A transfixation cast finite element model was developed from a computed tomography scan of the third metacarpal bone and modelled pin elements. The model was validated by comparing strain measured around a 6.3-mm transfixation pin in the third metacarpal bone with the finite element model. The pin parameters of diameter, number, location, spacing, orientation and material were evaluated by comparing a variety of pin configurations within the model.

Results Pin diameter and number had the greatest impact on BPI stress. Increasing the diameter and number of pins resulted in lower BPI stresses. Diaphyseal pin location and stainless-steel pins had lower BPI stresses than metaphyseal location and titanium alloy pins, respectively. Offset pin orientation and pin spacing had minimal impact on BPI stresses during axial loading.

Conclusion The results provide evidence that diameter and number are the main pin parameters affecting BPI stress in an equine distal limb transfixation cast. Configurations of various pin size and number may be proposed to reduce BPI stresses and minimize the risk of pin related complications. Further refinement of these models will be required to optimize pin configurations to account for pin hole size and its impact on overall bone strength.

Keywords
- orthopaedics
- external skeletal fixation
- horse
- finite element
- pin
- stress

Introduction

Transfixation casting is a technique used to treat distal limb fractures in the horse. Complications such as early pin loosening and secondary pin hole fracture impact clinical outcomes due to their common occurrence and potentially devastating consequences. Pin loosening is reported to occur in 68% of cases and secondary pin hole fractures occur in 14 to 20% of cases. Transfixation casting is similar to external skeletal fixation and the reliance of both methods on the stability of the transcortical pin results in comparable limitations related to the bone–pin interface (BPI). Pin loosening and pin hole...
fractures constitute a form of BPI failure, either insidiously for pin loosening or acutely for pin hole fracture. Local bone failure occurs when the yield stress threshold of the bone material is exceeded. A reduction in pin related complications could be achieved by understanding and mitigating the factors contributing to BPI stress during transfixation casting.

The effect of altering parameters of external skeletal fixation on BPI stress has been examined using analytical, finite element (FE), ex vivo and in vivo methods in humans and small animals. While some recommendations translate to transfixation casting, not all findings are expected to be applicable due to differences between the two techniques. Ex vivo studies of transfixation casting have evaluated parameters such as pin size, pin number, pin orientation, transcortical hole size, methods of cast attachment to pins and staged pin removal. These studies address specific questions related to transfixation casting and help guide current clinical practice. However, pin number and pin size were only evaluated in the radius, and transcortical hole size, pin orientation and staged pin removal have been evaluated in the third metacarpal bone. None of these studies examined the range of transcortical pin parameter values that could be modified nor did they evaluate the BPI. A systematic evaluation of specific transfixation pin parameters would provide clinicians with information regarding their effect on BPI stresses. We believe that similar to studies of external skeletal fixator systems, determining which transcortical pin configurations minimize BPI stresses during transfixation casting could be used to guide clinical practices and help reduce the occurrence of pin related complications.

Finite element analysis has been utilized in orthopaedics prior to or in parallel with ex vivo and in vivo testing. Utilizing FE analysis, the overall aim of our work was to evaluate a range of pin parameters and determine optimal configurations for the equine distal limb transfixation cast. The first objective of this particular study was to develop and validate an FE model representative of the equine distal limb transfixation cast. Our second objective was to utilize the model to determine the effect of six pin parameters on BPI stress and strain predictions in the equine third metacarpal bone. The results of this study will allow recommendations to be made regarding the effect of these pin parameters on anticipated BPI stresses during transfixation casting in the horse. The developed FE model will also provide a basis for future assessment of other parameters that determine the overall biomechanical performance of transfixation casts.

### Materials and Methods

#### Study Design

An FE model of the equine third metacarpal bone was developed from computed tomography (CT) images of a cadaveric forelimb from a 10-year-old Quarter Horse gelding weighing 465 kg. The horse was owned by the university, had not been lame and was euthanatized for reasons unrelated to this study. The CT was performed from the carpus to the foot using a 64 slice helical scanner (Lightspeed VCT, General Electric, Milwaukee, Wisconsin, United States) at a slice thickness of 3.75 mm. Validation was performed by comparing FE analysis results with measured bone surface strain values obtained during ex vivo testing of the same third metacarpal bone with a single 6.3 mm transcortical pin.

Individual FE models were generated by combining the third metacarpal bone geometry with specific pin combinations to determine the effect of six different pin parameters on BPI stress and strain during axial loading.

#### Finite Element Model Construction

Models combining the third metacarpal bone and transcortical pins were constructed within an FE software programme (Abaqus, v.6.12; Dassault Systemes Simulia Corp, Rhode Island, United States). Slice geometry from the CT images was used to create the shape of the bone directly within the FE software programme using geometric part construction features and Boolean operations. The length of the third metacarpal bone model was 156 mm spanning from the proximal diaphysis to the physeal scar of the distal metaphysis. The cortical thickness varied from 15 mm medially at the mid diaphysis to 7 mm laterally at the distal metaphysis. Pins were constructed to be 70 mm in length and were positioned within the bone model using Boolean operations. Non-linear surface to surface contact stiffness was applied at the BPI. This allowed separation of surfaces after contact, sliding between surfaces and prevented overclosure of surfaces under pressure. These conditions would be most representative of the BPI immediately after pin insertion. A 15 mm distance from the outer cortical bone margin to the fixed pin end was based on radiographic measurements from six previous clinical cases. To simulate standing and full weight shifting onto the limb, a 2500 N distributed axial compressive load was applied over the proximal surface of the third metacarpal bone. To simulate walking, a 7500 N distributed axial compressive load was applied to the proximal surface of the bone. The material properties of the bone and pins used for the models were based on previous studies and reference data obtained from metal suppliers for pins (Table 1).

Free meshing algorithms were used and all models were meshed using solid quadratic tetrahedral elements (type C3D10I). Adaptive remeshing was performed to refine the mesh for each model based upon the output variable von

<table>
<thead>
<tr>
<th>Material</th>
<th>Density (g/cm³)</th>
<th>Elastic modulus (GPa)</th>
<th>Poisson’s ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical bone</td>
<td>2,000</td>
<td>17</td>
<td>0.3</td>
</tr>
<tr>
<td>Cancellous bone</td>
<td>500</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>8,000</td>
<td>205</td>
<td>0.3</td>
</tr>
<tr>
<td>Titanium alloy</td>
<td>4,430</td>
<td>114</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Table 1 Material properties of bone and metals used for FE modelling of transfixation pin combinations within the equine third metacarpal bone

Abbreviation: FE, finite element.
Mises (VM) stress. Remeshing was continued until the maximum change in VM stress from one mesh to the next fell below 2%, resulting in a stable mesh for analysis. The cast was not modelled and pin to cast attachment was restrained in all three axes as a boundary condition. The distal end of the bone was unrestrained in the longitudinal axis while fully constrained in both transverse axes.

Model Validation
Validation was performed by comparing FE analysis to measured surface strains from ex vivo loading of the third metacarpal bone. A custom-made jig accommodated the bone and a single pin within the materials testing system (Qtest/50LP; MTS, Eden Prairie, Minnesota, United States) (► Fig. 1). A steel cap with a 5-mm deep, circular depression on the lower surface was placed over the proximal third metacarpal bone for loading. A solid steel cylinder 25 mm in length and 12 mm in diameter was positioned in a depression on the upper surface of the steel cap to transfer actuator load to the proximal bone surface.

A single smooth 6.3 mm diameter pin was inserted within the frontal plane 41 mm from the distal end of the bone segment following drilling of a transversely oriented 6.2 mm pin hole. Two rosette strain gauges (FRA-2-11; Texas Measurements, College Station, Texas, United States) were attached 5 mm from the hole margin at a proximal and a dorsal position for both lateral and medial holes. Longitudinally oriented single axis strain gauges (FLA-2-11) were placed in a palmar position 5 mm from the hole margins and on the dorsal midline 20 mm from the pin centre proximally and distally. Strain values in the longitudinal axis were obtained directly from the FE models and compared with those recorded during ex vivo testing (► Fig. 2). Axial compressive loads of 2500, 5000 and 7500 N were applied sequentially at a loading rate of 6 mm/min. Load-deformation curves were generated to determine that each cycle of testing was within the linear elastic range of the bone. Maximum and minimum principal strain values were calculated using the rosette gauge data proximal to the medial and lateral pin holes and compared directly to the corresponding values from the FE model.

Pin Parameters
Six parameters of transfixation pins and their positioning in the third metacarpal bone were examined. The parameters were pin diameter, number, location, spacing, orientation and material. The specific variables evaluated for each parameter are presented in ► Table 2. All possible variable combinations (a total of 3,168 models) were not created. Specific comparisons were made between parameter variables while keeping other parameters of the models being compared constant. The combinations of pin diameter and pin number specifically evaluated are presented in ► Table 3. Pin location was evaluated by comparing single pins of various diameters positioned in either the diaphyseal or the metaphyseal region of the third metacarpal bone. Pin spacing and orientation were evaluated using a 6.3 mm pin diameter. Pin spacing was defined as the closest edge to edge distance between pins. An angle of 20 degrees from the frontal plane was used for positioning pins in an offset or divergent orientation (► Fig. 3). Stainless steel and titanium alloy pin materials were compared using single pins with diameters of 5, 6, 7, 8 and 9 mm positioned in the distal metaphyseal region of the third metacarpal bone.

Data Analysis
Output database files were generated for each FE model constructed. Specific data values recorded included the bone maximum and minimum principal stress and strain, maximum cortical bone VM stress and maximum pin VM stress. Direct comparisons between models were made to assess the impact of individual parameters. von Mises stress was used to report single parameter comparisons as it is a common predictor of yielding or material failure. Stress and strain values were also examined for all parameter comparisons. Representative equations were developed to describe relationships between stress or strain and pin diameter or number. The Pearson product moment correlation coefficient was used to determine the best fitting equations describing the relationships observed.

Results
There were a total of 96 individual FE models constructed for the study. The number of models used to evaluate each of the pin parameters of interest are presented in ► Tables 2 and 3. The number of elements in the models ranged from approximately 25,000 up to 150,000; largely dependent upon the
number of pins included and the amount of remeshing required to achieve convergence of the models within the stated 2% limit for VM stress variation.

**Model Validation**

A similar linear response between the three load levels was observed in both the FE model and the bone-pin construct. Load, displacement and longitudinal strain values for both the ex vivo validation test procedure and the FE validation model are shown in Table 4. Comparison between the modelled and the measured strain values showed that measures were close to the \( x = y \) line representing complete agreement (Fig. 4). The greatest deviations from the line were at the highest magnitude strain values, where the FE model tended to underestimate the calculated maximum principal strain and overestimate the calculated minimum principal strain. The linear regression line of best fit for the measured versus modelled values was \( y = 0.962x - 88.7 \) \((R^2 = 0.99)\). Longitudinal strain values for the FE model varied from the corresponding measured values by a mean (±standard deviation) of 5.94 ± 5.88% across six measured sites (3 medial and 3 lateral). Maximum principal strain values, calculated from the rosette gauge measurements,
varied from the corresponding FE model values by a mean of 10.03 ± 8.31%. Minimum principal strain values varied from the corresponding FE model by a mean of 7.33 ± 3.96%.

**Pin Parameters**

Pin diameter had a consistent effect on cortical bone VM stress, as well as principal stresses and strains. Smaller pin diameters resulted in higher stresses at the BPI. Maximum stress and strain values were invariably observed at the outer proximal margin of the pin hole and fell sharply both from the outer cortex toward the inner cortex and radially away from the edge of the pin hole (Fig. 5). Pin number also had a consistent effect on maximum cortical bone VM stress. Increasing the number of pins resulted in a reduction in the maximum cortical bone VM stress values with a greater reduction for smaller pin diameters compared with larger pin diameters. The relationships between both pin diameter and pin number with maximum cortical bone VM stress are shown in Fig. 6.

Pin location was examined by comparing a range of pin sizes positioned in the diaphyseal region with corresponding pin sizes positioned in the distal metaphyseal region. Pin location in the distal metaphyseal region resulted in higher VM stress values than the diaphyseal region. This was more evident for smaller pin diameters (Fig. 7). Small differences were observed between locations for maximum principal stress or maximum principal strain, while minimum principal stress and strain were lower in the metaphyseal region (i.e. higher compressive stress and strain) when compared with the diaphyseal region.

Pin spacing between two adjacent pins did not appreciably change stress patterns or their magnitude. Maximum cortical bone VM stress values varied by less than 4%, ranging from 247.7 to 257.2 MPa, over pin spacing distances ranging from 10 to 50 mm. Qualitative examination of the stress and strain patterns surrounding the pin holes did not show stress concentrations between or around pins for these spacing distances.

Comparisons were made between pins oriented in a divergent position 20 degrees from the frontal plane (offset) and pins oriented solely within the frontal plane (inline).

---

**Table 4** Load (N), displacement (mm) and longitudinal strain (microstrain) measured from an ex vivo cadaveric test and the comparable FE analysis predictions used for model validation at three different loads. Negative values for strain represent compression.

<table>
<thead>
<tr>
<th>Load</th>
<th>Measured</th>
<th>Predicted</th>
<th>Measured</th>
<th>Predicted</th>
<th>Measured</th>
<th>Predicted</th>
<th>Measured</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,502</td>
<td>5,013</td>
<td>7,502</td>
<td>2,500</td>
<td>5,000</td>
<td>7,500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Displacement</td>
<td>0.48</td>
<td>0.79</td>
<td>1.23</td>
<td>0.43</td>
<td>0.80</td>
<td>1.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain-medial hole</td>
<td>−1.656</td>
<td>−2.788</td>
<td>−3.895</td>
<td>−1.376</td>
<td>−2.751</td>
<td>−4.131</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain-lateral hole</td>
<td>−1.539</td>
<td>−3.239</td>
<td>−4.436</td>
<td>−1.502</td>
<td>−3.008</td>
<td>−4.515</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: FE, finite element.
Offsetting the pin orientation in two and three pin models using 6.3 mm diameter pins resulted in similar values for maximum cortical bone VM stress, differing by less than 2% for both the two pin models (range from 243.4 to 247.7 MPa) and the three pin models (range from 163.3 to 166.3 MPa).

No consistent pattern of stress reduction or stress concentration was observed as a result of using an offset pin orientation.

Stainless steel and titanium alloy pins were compared using single pins positioned in the distal metaphyseal region. Maximum cortical bone VM stress with the stainless-steel pins was 26.9 to 37.0% lower than with the titanium alloy pins, while maximum pin VM stress for the titanium alloy pins was 4.7 to 9.1% lower than for the stainless-steel pins across the range of pin sizes examined.

**Discussion**

The purpose of this study was to develop and validate an FE model of the equine distal limb transfixation cast and use...
this model to systematically evaluate clinically relevant pin and pin positioning parameters to predict which combination(s) would result in reduced BPI stresses. The results show that the number of pins used in a transfixation cast, and their diameter, have the most profound effect on the BPI stresses and strains observed, consistent with previous studies examining external skeletal fixation parameters. In contrast, both the spacing between pins and their orientation had minimal impact on BPI stress during axial loading. Pins located in the metaphyseal region of the bone resulted in higher compressive BPI stress than pins located in the diaphysis, which we attribute to the thinner cortical bone width present in the metaphyseal region. Stainless-steel pins resulted in lower BPI stresses due to their higher...
stiffness; however, the titanium alloy pin stresses were lower than stainless-steel pins and as such may be less likely to break during cyclic loading, particularly as their yield stress is approximately four times higher than stainless-steel pins.25,27 These results provide a basis from which pin configurations may be proposed that reduce BPI stress and strain in an equine distal limb transfixation cast.

Finite element analysis was used in this study because it can utilize the mechanical conditions of a system, calculate the predicted stress and strain environment of the system and provide data on specific models that can be further developed and refined, either with further FE analysis or by cadaveric or in vivo testing. This method of screening pin parameters avoided the use of a large number of animals or cadaveric limbs. The conditions applied to the FE models in this study were designed to mimic the worst case-scenario of a horse walking with full weight on the cast limb with an axially unstable fracture.24 Validation of the current model was performed by comparison to ex vivo testing on the same third metacarpal bone from which the model was based. The differences between the cadaveric and FE models were generally low, with only four specific comparisons having a difference greater than 10%, and the mean percentage differences across each of the strain measures analysed less than or equal to 10%. The simple shape of the equine third metacarpal bone allows good reproduction of its mechanical performance using FE models. Several investigators have used simple models of the equine third metacarpal bone and shown good agreement with ex vivo results.24,32–34 These validation results support that the FE modelling approach had acceptable agreement with ex vivo testing.

The selection of parameters to evaluate in this study was based on current clinical practices. Pin diameters ranging in size from 4.7 to 9.5 mm have been reported clinically in adult horses.1,2 Larger pin diameters are more resistant to bending and result in reduced BPI stress.8 However, larger pins require larger holes in the bone cortex which has been shown to reduce the breaking strength of bone.19,35,36 The area moment of inertia of the pin increases with the fourth power of the diameter. The relationship demonstrated between pin diameter and maximum VM stress for a single pin appears to be consistent with the influence that pin area moment of inertia is expected to have on bending stiffness of the pin and consequently BPI stress. It is evident from examining pin diameter against maximum cortical bone VM stress in the FE models with an increasing number of pins that the influence of pin diameter lessens as the number of pins increases. Further evaluation of the relationship between the area moment of inertia of the pin and the pin number is warranted as these parameters had the greatest influence on BPI stresses and strains.

Recommendations on pin location, made based on clinical observations, have been to place pins as far from the top of the cast as possible to avoid secondary pin hole fracture.2,37 This approach results in pins located in the distal metaphysis of the third metacarpal bone. The results of the present study show that stress at the BPI would be expected to be lower in the diaphysis than the metaphysis. This suggests that previous clinical observations may be the result of factors other than high BPI stress contributing to an increased risk of secondary pin hole fracture for diaphyseal pin locations. The examination of pin orientation in this study failed to show a clear advantage of the method of offsetting pin positions from the frontal plane in the equine third metacarpal bone. However, our analysis used axial compression, while a previous study evaluating pin orientation in cadaveric bones used torsion and found that bone strength was greater with an offset orientation.17 We elected to test in axial compression because that is the predominant load experienced by the third metacarpal bone in the horse.24,33 The results of our study agree with clinical studies where neither pin loosening nor secondary pin hole fracture was found to be associated with an offset (divergent) pin orientation.1,2

There are several limitations of this study that merit discussion. Finite element analysis of mechanical behaviour requires the input of material properties, such as bone density and elastic modulus, and that several assumptions are made about the model. Bone is an anisotropic material and its density varies depending on the type of bone and its degree of porosity. A relationship between bone density and elastic modulus has been used to provide detailed material information on an elemental level to increase the accuracy of an FE model.38 However, this method of material assignment increases the computational complexity of the model substantially. The assumption that the pin ends are completely fixed is unlikely to reflect the true situation within a cast. Further evaluation of the effect of this assumption on the results of these models is warranted. Another limitation in this study was the fact that the BPI contact conditions were simplified by not accounting for BPI friction. Friction would be expected to have an effect on lateral to medial sliding of the pin even though the major loading direction is normal to the pin surface. In our models, sliding was unable to occur as the pin ends were fixed in position.

The main advantage of using the simplified modelling approach was the ability to make multiple comparisons across different pin parameters. Coupled with validation of the model, these findings provide a basis from which to investigate additional aspects of the equine transfixation cast which are likely to influence BPI stress using further ex vivo and in vivo testing. Ultimately, identifying ideal pin parameter combinations, such as pin size and number, which minimize BPI stresses and strains, should reduce the likelihood of both acute and chronic BPI failure and improve the safety of the equine distal limb transfixation cast during clinical use.

Authors’ Contributions
Timothy Lescun contributed to conception of study, study design, acquisition of data and data analysis and interpretation. Stephen Adams and Eric Nauman contributed to conception of study and study design. Russell Main contributed to acquisition of data and data analysis and interpretation. Gert Breur contributed to conception of study, study design and data analysis and interpretation. All authors drafted, revised and approved the submitted manuscript.

Veterinary and Comparative Orthopaedics and Traumatology
References

6. Aro HT, Markel MD, Chao EY. Cortical bone reactions at the interface of external fixation half-pins under different loading conditions. J Trauma 1993;35(05):776–785
27. Anonymous. Product Data Sheet - 316/316L Stainless Steel. Available at: www.aksteel.com
38. Schileo E, Taddei F, Cristofolini L, Viceconti M. Subject-specific finite element models implementing a maximum principal strain criterion are able to estimate failure risk and fracture location on human femurs tested in vitro. J Biomech 2008;41(02):356–367
Ketorolac tromethamine (KT) is a non-steroidal anti-inflammatory drug (NSAID) that has recently been evaluated in horses. This drug has been shown to reduce inflammation exhibited by equine white blood cells in the laboratory, and is well-tolerated in horses from a safety standpoint, but its efficacy in controlling pain has not been fully evaluated. As part of a larger project that compared the ability of KT to two commonly used NSAIDs in horses (“Bute” and Banamine®) to decrease lameness in an experimental model of foot pain, blood was collected to measure concentrations of each drug following intravenous (IV) administration. This was done to ensure that variation in blood concentrations of each drug did not differ enough among horses to affect the degree of pain relief conferred by each drug. Blood was collected to measure drug concentration immediately (within 5 minutes) following IV administration of each drug, and again 2, 4, 8 and 12 hours later. Results showed that there was minimal variation among horses in blood drug concentrations for each drug (KT, Bute and Banamine®) at each time point tested. Blood drug concentrations decreased as expected over time in all horses as the drug was metabolized and excreted from the body. Results from this project gave us assurance that the pain relief observed after drug administration in these horses was not affected by variations in the amount of drug found in the blood at various time points.

Manuscript preparation is nearly complete and we expect to submit the manuscript for publication to the Equine Veterinary Journal in January of 2019. These results will also be presented as an oral abstract at a national veterinary meeting in 2019 or 2020.
Pain management is an important aspect of equine medicine. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to manage pain through their anti-inflammatory mode of action, but currently-available NSAIDs are limited in their ability to mitigate severe pain. Ketorolac tromethamine (KT) is an NSAID that is commonly administered to post-operative human patients, but has not been fully evaluated in the horse. First, our laboratory measured blood levels of KT following various routes of administration in healthy horses. As part of a larger research program investigating potential treatments for sepsis in horses, we also investigated the ability of KT to decrease pro-inflammatory mediators, and found that KT is as effective as flunixin meglumine (Banamine®) in reducing these mediators in healthy horses. For the current project, we proposed to compare the ability of KT and 2 commonly used NSAIDs in horses (“Bute” and Banamine®) to decrease the severity of lameness in an experimental, reversible model of foot pain. For each of the 10 horses in the study, we applied a special shoe to one front foot that allowed induction of temporary foot pain. Following lameness induction, 1 of the 3 drugs or a saline placebo control was administered, and lameness was assessed. Each horse received each of the 4 treatments (KT, “Bute,” Banamine®, and saline/placebo) with a minimum of 2 weeks between treatments. The screw was loosened at the end of each of the 4 treatment trials, and soundness was confirmed. To measure the safety of repeated dosing of KT, “Bute,” and Banamine®, these drugs were administered every 12 hours for 5 days. For each of the 4 treatment trials, lameness induction, treatment, and lameness evaluations were done on Day 1, but safety was assessed regularly for a total of 5 days. A physical examination was done every 12 hours for 6 days, and laboratory testing was done every 24 hours for 6 days. These examinations and tests were done to detect early signs of drug toxicity. We expected that KT would be better than PB and FM in reducing foot pain, and that no side effects would be seen. In addition, we tested whether or not the foot pain model induced local inflammation, or just pain from pressure without inflammation. To assess inflammation, we measured the foot temperature using a heat-detecting camera, and we measured several inflammatory proteins from blood pooled in the affected limb. We expected that the foot temperature and the inflammatory proteins would increase if inflammation was present, and that NSAIDs would decrease these parameters.

Data collection for this project is complete, and preliminary results regarding the foot lameness model itself were presented at a national conference (Conference for Research Workers in Animal Diseases) in Chicago in December, 2017. Based on the inflammatory markers we tested, pain appears to be from pressure, rather than local inflammation. Although no adverse effects were observed after repeated dosing for any drug, we found that “Bute” achieved superior
analgesia compared to other drugs. However, this pain model has significant limitations; horses tended to compensate over time for the induced foot pain, which confounded results even among the horses receiving no drug (placebo). Modifications of this model should be considered prior to use in future studies. Final results from this study will be presented at a national meeting in 2019.
APPENDIX D

Refereed Scientific Articles:


Abstracts and Proceedings:


• Tinkler SH., Penny Lecca I., Weil A., Couétil LL. Cardiopulmonary Parameters in Field Anesthetized Working Equids at High Altitude in the Peruvian Andes. World Equine Veterinary Association Congress, Verona Italy. October 2019.
APPENDIX E

Refereed Scientific Publications:


Effects of high doses of levothyroxine sodium on serum concentrations of triiodothyronine and thyroxine in horses

François R. Bertin DVM, PhD
Lauren Eichstadt Forsythe PharmD
Janice E. Kritchevsky VMD, MS

Received April 16, 2018. Accepted October 19, 2018.

From the School of Veterinary Science, The University of Queensland, Gatton, QLD 4343, Australia (Bertin); and the Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, IN 47907 (Eichstadt Forsythe, Kritchevsky). Dr. Eichstadt Forsythe’s present address is School of Veterinary Medicine, University of California-Davis, Davis, CA 95616.

Address correspondence to Dr. Bertin (f.bertin@uq.edu.au).

OBJECTIVE
To investigate the effect of high doses of orally administered levothyroxine sodium (LT4) on serum concentrations of triiodothyronine (T3) and thyroxine (T4) in euthyroid horses.

ANIMALS
12 healthy adult horses.

PROCEDURES
10 horses initially received water (vehicle) or 240 mg (5X treatment) or 480 mg (10X treatment) of LT4, and blood samples were collected at baseline (0 hours) and 0.5, 1, 2, 4, 6, 8, 10, 12, 18, 24, 48, 72, 96, and 120 hours after treatment to measure serum T3 and T4 concentrations. Three horses then received 480 mg of LT4 for 14 days, and T4 concentration was measured on days 0, 14, 21, 28, and 35. Changes in T3 and T4 concentrations were compared over time and among treatments.

RESULTS
One-time administration of LT4 resulted in variable but significant increases in both T3 and T4 concentrations for up to 120 hours; however, T3 and T4 concentrations rarely exceeded reference intervals with either treatment. Prolonged administration of 480 mg of LT4 resulted in a 15-fold increase in T4 concentration after 14 days, but concentration returned to day 0 values within 21 days after LT4 administration was discontinued.

CONCLUSIONS AND CLINICAL RELEVANCE
In euthyroid horses, administration of a high dose of LT4 resulted in mild increases in thyroid hormone concentrations; however, prolonged administration of high doses of LT4 resulted in markedly increased thyroid hormone concentrations that returned to pretreatment values within 3 weeks after discontinuation of LT4 administration. These results indicated complex kinetics of LT4 and suggested a possible saturation of T4 excretion in euthyroid horses. (Am J Vet Res 2019;80:565–571)

The hormones T3 and T4 are involved in virtually all metabolic processes in horses, with their primary action being to promote oxygen consumption.1–3 Effects of these hormones are through their actions on cells, both directly (which results in rapid modulation of cellular activities) and indirectly through genomic stimulation (which results in the upregulation and downregulation of protein synthesis). Thyroid hormones enhance protein and lipid anabolism and catabolism, stimulate basal metabolic rate, and regulate body heat production.1 Secretion of thyroid hormones is regulated by thyrotropin-releasing hormone from the hypothalamus.4 After they are secreted, T3 and T4 in the bloodstream are primarily bound to proteins; however, the free fractions of T3 and T4 are the metabolically active forms, with free T3 being the most active.4 Although thyroid hormones are involved in many metabolic processes and stimulate organ growth and maturation in horses, they are not an essential requirement for life because thyroidectomized horses have only limited clinical signs, which include cold intolerance and hair coat abnormalities (coarse hair, mild alopecia, and delayed shedding).5–9

Naturally occurring hypothyroidism is rare in adult horses, and the existence of primary hypothyroidism in adult horses has been debated.3 However, extrapolation of the clinical signs described for humans or dogs with hypothyroidism has suggested a possible association between hypothyroidism and weight gain, low fertility, and impaired lipid metabolism in horses. More recently, those clinical signs have been associated with EMS rather than with actual hypothyroidism.10 The pathophysi-
ology of EMS is still unclear, but insulin dysregulation has been found to be at the center of this disorder. Insulin dysregulation encompasses hyperinsulinemia and insulin resistance in peripheral tissues; therefore, treatment options for EMS are aimed at reducing hyperinsulinemia and improving sensitivity to insulin. Management of hyperinsulinemia is mainly achieved by dietary modifications to limit ingestion of nonstructural carbohydrates. On the other hand, insulin sensitivity can be improved by exercise and weight loss. Unfortunately, exercise is often contraindicated for horses with laminitis, and efforts in such animals are usually concentrated on weight loss. Successful weight loss can be achieved by dietary modification; however, a weight loss–resistant phenotype has been described that necessitates pharmaceutical intervention with metformin or thyroid hormone analogs. Metformin possibly blunts postprandial insulin responses, and it has been suggested that LT4 (the synthetic analog of T4) can improve carbohydrate and fat metabolism and accelerate weight loss.

Long-term administration of LT4 to euthyroid horses has resulted in weight loss with no adverse effects, which suggests that even if horses with EMS do not have hypothyroidism, thyroid hormone supplementation could be beneficial. In addition, after receiving LT4 for 8 weeks, euthyroid horses had improvements in insulin sensitivity and insulin disposal. Although increasing the concentrations of thyroid hormones could be beneficial in horses with EMS, there is conflicting evidence regarding the dose and duration of treatment to obtain effects. In one study, an increase in the T4 concentration was observed only after administration of high doses for 16 weeks, whereas in another study, an increase in the T4 concentration was observed 30 minutes after administration of a low dose. Effects of thyroid hormones at replacement doses have been described, but to our knowledge, the short-term effects of supraphysiologic doses of LT4 have not been reported. Therefore, the purpose of the study reported here was to investigate effects of 1-time and prolonged administration of high doses of LT4 on serum concentrations of T3 and T4 in adult euthyroid horses.

**Materials and Methods**

**Horses**

The study population consisted of 12 adult horses of various breeds donated to or purchased by the Purdue University Veterinary Teaching Hospital. Eleven horses had been donated because of problems unrelated to clinical signs consistent with EMS or pituitary pars intermedia dysfunction, and none had received any treatments within the 4 weeks preceding the study. The procedures were approved by the Purdue University Animal Care and Use Committee.

**Procedures**

The study was conducted in 2 phases. The first phase of the study was designed to investigate the short-term effects of 1-time administration of 2 doses of LT4 on serum concentrations of T3 and T4. Ten horses were housed in stalls and allowed to acclimatize for 24 hours, during which they had free access to mixed-grass hay and water. Then, a catheter was asexually placed in a jugular vein. One hour after the catheter was placed, horses were randomly assigned (dice roll) to receive water (vehicle treatment), 240 mg of LT4 (5 times the daily recommended dose of 48 mg) in water (5X treatment), or 480 mg of LT4 (10 times the daily recommended dose of 48 mg) in water (10X treatment) through a nasogastric tube (5 horses/group). Blood samples (4 mL/sample) were then collected via the IV catheter immediately before (0 hours; baseline) and 0.5, 1, 2, 4, 6, 8, 10, 12, 18, 24, 48, 72, 96, and 120 hours after treatment and placed in heparinized tubes. After a 14-day washout period, the procedures were repeated, so each horse received 2 of the 3 treatments.

The second phase of the study was designed to investigate the effects of prolonged administration of a high dose of LT4 on serum concentrations of T4. For that purpose, 3 horses were again housed in stalls and allowed to acclimatize for 24 hours as described previously. Then, horses received 480 mg of LT4 orally for 14 days. The LT4 dose was divided and provided twice daily mixed with grain. The dose of 480 mg was based on results of the first phase of the study. Blood samples were collected via venipuncture into heparinized tubes immediately before (day 0) and 14 (end of treatment), 21, 28, and 35 (7, 14, and 21 days after end of treatment, respectively) days after the start of treatment.

**Measurement of serum T3 and T4 concentrations**

All blood samples were centrifuged (2,000 X g for 10 minutes at 4°C), and serum was harvested and used to measure tT3 and tT4 concentrations. The tT3 concentration was measured with a solid-phase competitive chemiluminescent immunoassay on a chemical analyzer. Analytic sensitivity of the assay was 19 ng/dL, and calibration range was 40 to 600 ng/dL. Mean coefficient of variation of the assay was 6.5%, mean intra-assay precision for equine samples was 5.5%, and mean percentage recovery for equine samples was 94.7%. The tT4 concentration was also measured with a solid-phase competitive chemiluminescent immunoassay by use of the same chemical analyzer. Analytic sensitivity of the assay was 0.3 µg/dL, and calibration range was 1.0 to 24 µg/dL. Mean coefficient of varia-
tion of the assay was 7.6%, mean intra-assay precision for equine samples was 8.7%, and mean percentage recovery for equine samples was 102.7%.

**Data analysis**

Normal distribution was determined by use of the Shapiro-Wilk test. Mean ± SD was calculated for normally distributed data, and median and range were calculated for nonnormally distributed data. For the first phase of the study, horses were grouped on the basis of treatment (vehicle, 5X, or 10X); however, each horse received only 2 of the 3 treatments. Initial (baseline and day 0 for the first and second phases, respectively) tT3 and tT4 concentrations were compared among treatments by use of a 1-way ANOVA, and changes in tT3 and tT4 concentrations resulting from LT4 administration were compared by means of a 2-way repeated-measures ANOVA and Tukey post hoc test, when relevant. For the second phase of the study, changes in tT4 concentrations resulting from LT4 administration were compared by use of a 1-way ANOVA and Dunnett post hoc test. Statistical analyses were performed with commercially available software. Values of P < 0.05 were considered significant.

**Results**

All horses tolerated both phases of the study well. The only adverse effects, which were observed in all horses, were an increase in nervous behavior and reluctance to stand still at day 14 of the second phase of the study.

At baseline, the tT3 concentration of 2 horses (1 for the vehicle treatment and 1 for the 10X treatment) was lower than the lower limit of the reference interval (30 to 80 ng/dL), but there were no significant differences (P = 0.11) in baseline tT3 concentrations among treatments. Similarly, the baseline tT4 concentration of 2 other horses (1 for the vehicle treatment and 1 for the 10X treatment) was lower than the lower limit of the reference interval (1.0 to 3.0 µg/dL), but there were no significant differences (P = 0.93) in baseline tT4 concentrations among treatments. None of the horses had baseline tT3 or tT4 concentrations greater than the upper limit of the reference intervals.

One-time administration of LT4 resulted in an increase in tT3 concentration (Figure 1); the concentration was significantly different from the baseline concentration at 4, 8, and 120 hours for the 10X treatment. Compared with concentrations for the vehicle treatment, tT3 concentration was significantly higher at 8 and 120 hours for the 5X treatment and at 4, 8, 12, and 120 hours for the 10X treatment. Administration of LT4 resulted in tT3 concentrations transiently greater than the reference interval in 2 horses for the 5X treatment and 1 horse for the 10X treatment.

One-time administration of LT4 also resulted in an increase in tT4 concentration (Figure 2). The concentration was significantly (P = 0.02) different from the baseline concentration at 4, 8, and 120 hours for the 10X treatment. Compared with concentrations for the vehicle treatment, tT4 concentration was significantly higher at 4, 8, 12, and 120 hours for the 10X treatment.

![Figure 1](image1.png)

**Figure 1**—Mean ± SD serum concentration of tT3 for 5 horses over 24 (A) and 120 (B) hours after receiving 1 dose of water (vehicle treatment; squares and dashed line), 240 mg of LT4 (white circles and solid line), or 480 mg of LT4 (black circles and solid line) by nasogastric intubation. Baseline (0 hours) was immediately before LT4 administration. The dotted lines indicate the reference interval. *Within a time point, value differs significantly (P < 0.05) from the value for the vehicle treatment. †Within a treatment, value differs significantly (P < 0.05) from the baseline value.
baseline concentration from 8 to 48 hours for the 5X treatment and from 2 to 120 hours for the 10X treatment. Compared with concentrations for the vehicle treatment, the tT3 concentration was significantly higher from 12 to 24 hours for the 5X treatment and from 4 to 120 hours for the 10X treatment. Administration of LT4 resulted in tT3 concentrations transiently greater than the reference interval in 5 horses for the 10X treatment.

Administration of LT4 for 14 days in the second phase of the study resulted in a marked increase in tT4 concentration (Figure 3). The concentration on day 14 was significantly different from the concentration on day 0, but the concentration on day 21 (7 days after cessation of administration) was not significantly different from the concentration on day 0. Discontinuation of treatment resulted in a return to tT3 concentrations to within the reference interval by day 35 (21 days after cessation of administration).

**Discussion**

The main result of the study reported here was that although 1-time administration of high doses of LT4 resulted in a significant increase in serum tT3 and tT4 concentrations, those increases were mild and rarely resulted in concentrations above the reference intervals. In contrast, administration of a high dose of LT4 for 14 days resulted in a marked increase in the tT4 concentration, which returned to preadministration concentrations gradually when treatment was discontinued.

The limited effect of LT4 administration on tT3 concentrations has been previously described. In 1 of those studies, administration of LT4 at a lower dose (48 mg/d) but for a longer period (48 weeks) resulted in a decrease in tT3 concentrations by 32 weeks, with mean values as low as 20 ng/dL. On the other hand, in another study, a lower dose of LT4 (10 mg, once) resulted in marked increases in tT3 concentrations within 2 hours, with mean values as high as 100 ng/dL. These changes were not detected after 1-time administration in the study reported here because, despite the higher doses used in the present study, mean values remained within the reference intervals, and only 3 horses had values transiently and mildly greater than the reference intervals. The effect of LT4 administration on tT3 concentrations depends on dose and duration of LT4 administration, with possible paradoxical effects. On the one hand, LT4 could decrease tT3 concentrations because LT4 would be converted to free T4 and activate negative feedback on thyroid-stimulating hormone secretion, but on the other hand, LT4 could increase tT3 concentrations because most of tT3 comes from peripheral deiodination of tT4. Measurement of free T3 concentrations in the present study and in other studies might have been a more accurate estimation of the effect of LT4 administration; however, total and free fractions supposedly are correlated in healthy subjects.

In 1 study, LT4 administration (10 mg, once) induced an increase in tT3 concentration by 1 hour after...
administration, and the increase was sustained for 24 hours. Consistent with results of that study, a more moderate but acute increase in tT₄ concentration was detected in the study reported here. The increases detected in that other study were mild, with a mean peak concentration of 2.0 µg/dL, whereas in the present study, a mean peak of 3.5 µg/dL was achieved for the 10X treatment. This difference could be attributable to the dose used (10 mg vs 480 mg); however, it could be expected that a 48-fold increase in drug dose would result in larger changes. A possible explanation for a limited dose effect is the poor absorption of LT₄ after oral administration to horses. In human medicine, fiber and bran cereal decrease oral absorption of thyroid hormones. Considering the diet of horses, it could be expected that orally administered LT₄ would be poorly absorbed, which suggests that even large increases in drug doses would result in only minimal increases in blood hormone concentrations and that for maximal effects, food should be withheld from horses prior to administration of thyroid hormones. In the present study, the difference between the 5X and the 10X treatments was less than could be expected, which suggested a possible saturation of LT₄ absorption when administered to horses that did not have food withheld prior to LT₄ administration. This poor absorption could also explain the reason that no results are detected during the first few weeks of administration and only limited increases in concentrations are evident, even with long-term administration to horses. However, even if LT₄ is absorbed in horses from which food has been withheld, administering the drug to horses with an empty stomach is impractical in most on-farm settings. Further investigations of LT₄ absorption in horses from which food has been withheld prior to LT₄ administration are warranted, even if the practice would potentially have limited clinical relevance.

Despite the fact there is poor drug absorption of LT₄, long-term administration of LT₄ has been used clinically for the treatment of various conditions. Immune-mediated thyroid gland disease has been reported in humans and in dogs but is rare in horses; therefore, long-term treatment has mainly been recommended for euthyroid horses with obesity-related endocrine disorders such as EMS. In those cases, common practice has been to adapt the dose of LT₄ on the basis of tT₄ concentrations. In the study reported here, administration of 480 mg of LT₄/d for 14 days resulted in a 15-fold increase in the tT₄ concentration, with a mean value of approximately 21 µg/dL. These results are in agreement with those of another study in which administration of 48 mg of LT₄/d resulted in a 5-fold increase in the tT₄ concentration to achieve a mean value of approximately 9 µg/dL within 16 weeks.

The tremendous differences in concentrations achieved between 1-time administration and administration of LT₄ for 14 days indicated that thyroid hormones have extremely complicated kinetics. Furthermore, these results suggested that there would be a saturation effect of LT₄ excretion and that horses could physiologically manage a high dose of LT₄ for a short time but not for prolonged periods of administration.

The awareness of EMS is increasing, and LT₄ is mainly used for treatment of obesity in horses. Although the precise mechanism of EMS has not been determined, obesity is one of its most common yet nonessential traits. In euthyroid horses, prolonged treatment with LT₄ results in weight loss and improved insulin sensitivity. Within 16 weeks after the start of treatment, substantial weight loss associated with an increase in insulin sensitivity, as measured via a frequently sampled IV glucose tolerance test, has been reported. These findings suggest a beneficial effect for the treatment of obesity in horses with insulin dysregulation in the context of EMS. A positive correlation between thyroid gland function and insulin sensitivity has been described for humans, and the administration of low doses of T₃ has been recommended for specific cases of obesity and human metabolic syndrome. The effect of an increased dose of LT₄ on glucose and insulin dynamics was beyond the scope of the study reported here; however, the data reported here support the use of a higher dose of LT₄ for prolonged periods in horses because it resulted in a significant increase in tT₄ concentrations. In addition, there are possible effects on weight loss. Although no clinical complications were observed in the present study, a more thorough investigation of the safety of higher doses is warranted.

In the study reported here, 4 horses had low thyroid hormone concentrations at baseline, which suggested that those horses could have been misdiagnosed with hypothyroidism on the basis of analysis of
a sample obtained at a single time point. Measurement of thyroid hormones has been both physiologically and technically challenging, which has led to a false-positive diagnosis of hypothyroidism in horses and also has led to some authors questioning the validity of the reference intervals used. Therefore, a low hormone concentration at a single time point would be a weak indicator of hypothyroidism because factors such as weather, diet, feed availability, exercise, drug administration, stage of the reproductive cycle, and time of day have all been found to decrease thyroid hormone concentrations.

In addition to differences between measurement of total or free fractions of thyroid hormone concentrations when hormonal protein binding can be altered by disease state, drugs, or other hormones, measurement by direct methods reportedly underestimates T4 concentrations, compared with concentrations obtained by use of dialysis or ultrafiltration methods. In the present study, different, although not significantly so, initial (baseline) results obtained from the same horses were in agreement with the low diagnostic value of a result obtained at a single time point when assessing thyroid gland function. Stimulation tests to assess thyroid gland function have been described and could have been used in the study reported here to confirm the absence of hypothyroidism; however, given the low prevalence of hypothyroidism in horses, the limited description of those tests, and the poor availability of drugs such as synthetic thyroid-stimulating hormone, stimulation tests were not undertaken.

Results of the present study indicated that 1-time administration of LT4, even at high doses, induced limited increases in serum tT3 and tT4 concentrations but that prolonged administration of high doses of LT4 significantly increased the serum tT4 concentration, which could be relevant in horses with weight loss resistance. Further studies are warranted to evaluate the effect of high doses of LT4 on insulin dynamics of horses.

Acknowledgments
The authors received no funding for the study. The authors declare that there were no conflicts of interest.

Presented in part as an oral presentation at the 2015 Forum of the American College of Veterinary Internal Medicine, Indianapolis, June 2015, and at the 35th Annual Meeting of the Society of Veterinary Hospital Pharmacists, Manhattan, Kan, June 2015.

Footnotes
c. Prism, GraphPad Software Inc, La Jolla, Calif.

References
Comparison of the racing performance of Thoroughbreds with versus without osteochondral fragmentation of the accessory carpal bone identified on yearling sales repository radiographs

Alec J. Davern DVM, MS  
John G. Peloso DVM, MS  
Jan F. Hawkins DVM  
George E. Moore DVM, PhD  
James P. Morehead DVM

From Equine Medical Center of Ocala, 7107 W Hwy 326, Ocala, FL 34482 (Davern, Peloso); the Departments of Veterinary Clinical Sciences (Hawkins) and Veterinary Administration (Moore), College of Veterinary Medicine, Purdue University, West Lafayette, IN 47907; and Equine Medical Associates PSC, 996 Nandino Blvd, Lexington, KY 40583 (Morehead). Dr. Davern’s present address is Centaur Equine Specialty Hospital, Purdue University, 350 W Bassett Rd, Shelbyville, IN 46176.

Address correspondence to Dr. Davern (adavernf@purdue.edu).

OBJECTIVE
To evaluate 2- and 3-year-old and career race performance of Thoroughbred racehorse prospects with and without osteochondral fragmentation of the accessory carpal bone (ACB) identified on yearling presale radiographs.

DESIGN
Retrospective, matched cohort study.

ANIMALS
47 nonlame Thoroughbreds with (exposed cohort) and 94 nonlame Thoroughbreds without (unexposed cohort) osteochondral fragmentation of ACB fracture identified on yearling sales repository radiographs.

PROCEDURES
Repository radiographic interpretation reports for September yearling sales of a large Kentucky auction house from 2005 through 2012 were reviewed, and race records were collected and analyzed. Race performance was compared between horses with and without ACB fracture chosen from the same sale to identify associations between racing performance and ACB fracture.

RESULTS
No significant differences were identified between horses with or without ACB fracture in their incidence of starting a race as a 2- or 3-year-old and the number of races started, earnings, or earnings per start for 2- or 3-year-old or career race performance. There was no significant difference in performance between horses with or without concurrent carpal osteoarthritis, nor did performance differ between horses with ACB fracture alone and those with ACB fracture and other radiographic abnormalities found to be associated with poorer performance in previous studies.

CONCLUSIONS AND CLINICAL RELEVANCE
ACB fracture with or without carpal osteoarthritic changes identified on repository radiographs of Thoroughbred yearlings was not associated with poorer racing performance or lower likelihood of starting a race as a 2- or 3-year-old, compared with outcomes for unaffected horses. (J Am Vet Med Assoc 2019;254:501–507)

Approximately 8,400 yearling Thoroughbreds in the United States were offered at public auction annually between 2000 and 2015, according to the Jockey Club.1 Prepurchase examinations are an important aspect of the equine veterinary profession and are a source of professional liability.2 For yearling Thoroughbreds, prepurchase assessment at public auction often consists of brief visual inspection, observation of the horse walking in hand, endoscopic examination of the upper respiratory tract, and review of a standardized set of survey radiographs, as regulated by the specific sales company. Radiographic images are stored in repositories for review by prospective buyers and their veterinarian prior to the auction.

ABBREVIATIONS
ACB  Accessory carpal bone

Given the results of the prepurchase examination, veterinarians are asked to make an accurate assessment of findings that may affect future racing or resale prognosis.3,4 The findings of prepurchase examinations and subsequent recommendations to clients should be based on objective, scientific evidence when possible. However, published objective data are lacking on the clinical importance of some common radiographic findings.

Researchers have attempted to identify normal and abnormal radiographic findings in yearling Thoroughbreds that could impact future racing performance. In research reported in 2003,3,4 radiographic data on 1,162 yearlings offered for public auction were analyzed, and the incidence of abnormalities as well as their correlations to racing performance were determined. Four abnormalities were significantly associated with a decreased incidence of horses starting a race as a 2- or 3-year-old: moderate to extreme supra-
condylar lysis of the third metacarpal bone, enthesophytosis of the proximal sesamoid bones, dorsomedial intercarpal joint disease, and distal intertarsal or tarso-metatarsal osteophytosis or enthesophytosis. A significant decrease was also identified in the percentage of race starts in which horses placed, total earnings, and money earned per race start for horses with (vs without) proximal sesamoid bone enthesophytes. In contrast, in another study in which radiographs of 597 yearlings offered for sale in Kentucky were reviewed, only enthesophytosis or enthesophytosis of the forelimb proximal sesamoid bones was significantly associated with a decreased likelihood of starting a race as a 2-year-old. Although both research groups found that osteochondral fragmentation (vs no fragmentation) of the proximal phalanx was associated with a decreased incidence of starting a race as a 2- or 3-year-old, this association was not significant.

An additional study involving 2-year-old Thoroughbreds in training revealed lower odds of starting a race when horses had (vs did not have) a diagnosis of dorsoproximal osteochondral fragmentation of the proximal phalanx, proximal sesamoid bone sesamoiditis or fracture, or wedge-shaped central or of the proximal phalanx, proximal sesamoid bone sesamoiditis or fracture, or wedge-shaped central or thirplex proximal sesamoid bones. Other research showed poorer 2- and 3-year-old racing performance for yearlings with (vs without) radiographic evidence of sesamoiditis. Finally, in a study involving 548 yearling Thoroughbreds offered for sale in Texas, no single abnormal radiographic finding was significantly associated with the ability to start a race.

Fracture of the ACB in horses has been uncommonly identified in repository radiographs in previous studies. The overall prevalence of ACB fracture in all 4 studies combined was 4 of 2,176 (0.2%). Although this prevalence is low, ACB fracture does appear on repository radiographs, and to our knowledge, no peer-reviewed, objective data exist of its impact on future racing performance of Thoroughbreds.

The aim of the study reported here was to provide objective data regarding the prognostic value of identification of ACB fractures, specifically osteochondral fragments originating from the ACB, on the prepurchase radiographs of yearling Thoroughbreds. We hypothesized that there would be no significant difference in race performance between horses with ACB fracture diagnosed on yearling sale repository radiographs and their unaffected counterparts. Our second hypothesis was that, in horses with ACB fracture, concurrent osteoarthritic change (vs no such change) noted at the dorsal aspect of the affected carpus would be associated with poorer racing performance.

**Materials and Methods**

**Study design**

A retrospective matched cohort study was performed. Repository radiographic interpretation reports from the Keeneland September sale of yearlings in Kentucky (2005 through 2012) generated by any 1 of 3 experienced observers from 1 Kentucky-based private practice or 1 experienced observer from a Florida-based private practice were searched. Experienced was defined as having reviewed a minimum of 1,000 sets of repository radiographs. The following radiographic images were included in the repository: metacarpophalangeal joints (dorsal 15° proximal-palmarodistal oblique, flexed lateromedial, standing lateromedial, dorsal 30° lateral 15° proximal-palmaromedial distal oblique, and dorsal 30° medial 15° proximal-palmarolateral distal oblique views), carpi (flexed lateromedial, dorsal 35° lateral-palmaromedial oblique, and dorsal 25° medial-palmarolateral oblique views), metatarsophalangeal joints (dorsal 15° proximal-plantarodistal oblique, dorsal 15° proximal 30° medial-plantarolateral oblique, dorsal 15° proximal 30° lateral-plantaromedial oblique, and standing lateromedial views), tarsi (dorsal 10° lateral-plantaromedial oblique, lateromedial, and dorsal 65° medial-plantarolateral oblique views), and stifle joints (lateromedial, caudal 20° lateral-cranioomedial oblique, and caudal 15° proximal-craniodistal views).

**Animals**

The exposed cohort was comprised of yearlings in which a fracture of 1 or both ACBs was noted on the radiographic interpretation report but that were presumed to be nonlame at the time (as per conditions of the sale). All horses with ACB fractures were initially included in this cohort; however, the cohort was further refined prior to analyses to include only those with osteochondral fragmentation specifically (referred to henceforth simply as ACB fracture). To select horses for the unexposed cohort, a 2:1 matching scheme was used by which 2 horses without any ACB fracture (1 yearling sold before and 1 sold after the matched horse with ACB) were included for every horse with ACB fracture. Specifically, unexposed horses were chosen from the yearlings with the closest hip number before and after the horse with the ACB fracture in the sale catalog, for which radiographic interpretation reports were available. This selection protocol was similar to that used in a previous study, except that the number of horses selected for the unexposed cohort was greater to increase statistical power while including representative individuals. This strategy allowed confirmation of the absence of ACB fracture in unexposed horses while providing age-matched counterparts with similar presale appraisal value and athletic potential for comparison.

At the Keeneland September sale, location in the sale catalog is based on presale analysis of pedigree power as well as appraisal of physical appearance and conformation performed by the sale company. For consistency, all horses selected for the unexposed cohort were identified from reports generated by 3 veterinarians at the Kentucky-based practice. These horses were selected without replacement, so no unexposed horse was included in the data analyses more than once.
Categorization of lesions

Horses were first grouped for data analysis by exposure status (ACB fracture or no ACB fracture). The exposed cohort was then subdivided into yearlings with or without other radiographic findings previously identified in the literature as associated with a significant reduction in the likelihood of starting a race as a 2- or 3-year-old (ie, osteoarthritic change at the dorsal aspect of the carpus, moderate or severe tarsal osteoarthritis, forelimb proximal sesamoid bone enthesophytosis or osteophytosis, moderate or severe supracondylar lysis of the third metacarpal bone, or moderate to severe sesamoiditis). The exposed cohort was also subdivided into yearlings with ACB fracture and concurrent osteoarthritic change visible at the dorsal aspect of the affected carpus and those with ACB fracture and no such concurrent osteoarthritic change.

Radiographic findings considered to indicate osteoarthritis of the carpal joints included periartricular enthesophytosis or osteophytosis or remodeling at the dorsal aspect of the carpal bones. Likewise, radiographic findings consistent with osteoarthritis of the distal aspect of the tarsal joints included periartricular new bone formation at the dorsal aspect of the distal intertarsal or tarsometatarsal joint with or without a wedge shape to the central or third tarsal bones.

Data regarding descriptors similar to “mild osteoarthritis of the lower hock joint” and “mild spavin” indicating a mild osteoarthritic change at the dorsal aspect of the distal tarsal joints were extracted as a separate variable and excluded from the definition of clinically important lesions because these findings were identified more commonly in horses without ACB fractures than in those with ACB fractures.

Racing performance analysis

Racing performance data were collected via an online third-party database of equine racing records, accessed on December 21, 2014, and May 15, 2016. The database was searched by the dam’s name and horse’s birth year. If no record existed, the horse in question was presumed to have failed to start a race. Collected performance data included the number of starts as well as earnings per year and earnings per start for the races performed as a 2- and 3-year-old. Data on career total starts, earnings, and mean earnings per start were also collected and assessed. Analyses of career performance data was performed, including only horses that had started at least 1 race and had not raced within 6 months before data collection.

Statistical analysis

Horses with bilateral lesions were included only once in the statistical analyses. The χ² test of independence was used for comparisons of proportions of horses with versus without ACB fracture regarding certain binary characteristics (eg, raced vs did not race as a 2- or 3-year-old, presence vs absence of other radiographic lesions previously deemed important, and presence vs absence of radiographic evidence of carpal osteoarthritis). For 2- and 3-year-old horses, owing to the large number of 0 values for starts and therefore earnings, zero-inflated Poisson regression models and zero-inflated negative binomial regression models were used to compare horses with and without ACB fracture regarding the number of starts as well as earnings and earnings per start, respectively, while controlling for sex. The choice of zero-inflated model was based on a significant Vuong test result for comparisons of zero-inflated models with standard Poisson and negative binomial regression models. Because career analyses included only horses that had started a race (ie, no 0 values), standard linear regression modeling was used for starts, earnings, and earnings per start after logarithmic transformation of these variables.

The exposed cohort was then subdivided, and analyses were repeated to compare horses that had ACB fracture and other radiographic abnormalities previously deemed important with horses that had ACB fracture and none of these abnormalities. In a final analysis, horses with ACB fracture and concurrent carpal osteoarthritis were compared with those with ACB fracture and no such osteoarthritis. For all tests, values of P < 0.05 were considered significant. All statistical analyses were performed with statistical software.

Results

Horses and fractures

During the study period, 52 ACB fractures were identified in 50 Thoroughbred yearlings. Two horses had unilateral vertical fractures in the frontal plane of the ACB and were subsequently excluded, and another horse was excluded because it would have shared the same matched horses without ACB fracture as another horse with ACB fracture, violating the assumption of independence of the horses. Consequently, 47 yearlings (2 with bilateral fractures; 27 [57%] males and 20 [43%] females) with 49 fractures (27 of the right ACB and 22 of the left ACB) were included in the exposed cohort, and 94 yearlings without ACB (55 [59%] males and 39 [41%] females) were included in the matched unexposed cohort.

Of the osteochondral fragments noted in the repository radiographic interpretation reports for horses with ACB fracture, most (29/49 [59%]) were in a dorsoproximal location. Other fragments were described as dorsal (5 [10%]), proximal (3 [6%]), dorsal nonarticular (2 [4%]), and dorsodistal articular, articular, axial, axial articular, and proximopalmar (1 [2%] each). Five (10%) osteochondral fragments had undescribed locations.

Twenty-four of the 47 (51%) horses with ACB fracture had concurrent osteoarthritis at the dorsal aspect of the affected carpus. In 16 of these horses, this concurrent carpal osteoarthritis would have been the only finding considered a clinically impor-
tant radiographic abnormality given the predefined criteria. When osteoarthritic change in the carpus with the ACB fracture was removed from the definition of important radiographic abnormalities, there were 33 (70%) horses with ACB fracture but no other important radiographic abnormalities, whereas 68 of the 94 (72%) horses without ACB fracture were free of important radiographic abnormalities. These proportions were statistically similar ($P = 0.79$).

Overall, 54 of all 141 (38%) included horses had a diagnosis of mild tarsal osteoarthritis (11/47 [23%] with and 43/94 [46%] without ACB fracture). For 9 of 11 horses with ACB fracture and 33 of 43 horses without ACB fracture, this would have been the only clinically important lesion noted; therefore, given the predefined criteria, these horses were considered to have no radiographic abnormalities. Of the 47 horses with ACB fracture, 24 (51%) had concurrent carpal osteoarthritis at the dorsal aspect of the affected carpus and 23 (49%) had no such changes. For purposes of analyses, the carpus was viewed as a single unit; thus, horses were not separated on the basis of whether the concurrent carpal osteoarthritis was identified in the antebrachiocarpal joint (21/47), middle carpal joint (1/24), or both joints (2/24). Carpometacarpal joint disease was not reported for any horse. Of the horses with bilateral ACB fractures, one had a bilateral antebrachiocarpal joint osteoarthritic change and the other had unilateral antebrachiocarpal joint changes.

**Performance**

Of horses with ACB fracture as yearlings, 28% (13/47) started a race as a 2-year-old and 60% (28/47) started a race as a 3-year-old. Six (13%) horses with ACB fracture never started a race. Of horses without ACB fracture, 37% (35/94) started a race as a 2-year-old and 73% (69/94) started a race as a 3-year-old. The incidence of a horse starting at least 1 race during either of these years did not differ significantly between the 2 cohorts (Table 1). Additionally, 66% (31/47) of horses with ACB fracture and 79% (74/94) of horses without ACB fracture started a race at some point in their careers, and the difference in these proportions was not significant (Table 2). No significant differences were identified in the number of races started, earnings, or earnings per start as a 2- or 3-year-old between horses with and without ACB fracture.

Of the 47 horses with ACB fractures, 24 were included in the career performance analyses because they had successfully started a race but had not started a race within 6 months prior to the date of data collection. Of the 94 horses without ACB fractures, the same criteria resulted in 53 horses being included in the career performance analyses. Sixteen horses with ACB fracture and 20 without ACB fracture failed to start a race, and another 7 horses with ACB fracture and 21 without ACB fracture were excluded from career performance analyses because of having raced within 6 months prior to the date of data collection. These exclusions resulted in inclusion of fewer horses in the career data analyses than the number that raced as a 2- or 3-year-old. No significant differences were identified between cohorts in total career starts, earnings, or earnings per start (Table 2). However, a post hoc power analysis indicated that to have 80% power to detect a significant difference ($\alpha$...)

---

### Table 1—Comparison of race performance as a 2- or 3-year-old between Thoroughbred yearlings with (n = 47) and without (94) osteochondral fragmentation of the ACB (ACB fracture) diagnosed on yearling sale repository radiographs.

<table>
<thead>
<tr>
<th>Performance variable</th>
<th>With ACB fracture</th>
<th>Without ACB fracture</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) that started ≥ 1 race</td>
<td>13 (28)</td>
<td>35 (37)</td>
<td>0.26*</td>
</tr>
<tr>
<td>Median (range) No. of starts, if raced</td>
<td>3 (1–8)</td>
<td>2 (1–8)</td>
<td>0.17†</td>
</tr>
<tr>
<td>Median (range) annual earnings ($), if raced</td>
<td>7,908 (0–157,267)</td>
<td>9,318 (200–126,000)</td>
<td>0.94‡</td>
</tr>
</tbody>
</table>

*Value derived from the $\chi^2$ test of independence. †Value derived from a zero-inflated Poisson regression model adjusted for horse sex. ‡Value derived from a zero-inflated negative binomial regression model adjusted for horse sex.

### Table 2—Comparison of career race performance between Thoroughbreds with (n = 47) and without (94) ACB fracture diagnosed on yearling sale repository radiographs.

<table>
<thead>
<tr>
<th>Performance variable</th>
<th>With ACB fracture</th>
<th>Without ACB fracture</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) that started ≥ 1 race</td>
<td>31 (66)</td>
<td>74 (79)</td>
<td>0.10*</td>
</tr>
<tr>
<td>Median (range) No. of starts</td>
<td>13 (1–48)</td>
<td>15 (2–43)</td>
<td>0.62‡</td>
</tr>
<tr>
<td>Median (range) annual earnings ($)</td>
<td>23,967 (3,153–1,719,621)</td>
<td>34,130 (680–2,015,893)</td>
<td>0.53‡</td>
</tr>
<tr>
<td>Median (range) earnings per start ($)</td>
<td>3,095 (978–101,154)</td>
<td>3,095 (171–95,995)</td>
<td>0.20‡</td>
</tr>
</tbody>
</table>

*Value derived from the $\chi^2$ test of independence. †Value derived from a linear regression model with a logarithmically transformed independent variable, adjusted for horse sex.

For number of starts, earnings, and earnings per start, horses that had never raced or were suspected to be actively racing at the time of data acquisition were excluded, thereby leaving 24 horses with ACB fracture and 53 horses without ACB fracture in those analyses.
\[P = 0.05\] between the observed proportions of horses that raced, the exposed cohort would have required 136 horses and the unexposed cohort 272 horses.

Statistical analyses revealed no association between concurrent carpal osteoarthritis in horses with ACB fracture and any performance variable. To further isolate the effect of the ACB fracture, the racing performance of horses with ACB fracture and other clinically important radiographic lesions was compared with the performance of horses with ACB fracture and radiographic lesions limited to the fracture (with horses with concurrent osteoarthritis of the affected carpi excluded), again revealing no significant differences between groups (Table 3).

### Discussion

Findings of the present study indicated that nonlame Thoroughbred yearlings with ACB fractures, specifically osteochondral fragmentation, diagnosed on prepurchase radiographs could achieve a level of race performance similar to their matched, unaffected counterparts, supporting our first hypothesis that there would be no difference between these 2 cohorts. No significant difference was identified in the likelihood of a horse starting a race or any other performance variable assessed, suggesting that although the ability to race successfully was influenced by no single factor, the identification of ACB fracture in yearling Thoroughbreds sold for racing did not appear to adversely impact their subsequent likelihood of racing.

The ACB is 1 of 7 carpal bones comprising the carpus. Although not directly weight bearing, the ACB acts as a sesamoid bone that serves as the attachment site for several short ligaments and the tendons of insertion of the flexor carpi ulnaris and ulnaris lateralis muscles. The ACB has dorsal articular surfaces that form a portion of the palmar aspect of the antebrachiocarpal joint, and the medial surface of the ACB is intimately involved in the carpal canal.11-13

Fractures of the ACB can differ in morphology, although those described herein were osteochondral fragments. The most common form of ACB fracture reported in the veterinary literature is a complete, mildly displaced fracture in the frontal plane. This type of ACB fracture is often diagnosed in jumping or steeplechase horses that are evaluated for lameness, commonly after a traumatic event.13,14 The horses included in the present study were those brought for routine prepurchase examination at public auction, and in accordance with yearling sales protocol, yearlings brought for sale are expected to be free of lameness at the walk.

Clinical outcomes of ACB fractures of various morphologies have been reported and include so-called carpal canal syndrome15 and carpal osteoarthritis. However, dorsoproximal fragments and vertical fractures can carry a good prognosis when treated conservatively, and secondary osteoarthritis of the antebrachiocarpal joint is seemingly uncommon.14 Although uncommon in sport horses following ACB fracture, the potential for development of antebrachiocarpal synovitis or osteoarthritis following

---

**Table 3** — Comparison of race performance as a 2- and 3-year-old between Thoroughbreds with ACB fracture with versus without concurrent carpal osteoarthritis.

<table>
<thead>
<tr>
<th>Performance variable</th>
<th>With carpal osteoarthritis (n = 24)</th>
<th>Without carpal osteoarthritis (n = 23)</th>
<th>P value</th>
<th>ACB fracture with other lesions (n = 14)</th>
<th>ACB fracture without other lesions (n = 17)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-year-old</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range) No. of starts</td>
<td>0 (0–7)</td>
<td>0 (0–8)</td>
<td>0.93*</td>
<td>0 (0–6)</td>
<td>0 (0–8)</td>
<td>1.00*</td>
</tr>
<tr>
<td>No. (%) that started ≥ 1 race</td>
<td>6 (25)</td>
<td>7 (30)</td>
<td>0.68‡</td>
<td>5 (36)</td>
<td>4 (24)</td>
<td>0.46‡</td>
</tr>
<tr>
<td>Median (range) No. of starts, if raced</td>
<td>3.5 (3–7)</td>
<td>3 (1–8)</td>
<td>0.38*</td>
<td>3 (1–6)</td>
<td>2.5 (1–8)</td>
<td>0.95*</td>
</tr>
<tr>
<td>Median (range) earnings ($)</td>
<td>(1,189–157,267)</td>
<td>(0–14,480)</td>
<td>0.20†</td>
<td>(30–32,760)</td>
<td>(4,600–11,252)</td>
<td>0.08‡</td>
</tr>
<tr>
<td>Median (range) earnings per start ($)</td>
<td>3,057</td>
<td>2,475</td>
<td>0.34‡</td>
<td>1,789</td>
<td>2,697</td>
<td>0.49‡</td>
</tr>
<tr>
<td>3-year-old</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range) No. of starts</td>
<td>2.5 (0–14)</td>
<td>2 (0–10)</td>
<td>0.63*</td>
<td>2.5 (0–14)</td>
<td>2 (0–10)</td>
<td>0.47*</td>
</tr>
<tr>
<td>No. (%) that started ≥ 1 race</td>
<td>14 (58)</td>
<td>14 (61)</td>
<td>0.86‡</td>
<td>10 (71)</td>
<td>9 (53%)</td>
<td>0.29‡</td>
</tr>
<tr>
<td>Median (range) No. of starts, if raced</td>
<td>5 (1–14)</td>
<td>4 (2–10)</td>
<td>0.08*</td>
<td>6 (1–14)</td>
<td>4 (2–10)</td>
<td>0.50*</td>
</tr>
<tr>
<td>Median (range) earnings ($)</td>
<td>(732–195,711)</td>
<td>(480–170,433)</td>
<td>0.91‡</td>
<td>15,408</td>
<td>33,876</td>
<td>0.29‡</td>
</tr>
<tr>
<td>Median (range) earnings per start ($)</td>
<td>3,507</td>
<td>2,844</td>
<td>0.61†</td>
<td>2,658</td>
<td>6,775</td>
<td>0.17‡</td>
</tr>
<tr>
<td>Career</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range) starts, if raced</td>
<td>14 (1–48)</td>
<td>12 (1–42)</td>
<td>0.82§</td>
<td>8 (1–48)</td>
<td>19 (1–42)</td>
<td>0.20§</td>
</tr>
<tr>
<td>Median (range) career earnings ($), if raced</td>
<td>21,658</td>
<td>26,275</td>
<td>0.92†</td>
<td>21,170</td>
<td>164,262</td>
<td>0.18§</td>
</tr>
<tr>
<td>Median (range) career earnings per start ($)</td>
<td>(3,153–1,719,621)</td>
<td>(3,383–758,338)</td>
<td></td>
<td>(3,153–1,719,621)</td>
<td>(4,600–758,338)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3,037)</td>
<td>3,903</td>
<td>0.96‡</td>
<td>2,628</td>
<td>5,416</td>
<td>0.34‡</td>
</tr>
</tbody>
</table>

*Value derived from a zero-inflated Poisson regression model adjusted for horse sex. †Value derived from the \( \chi^2 \) test of independence. ‡Value derived from a zero-inflated negative binomial regression model adjusted for horse sex. §Value derived from a linear regression model with a logarithmically transformed independent variable, adjusted for horse sex.

Career data were unavailable for horses that were still racing or that had never started a race.
osteochondral fragmentation of the ACB is a concern for Thoroughbred racehorse prospects.\textsuperscript{13,14} Osteochondral fragments arising from the palmar aspects of the cuboidal carpal bones in the antebrachiocarpal and middle carpal joints secondary to trauma can carry a poorer prognosis for return to function than dorsal carpal osteochondral fragments.\textsuperscript{16} This poorer prognosis is attributable to the development of carpal osteoarthritis or degenerative joint disease. At the time of sale, 23 of 47 (49\%) yearlings with osteochondral fragmentation of the ACB in the present study had radiographic evidence of carpal osteoarthritis; however, its presence was not associated with poorer performance and thus our second hypothesis was rejected. However, results of previous studies\textsuperscript{3,e} indicating poorer athletic performance in horses with (vs without) ACB fracture and concurrent antebrachiocarpal joint osteoarthrosis as well as in horses with (vs without) dorsal middle carpal disease alone illustrate the importance of carpal health in racehorses.

The results of the present study regarding similar racing performance of horses that have ACB fracture as yearlings with or without concurrent radiographic evidence of concurrent carpal osteoarthritis conflict with preliminary results in another study.\textsuperscript{e} Those data indicate lower earnings per start and total earnings as a 2- and 3-year-old for yearlings with osteochondral fragments arising from the ACB with versus without corresponding dorsal antebrachiocarpal joint disease. That study\textsuperscript{e} was based on repository radiographic evaluation of a similar group of yearlings from 2004 through 2007 and maternal sibling race records for comparison. Because both studies had limited statistical power given the low numbers of included horses, analysis of a larger cohort of horses with ACB fracture might yield different results. Furthermore, differences in the unexposed cohorts used in the 2 studies could have influenced the results. Recognizing that there is a conceivable association between fracture of the ACB with subsequent antebrachiocarpal arthritis and potential progression of disease once these yearlings enter race training, we believe our data indicated that in horses with osteochondral fragmentation of the ACB identified on prepurchase radiographs, the incidence of starting a race is not significantly lower than that for horses without such fractures.

Although no differences in race performance were identified in the present study between horses with ACB fracture identified on yearling repository radiographs and their unaffected counterparts, in contrast to previous findings,\textsuperscript{3} we assert that this discrepancy is validating evidence that radiographic examination of a yearling has limited value in predicting its future athletic potential. In all prepurchase examinations, emphasis should be made that the findings indicate the horse’s condition at the time of examination. Future racing performance depends on a multitude of influences beyond the veterinarian’s control and unknown at the time of examination, including but not limited to individual athletic capability, future injury, training differences, owner investment, and surgical interventions.

Because of the retrospective nature of the present study, the cause of the identified fractures could not be established. Our reliance on radiographic interpretation reports prevented full characterization of the location of these fragments in some horses. Because the small sample size was recognized as a further limitation, horses with osteochondral fragments originating from the ACB were grouped together regardless of fragment location. Had the radiographs rather than the reports been available, more definitive descriptions could have been obtained; however, the original observers assessed the descriptions as adequate at the time of interpretation for the intended purpose, which was to assess the horse for radiographic findings that might be relevant to future racing or resale value.

The approach used in the present study contributed a further degree of separation between our data and previously reported preliminary data that were derived from analysis of horses with the specific diagnosis of dorsoproximal fragmentation of the ACB.\textsuperscript{3} Small dorsoproximal osteochondral fragments from the ACB are often considered similar to osteochondrosis dissecans lesions or potentially avulsion fractures, whereas fractures in the frontal or horizontal planes are consistent with traumatic events.\textsuperscript{14} It is also common for ACB fractures to heal with fibrous or fibrocartilaginous unions with medical management, and this may result in observation of persistent fracture lines on radiographs obtained after healing has completed.\textsuperscript{17} However, the lack of medical history available for the horses included in the present study and the lack of lameness at the time of examination prevented the prognostic application of our findings to horses evaluated clinically for lameness associated with acute fracture of the ACB.

An additional limitation was that no follow-up medical information was available for the included horses to establish which, if any, of the ACB fractures required treatment after horses were sold. It is conceivable that some horses may have had these ACB fragments surgically removed or had other treatments to address the potential synovitis resulting from the presence of the osteochondral fragment.\textsuperscript{18,19}

In the study reported here, horses without ACB fracture were not purposefully matched with those with osteochondral fragmentation of the ACB on the basis of sex; however, sex distributions were similar between these cohorts. At the Keeneland September sale of yearlings, catalog placement involves no accounting for horse sex because sire power and racing prowess of the dam carry more weight as analyzed by the Jockey Club. In the present study, the median distance in hip number (identification method denoting sale order and location in the sale catalog) between horses with ACB and their respective counterparts in the study population was 1, leading to the conclusion that in a sale that averaged 4,919 catalog entries annually during the study period, these horses were fairly closely matched. Our study was based on catalog entries, and no effort was made to assess price obtained in the sale ring nor whether horses were withdrawn.
from the sale, deferring to presale appraisal as the basis for determining peer relationships between horses with and without ACB fracture.

The prevalence of mild tarsal osteoarthritis in both cohorts of horses in the present study was higher than expected given findings of previous reports2,20,21 indicating a prevalence of 9.9% to 27% for tarsal osteoarthritis in yearling Thoroughbred race prospects; however, it should also be recognized that the statistical effect of this radiographic finding on subsequent racing performance was ascertained in only 1 previous study.2 Given the higher prevalence of this finding, compared with expected prevalence, as well as the higher prevalence in the nonexposed versus exposed cohorts in the present study, only the findings of moderate or severe distal tarsal osteoarthritis were included in the classification of clinically important radiographic lesions.

Reduction of bias and identification of confounders are common challenges in retrospective investigations. In the present study, which was based on radiographic interpretation reports, we believe the bias associated with the radiographic findings was minimal because none of the observers had knowledge of the study at the time of interpretation. We also believe that these reports were primarily generated for potential buyers, rather than consignors, which might have led to exaggeration of radiographic findings, if any bias existed. However, this bias would have been similar between cohorts. Additionally, although we recognize that substantial bias may have existed in the order of placement of these horses in the sale catalog, the inclusion of 2 horses without ACB fracture (1 horse on either side in the catalog) for each horse with ACB fracture would have minimized this bias.

The clinical importance of radiographic findings in young, untrained Thoroughbreds can be difficult to ascertain, despite multiple studies having been conducted to investigate correlations with future performance. Veterinarians are expected to make assessments and predictions of the importance of their examination findings on the basis of the available literature as well as their own experiences. We found no association between the radiographic identification of osteochondral fragmentation of the ACB in yearling Thoroughbreds and the incidence of starting a race as a 2- or 3-year-old in the present study.

Acknowledgments

The authors declare that there were no financial or ethical conflicts of interest.

The authors thank Drs. Patrick Worden, Michael Prichard, Jeffrey Berk, and Elizabeth Santschi for their contribution of medical records to the study.

Footnotes

b. Dr. Patrick Worden, Equine Medical Center of Ocala, Ocala, Fla.
c. Equibase [database online], Lexington, Ky; Equibase Company

References

Viral testing of 18 consecutive cases of equine serum hepatitis: A prospective study (2014-2018)


1Department of Microbiology and Immunology, Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York
2Center for Vaccines and Immunity, Research Institute at Nationwide Children's Hospital, Columbus, Ohio
3Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York
4New York State Animal Health Diagnostic Center, Cornell University, Ithaca, New York
5Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas
6Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan
7Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, Urbana, Illinois
8Department of Veterinary Clinical Sciences, Oklahoma State University, Stillwater, Oklahoma
9Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, Missouri
10Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana
11Department of Clinical Sciences, Oregon State University, Corvallis, Oregon
12Oklahoma Equine Hospital, Washington, Oklahoma
13Tryon Equine Hospital, Columbus, North Carolina
14Equine Medicine Specialists of South Florida, Wellington, Florida
15Oakridge Equine Hospital, Edmond, Oklahoma
16Lloyd Veterinary Medical Center, Iowa State University, Ames, Iowa
17Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York

Correspondence
Joy E. Tomlinson, Department of Microbiology and Immunology, Baker Institute for Animal Health, Cornell University College of Veterinary Medicine, 235 Hungerford Hill Road, Ithaca, NY 14853.
Email: jet37@cornell.edu

Funding Information
Harry M. Zweig Memorial Fund for Equine Research

Background: Three flaviviruses (equine pegivirus [EPgV]; Theiler’s disease-associated virus [TDAV]; non-primate hepacivirus [NPHV]) and equine parvovirus (EqPV-H) are present in equine blood products; the TDAV, NPHV, and EqPV-H have been suggested as potential causes of serum hepatitis.

Objective: To determine the prevalence of these viruses in horses with equine serum hepatitis.

Animals: Eighteen horses diagnosed with serum hepatitis, enrolled from US referral hospitals.

Methods: In the prospective case study, liver, serum, or both samples were tested for EPgV, TDAV, NPHV, and EqPV-H by PCR.

Abbreviations: ACVIM, American College of Veterinary Internal Medicine; EqPV-H, equine parvovirus-hepatitis; EPgV, equine pegivirus; NPHV, non-primate hepacivirus; TAT, tetanus antitoxin; TDAV, Theiler’s disease-associated virus.
1 | INTRODUCTION

Equine serum hepatitis, also known as Theiler's disease or idiopathic acute hepatitis, is a serious and often life-threatening disease of adult horses that was first described in 1918 in South Africa by Sir Arnold Theiler. The 1st cases of serum hepatitis in horses reported in the United States occurred during the pandemic of western equine encephalomyelitis in the 1930s. The incidence of fulminant hepatitis among horses receiving antiserum in these outbreaks was between 1.4% and 18%. Serum hepatitis has since been described in horses worldwide after treatment with a variety of equine serum products, including tetanus antitoxin (TAT), botulinum antitoxin, Streptococcus equi antiserum, pregnant mare's serum, and equine plasma. Of these equine biologic products, the disease has been most commonly associated with TAT, possibly because this is the most frequently administered equine blood origin biologic product. The incubation period for clinical disease usually ranges between 4 and 10 weeks after administration of an equine origin biologic product. More recently, a novel equine parvovirus (equine parvovirus-hepatitis, EqPV-H) was discovered in the liver and serum of a horse that died of Theiler's disease. EqPV-H nucleic acids were also found in the TAT administered to this horse 9 weeks before onset of hepatitis. Experimental administration of EqPV-H-positive TAT samples to 2 horses resulted in EqPV-H viremia 6.4 weeks later, followed by marked biochemical evidence of liver disease in both horses and clinical disease in one of the horses.

After the discovery of these viruses, a prospective study on field cases of Theiler's disease involving American College of Veterinary Internal Medicine (ACVIM) Diplomates was initiated to investigate the association of these viral infections with naturally occurring cases of serum hepatitis. This report details case information and virus testing of 18 consecutive cases of serum hepatitis.

2 | MATERIALS AND METHODS

2.1 | Prospective clinical case study

In collaboration with North American academic and private referral equine hospitals, we initiated a prospective clinical case study to assess the possible role of the newly identified viruses in the etiology of acute serum hepatitis via a letter sent to ACVIM Large Animal Diplomates at teaching and large referral hospitals. Case definition included (1) acute onset of clinical signs of hepatic failure with laboratory or liver histopathologic findings characteristic of serum hepatitis (Theiler's disease) and (2) a history of receiving an equine biologic product 4-14 weeks earlier. For each case, a diagnosis of Theiler's disease or serum hepatitis was made at the referral practice before submitting samples to the New York State Animal Health Diagnostic Center for viral testing. Cases were enrolled between January 2014 and February 2018, and all submitted (consecutive) cases were included in the study.

2.2 | Sample collection for prospective study

Serum samples collected from horses in the prospective clinical case study were frozen or kept on ice before being shipped from the clinic.
2.3 | Polymerase chain reaction

Viral nucleic acids were extracted from serum or liver with Qiagen Viral RNA Mini kit (catalog no. 52906) according to the manufacturer’s instructions. No DNAase treatment was applied. All PCR mixtures used the Path ID multiplex RT-qPCR kit (catalog no. 4442137; Thermo Fisher Sci, Waltham, MA, USA) and 4 μL of extracted nucleic acids in a 25 μL reaction volume. The primers are listed in Table 1. Two primer pairs were used for EPgV; a positive result in either pair was considered positive. Primers were used at 0.4 μM concentration and probes at 0.12 μM. All PCR reactions were run on the ABI Step-One-Plus Real-Time System and analyzed with StepOne software (Thermo Fisher Sci, Waltham, MA, USA). Real-time PCR conditions included an initial incubation at 48°C for 10 minutes, then 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. PCR methods were validated using the AAVLD accreditation guidelines (data not shown).

2.4 | Statistics

Descriptive statistics of demographic data are provided. Continuous variables are reported as median and range because of non-Gaussian distribution of some variables, as assessed by the examination of skewness, kurtosis, and Q-Q plots.

3 | RESULTS

3.1 | Signalment and biologic product history

Eighteen cases of serum hepatitis were enrolled between December 2014 and February 2018. Demographic data and virologic testing are summarized in Table 2, and greater individual case details are included in Supporting Information Supplemental Table 1. Multiple breeds of horses were affected. There were 6 mares, 1 stallion, and 11 geldings. The ages ranged from 2 to 18 (median 12) years. Of the 18 cases, 12 horses received commercial TAT 4-13 weeks (median 8 weeks) before acute onset of signs of liver failure. The antitoxin (same vial or lot number) was available for testing in 11 cases. In 4 cases, the TAT lot number was narrowed to 2 possibilities and both were tested (Supporting Information Supplemental Table 1). In 2 of these cases, PCR results were the same for both lots. In the other 2 cases, there was a discrepancy between the 2 lots in either NPHV (1 case) or EqPV-H (1 case) status. Therefore, we are confident that EqPV-H-positive TAT was administered to at least 10 of the 12 cases. Of the remaining 6 horses, 3 had received allogenic stem cells as a treatment for soft tissue orthopedic injuries 6.4, 6.7, and 7.6 weeks earlier, and 3 horses received equine plasma 6, 6.4, and 8.6 weeks earlier as colloid treatment, after abdominal surgery in 1 horse and for diarrhea in 2 other horses. Stem cell inoculum (frozen) was available for virus testing from 1 case (case 17) only, and the inoculum was EqPV-H positive. Sera from the donor horses of the remaining 2 cases were tested 20 weeks after inoculation (case 8) and 15 weeks before inoculation (case 12), and both donor horses were PCR negative for EqPV-H. Samples from the commercial plasma given to the other 3 horses were not available for virus (PCR) testing. This commercial plasma was from 2 separate vendors, although 2 cases (cases 13 and 14) received plasma with an identical lot number.

Abbreviations: EPgV, equine pegivirus; EqPV-H, equine parvovirus-hepatitis; NPHV, non-primate hepacivirus; TDAV, Theiler’s disease-associated virus; qRT-PCR, real-time PCR.
TABLE 2  Demographic data and virologic testing results for 18 cases with equine biologic-product associated serum hepatitis

<table>
<thead>
<tr>
<th>Biologic administered</th>
<th>TAT (12)</th>
<th>Plasma (3)</th>
<th>Allogenic stem cells (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) (median [range])</td>
<td>11 (2-17)</td>
<td>15 (12-16)</td>
<td>13 (9-18)</td>
</tr>
<tr>
<td>Breed</td>
<td>Aqh, 6; Wb, 2; others, 4</td>
<td>Wb, 2; UNK, 1</td>
<td>Aqh, TB, Wb</td>
</tr>
<tr>
<td>Sex</td>
<td>Mare, 6; Stallion, 1; Gelding, 5</td>
<td>Gelding, 3</td>
<td>Gelding, 3</td>
</tr>
<tr>
<td>Incubation period (wk) (median [range])</td>
<td>8 (4-13)</td>
<td>7 (6-8)</td>
<td>6 (5-8)</td>
</tr>
<tr>
<td>Survival</td>
<td>4/12</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Serum qRT-PCR*</td>
<td>EqPV-H 9/9</td>
<td>2/2</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>NPHV 2/9</td>
<td>0/2</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>TDAV 0/9</td>
<td>0/2</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>EPGV 2/9</td>
<td>2/2</td>
<td>2/3</td>
</tr>
<tr>
<td>Liver qRT-PCR*</td>
<td>EqPV-H 6/6</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>NPHV 0/6</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>TDAV 0/6</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>EPGV 0/6</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Biologic product</td>
<td>EqPV-H 9/9</td>
<td>NA</td>
<td>1/1</td>
</tr>
<tr>
<td>qRT-PCR*</td>
<td>NPHV 7/9</td>
<td>NA</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>TDAV 0/9</td>
<td>NA</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>EPGV 9/9</td>
<td>NA</td>
<td>0/1</td>
</tr>
</tbody>
</table>

Virology testing (in rows indicated by *) is shown as the number of positive samples out of the number of samples tested. Biologic products tested were mainly aliquots of the same lot administered to the actual cases. Four horses had the TAT lot narrowed to 1 of 2 lots; 2 sets had identical virology results and are included in this table; 2 sets had discrepant results reported in Supporting Information Supplemental Table 1 and are not included in this table. Abbreviations: Aqh, American Quarter Horse; EPGV, equine pegivirus; EqPV-H, equine parvovirus-hepatitis; NA, not available; NPHV, non-primate hepavivirus; qRT-PCR, real-time PCR; TAT, tetanus antitoxin; TB, Thoroughbred; TDAV, Thielers disease-associated virus; WB, Warmblood; UNK, unknown.

3.2 | Virology

All 18 horses were positive for EqPV-H infection (Table 2). Serum and liver samples were available for testing in 6 of the 18 cases; serum only was available in 8 cases and liver only in 4 cases; all these samples were positive for EqPV-H. Of the 14 serum samples, 5 were also positive for EPGV, 2 were positive for NPHV, but none of the 14 samples were positive for TDAV. All 10 liver samples were only positive for EqPV-H. Twelve of the 18 horses died (n = 6) or were euthanized (n = 6) because of the severity of liver failure. One of the horses (case 7) that survived acute fulminant hepatitis was tested 13 months later and still had detectable EqPV-H viremia without any biochemical evidence of hepatic disease.

3.3 | In-contact horses

The newborn foal of case 4 received the same TAT as her dam at foaling. Serum from the foal was also EqPV-H positive (viremia was 100-fold higher than the dam) when tested at 8 weeks of age; the foal was clinically normal; however, no biochemical analysis was performed. After case 5 recovered and returned to the farm, an in-contact horse developed clinical and biochemical findings of liver failure 6 weeks later. This horse was also sent to the university (Missouri) referral hospital, diagnosed with acute hepatic failure, successfully treated, and returned to the farm 1 week later. The serum of this in-contact horse also tested positive for EqPV-H but no biologic product had been administered to this horse, suggesting the possibility of horse-to-horse transmission (from case 5) as has sporadically been observed in serum hepatitis outbreaks. In case 6, the field veterinarian reported that 2 horses had been inoculated with the same lot of TAT after castration and both developed signs of liver failure, although only 1 of the horses (case 6) was referred for hospitalization and included in our study. Case 6 died, although the other horse recovered on the farm and a blood sample from that horse was positive for EqPV-H.

3.4 | Clinical data

Although not a primary aim of the study, information on clinical signs, biochemical findings, and necropsy findings was available for many of the cases and are reported here for clinical interest. The clinical findings in horses that necessitated the initial veterinary examination were reported to be acute onset of neurologic signs in 12 of 16 cases where the initial clinical findings were available. Ten of the 12 horses were reported as having predominantly cerebral signs, including blindness, head pressing, and obtundation, whereas 2 horses had acute ataxia that preceded the cortical signs. Other initial clinical findings noted in the case records that were provided included icterus (n = 9), discolored urine (n = 5), colic signs with gastric reflux (n = 2), and recumbency in 1 horse that was severely hypoglycemic. Pyrexia was only reported in 2 cases. In 7 cases, the owners reported decreased appetite and dullness for 1-2 days before the onset of neurologic signs. Serum biochemistry findings at referral admission are summarized in Table 3. Median percentage direct to total bilirubin was 17% (13%-27%, n = 7). Glucose values were reported for 9 horses. Two values were moderately low (60 and 52 mg/dL), and 2 values were severely low (<20 mg/dL) in recumbent horses. Duration of clinical signs before death or euthanasia (median 3 days, range 1-7 days) was available in all 12 horses that died. Information regarding time to clinical improvement after hospitalization was available for 4 of 5 surviving horses, and number of days after hospitalization to clinical improvement was 3, 3, 4, and 7 days. One horse (case 16) was being treated for a chronic respiratory disease and had a complete blood chemistry panel (which was normal) 8 days before onset of liver failure. This was the only horse in the study receiving medical treatment for another condition at the time of onset of liver failure.

Gross findings of the liver were noted on 7 of the necropsy reports, and in all but 1, the liver was reported to be small (normal >1.5% of body weight) and friable. A reticular pattern was noted in 3 cases. Reports on the microscopic findings in the liver were available in 15 cases that had either biopsy (4 cases) or necropsy reports (11) submitted; 3 horses that survived did not have liver biopsies performed because only a small amount of liver could be visualized on
TABLE 3  Clinical pathology of 18 Theiler’s disease cases

<table>
<thead>
<tr>
<th></th>
<th>Number of cases</th>
<th>Median (range)</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>12</td>
<td>2097 (706-4078)</td>
<td>222-489</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>13</td>
<td>129 (68-314)</td>
<td>8-33</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>13</td>
<td>13.4 (7.6-24.3)</td>
<td>0.5-2.1</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dL)</td>
<td>7</td>
<td>1.9 (1.3-5.8)</td>
<td>0.1-0.3</td>
</tr>
<tr>
<td>Ammonia (mmol/L)</td>
<td>6</td>
<td>249.5 (30.6-692)</td>
<td>Not determined</td>
</tr>
<tr>
<td>Bile acids (μmol/L)</td>
<td>6</td>
<td>118.5 (98.7-171)</td>
<td>2-10</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>9</td>
<td>87 (19-121)</td>
<td>71-113</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>13</td>
<td>48 (36-58)</td>
<td>34-46</td>
</tr>
</tbody>
</table>

Data are from the 1st serum biochemistry performed on each horse at hospital admission. Reference range provided is a general range from the New York State Animal Health Diagnostic Center. Tests were run at multiple laboratories and laboratory-specific reference ranges varied. Abbreviations: AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

ultrasonographic examination. Microscopic findings reported in affected horses consistently included acute centrlobular to massive necrosis, collapse of the lobular architecture, and replacement with cellular debris and sometimes hemorrhage. Lesser affected periportal hepatocytes were often described as degenerate, swollen, and containing cytoplasmic vacuoles. In all but 1 case, a mild to moderate lymphocytic/plasmacytic perportal infiltration was noted. Bile stasis and biliary proliferation were noted less commonly. Alzheimer type 2 cells in the brain, consistent with hyperammonemia, were present in 4 of 5 reports that included microscopic examination of the brain. All necropsy and biopsy reports summarized the histologic features as being most suggestive of, presumptive for, or compatible with serum hepatitis.1,8,11,12

4 | DISCUSSION

The 18 cases in our study were (1) clinically and clinicopathologically consistent with previous descriptions of serum hepatitis in horses, (2) all infected with EqPV-H, and (3) rarely infected with the equine flaviviruses that have recently been suspected of causing the disease.13,19 When samples of the biologic product or their same lot number were available for virus testing, EqPV-H was found in the products administered to the horses before onset of hepatitis. Despite the limitation of a lack of controls, the 100% EqPV-H prevalence among these 18 cases compared to the low prevalence of 13% EqPV-H viremia among normal horses30 is highly suggestive that this association is significant. These findings are indicative that EqPV-H can be transmitted by administration of equine biologic products and is the likely cause of equine serum hepatitis.

The clinical and histopathologic findings in these cases, along with the knowledge of administration of an equine origin blood product 4-12 weeks earlier, were considered diagnostic for serum hepatitis.6-9,11,12,15 Therefore, additional testing (eg, heavy metals and other hepatotoxins) was limited. All except 1 horse in our study were in the “typical” 4- to 10-week incubation period for serum hepatitis.8,10-12,15 And the longest incubation period was 12.7 weeks. One of 2 adult horses inoculated with TAT containing EqPV-H had clinical signs of liver failure and abnormal liver function test results 12.7 weeks after inoculation, supporting the possibility that some cases of serum hepatitis can develop outside the normal 4- to 10-week incubation period.30 Some potential explanations for differences in incubation time for disease after administration of virus-laden blood products could include (1) different viral loads or specific antibody titers in the biologic products, (2) individual horse differences in the immune responses to infection, (3) partial protective immunity from previous exposure, or (4) concurrent liver injury of another etiology.

Although the overall incidence of clinically recognized serum hepatitis in adult horses receiving TAT is low, TAT has been the most common blood product associated with the disease in the United States for the past 50 years.6,7,9,11,12 Our findings concur with those reports as 12 of the 18 cases with serum hepatitis received TAT. Serum hepatitis could be more commonly associated with TAT administration than with administration of equine plasma because of the more frequent administration of TAT to horses or because TAT is produced as a pooled donor product. The latter could increase the risk of virus contamination of TAT compared to plasma, whole-blood products, and allogenic stem cell inoculations, which are more commonly single donor products. Commercial TAT is usually heat treated (60°C for 1 hour) for the purpose of virus inactivation, and both phenol and thimerosol are added as preservatives. If effective in sterilizing the product, such treatments could leave detectable viral nucleic acids in TAT that are no longer infectious. However, although this form of heat treatment of blood products is known to inactivate heat-labile viruses such as lentiviruses,32 the parvoviruses (and especially animal parvoviruses) are resistant to both heat inactivation and solvent detergent treatments.32-35 Indeed, EqPV-H can be successfully transmitted using heat-treated, commercially available TAT.30 In contrast to EqPV-H, it appears that transmission of the flaviviruses NPHV and EpgV might have been effectively reduced or eliminated by heat and chemical treatment of equine TAT used for the cases in the present study. This is supported by the fact that among 9 cases that had serum and the administered lot number of TAT tested, TAT was positive for EpgV in all 9 lot inocula and NPHV was positive in 6 horses, but only 2 horses were positive for EpgV and 1 positive for NPHV in serum samples. Those flavivirus-positive cases might have been infected either by receiving contaminated TAT that was not properly heat-inactivated or by exposure to these viruses via another source before antitoxin administration. Because the virus prevalence rate of both NPHV and EpgV in the adult horse population is approximately 15%, the latter is a plausible explanation.26-28,36
The association of equine plasma administration and serum hepatitis is also well documented. The time between plasma administration and development of hepatic failure in the 3 cases in our study is typical of previous plasma-associated cases of serum hepatitis.\textsuperscript{13,15} The 2 plasma products (2 horses received the same lot number) administered to these horses were not available for virus testing; therefore, the spread of infection by commercial plasma in these 3 plasma-related cases remains presumed.

An association between the allogenic stem cell treatment and serum hepatitis, as occurred in 3 horses in our study, has not been previously reported. The incubation period between the stem cell inoculation and the onset of disease in these 3 horses was typical for serum hepatitis. In only 1 horse (case 17) was the stem cell inoculum available for testing, and although this sample was EqPV-H positive, transmission via this method remains suppositional. If the stem cell inoculation was responsible for EqPV-H transmission, the contamination might have occurred from either EqPV-H infection of the stem cells themselves or carryover of donor serum used to culture the stem cells. Viral testing results of stem cell donor horses, albeit more than 14 weeks distant from the inoculation of cases 8 and 12, did not support transmission of EqPV-H by this route. Although the incubation time in these 2 cases was typical for serum hepatitis, EqPV-H infection might have occurred by another method.

The virologic testing in our study clearly links EqPV-H, but not the flaviviruses, with serum hepatitis. We found no evidence that infection or coinfection with the other known hepatotropic virus, NPHV, was associated with clinical disease. Although an original study by 2 of the current authors (T.J.D., B.C.T.) and others found an association between TDAV and plasma-associated hepatitis in a group of horses,\textsuperscript{13} the prospective study described here could not find TDAV in any of these field cases. Importantly, retrospective analysis of the commercial plasma botulinum antitoxin and experimental pony infection samples from the 2011 outbreak of Theiler’s disease in which TDAV was discovered\textsuperscript{13} showed that EqPV-H was also present in the antitoxin, in diseased horses on the farm, and in the 4 experimental horses inoculated with the same plasma antitoxin lot.\textsuperscript{30} Parvovirus was likely not detected in the original investigation because sequencing in that study focused on RNA viruses with proximity to hepatitis C virus, and so a DNAse treatment was performed on the RNA pellet before sequencing.\textsuperscript{13} Although TDAV and EPG\textsubscript{v} nucleic acids have been found in commercial plasma and serum products,\textsuperscript{13,29} pegiviruses are neither believed to be hepatotropic nor have they been documented to cause liver disease in any mammalian species.\textsuperscript{17,26,37} Our findings also suggest that they are rarely transmitted via TAT administration.

Epidemiologic data regarding Theiler’s disease and virologic testing for EqPV-H are both consistent with the theory that subclinical or silent infection is likely common. This is supported by the low incidence of clinical disease after inoculation with the same biologic product.\textsuperscript{1,4,11,13} In addition, subclinical disease has been documented in multiple horses in 2 studies of biologic-associated serum hepatitis outbreaks,\textsuperscript{11,13} and in one of these outbreaks, disease was demonstrated to be associated with EqPV-H.\textsuperscript{13,30} Similarly, 2 horses experimentally inoculated with EqPV-H developed only mild clinical or subclinical disease.\textsuperscript{30} Finally, a seroprevalence survey of 100 horses found that 13% of horses without biochemical evidence of liver disease were EqPV-H positive,\textsuperscript{30} suggesting that most horses that become infected with EqPV-H do not develop clinical disease. Taken together, these findings suggest that many horses infected with EqPV-H could have a short period of subclinical disease followed by complete recovery. Why some horses develop severe and often fatal disease after EqPV-H infection and others do not is unknown. Hepatic cell damage related to the high level of viremia and direct cytopathic effects is one possibility. Alternatively, injury might result as an indirect consequence of the immune response directed against the virus or injured hepatocytes, as occurs with hepatitis B virus in people.\textsuperscript{38} The lymphocytic infiltration seen in many of these cases and in the experimentally EqPV-H–infected horses\textsuperscript{30} could be consistent with an immune-mediated mechanism of liver damage.

Taken together, this prospective study lends additional support for a causative association between EqPV-H and equine serum hepatitis. This information should encourage blood product manufacturers to eliminate EqPV-H–infected horses from their facilities.

**ACKNOWLEDGMENTS**

Dr Mark McMahan, South Lyon, Michigan; Dr William Rhoads, Whitesboro, Texas; Dr Kelly Whitesel, Eaton, Indiana; Alec Darvin, Shelbyville, Indiana; Dr Kent Cooper, Independence, Kansas; Dr Paul Cotterill, Cherrycove, Kansas; Dr Jason Wooderson, Bolivar, Missouri; Dr Donald St. Ledger, Albion, Illinois; Dr George Eales, San Jose, Illinois are thanked for providing case information and samples for virus testing. The viral testing and data analysis were performed at Cornell University, Ithaca, New York, and Nationwide Children’s Hospital, Columbus, Ohio. Parts of this work presented at the 2018 ACVIM Forum, Seattle, Washington. While the viral testing in this case series was performed on a prospective basis, the individual case management was not determined by the study parameters and was performed by the attending clinicians.

**CONFLICT OF INTEREST DECLARATION**

Melissa Laverack, Randall Renshaw, and Edward Dubovi are employees of the New York Animal Health Diagnostic Center where equine hepatitis panel PCR testing is offered as fee-for-service. These authors were instrumental in viral testing development, validation, and performance but did not contribute to the specific analysis of results. Joy E. Tomlinson received speaker honoraria for presenting parts of this data at the 2018 ACVIM Forum, Seattle, Washington.

**OFF-LABEL ANTIMICROBIAL DECLARATION**

Authors declare no off-label use of antimicrobials.

**INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION**

All work was approved by the Cornell University IACUC #2012-0154.
HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Joy E. Tomlinson https://orcid.org/0000-0001-7365-3967
Katie Delph https://orcid.org/0000-0003-0259-7210
Harold Schott II https://orcid.org/0000-0002-7728-5409
Rebecca Ruby https://orcid.org/0000-0002-1113-6566
Thomas J. Divers https://orcid.org/0000-0001-7125-636X

REFERENCES

1. Theiler A. Acute liver-atrophy and parenchymatous hepatitis in horses. Union of South Africa Dept. of Agriculture 5th and 6th Repts. of the Director of Veterinary Research; 1918.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

CASE REPORT

Presumptive tick paralysis in 2 American Miniature horses in the United States

Kelsey M. Trumpp | Ashley L. Parsley | Melissa J. Lewis | Joseph W. Camp Jr. | Sandra D. Taylor

1Department of Veterinary Clinical Sciences, Purdue University College of Veterinary Medicine, West Lafayette, Indiana
2Department of Comparative Pathobiology, Purdue University College of Veterinary Medicine, West Lafayette, Indiana

Correspondence
Sandra D. Taylor, Purdue University College of Veterinary Medicine, 625 Harrison Street, West Lafayette, IN 47907.
Email: taylo248@purdue.edu

Abstract

Rationale: Tick paralysis has not been reported in horses in North America.
Clinical Findings: Two American Miniature horses were examined for progressive weakness and recumbency. Numerous ticks (Dermacentor variabilis) were found on both horses. Horse 1 was recumbent (grade 5/5 gait deficit) on presentation, whereas Horse 2 was standing but ataxic (grade 4/5 gait deficit) and tetraparetic. Both horses had decreased tongue and tail muscle tone, and had normal spinal reflexes. Cerebrospinal fluid cytology was normal. Equine herpesvirus-1 testing was negative.

Pertinent Interventions: Ticks were removed within 24 hours of presentation. Both horses were treated topically with permethrin. Supportive care included fluid therapy, treatment for corneal ulceration, and frequent repositioning during recumbency.
Outcome: Within 48 hours of tick removal, both horses were neurologically normal.

Clinical Relevance: Ours is the first reported case of presumptive tick paralysis in horses in North America. Although rare, tick paralysis should be considered in horses presented with acute-onset weakness progressing to recumbency.

KEYWORDS
horse, recumbency, tetraparesis, tick

1 | CASES

A 3-year-old 93.6-kg American Miniature horse filly (Horse 1) was referred in May 2018 to the Purdue University Veterinary Teaching Hospital (PUVTH) for recumbency. The owner reported the horse had 12 hours of difficulty walking and weakness that progressed to recumbency. The filly had no known vaccination history and was housed with another female American Miniature horse (Horse 2) in a heavily wooded pasture. Both horses had been purchased from a nonlicensed petting zoo 9 days before the onset of clinical signs, where they had been housed on pasture and fed round bale grass hay. They were evaluated by the referring veterinarian approximately 4 hours before presentation, and did not receive medication at that time. On examination at the PUVTH, Horse 1 was recumbent in the trailer and unable to rise (grade 5/5 gait deficit). The horse was bright, alert, and responsive but anorexic with a body condition score (BCS) of 7/9. Rectal temperature was 37.8°C (reference range, 37.2°C-38.6°C), heart rate was 80 beats/min (reference range, 28-40 beats/min), and respiratory rate was 30 breaths/min (reference range, 8-20 breaths/min). In addition to recumbency, the filly also had decreased tongue and tail muscle tone. Aside from decreased tongue muscle tone, the cranial nerve

Abbreviations: BCS, body condition score; CNS, central nervous system; CSF, cerebrospinal fluid; EHM, equine herpes myeloencephalitis; EHV, equine herpesvirus; LMN, lower motor neuron; PUVTH, Purdue University Veterinary Teaching Hospital; qPCR, quantitative polymerase chain reaction; UMN, upper motor neuron.

Kelsey M. Trumpp and Ashley L. Parsley are first authors.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2019 The Authors. Journal of Veterinary Internal Medicine published by Wiley Periodicals, Inc. on behalf of the American College of Veterinary Internal Medicine.
The horse was bright, alert, and responsive but anorexic with a BCS noted to be tetraparetic and ataxic (grade 4/5 gait deficit in all 4 limbs) and able to walk off the trailer with assistance, but the horse was that of Horse 1. On examination at the PUVTH, Horse 2 was standing approximately 12 hours before referral. Otherwise, the history was similar to intermittently recumbent (but able to rise voluntarily) for approximately 14 hours before referral and was shown incoordination and weakness 24 hours before referral and was referred to the PUVTH with Horse 1 for weakness progressing to 5/9. Rectal temperature was 38.4°C (reference range, 37.2°C-38.6°C), heart rate was 100 beats/min (reference range, 28-40 beats/min), and respiratory rate was 52 breaths/min (reference range, 8-20 breaths/min). Approximately 100 embedded and engorged ticks (D. variabilis) were observed, primarily concentrated at the base of the mane and tail. Horse 2 demonstrated a grade 4/5 gait deficit in all 4 limbs (ie, stumbling, tripping, and falling spontaneously), as well as decreased tongue and tail muscle tone. Postural reactions and spinal reflexes were normal. Abnormal results of hematologic and serum biochemical analyses included leukocytosis (12.7 K/μL; reference range, 6.0-12.0 K/μL), mature neutrophilia (7.9 K/μL; reference range, 3.0-7.0 K/μL), hypocalcemia (9.5 mg/dL; reference range, 10.7-13.4 mg/dL), and an increase in creatine kinase activity (2864 IU/L; reference range, 88-453 IU/L).

A 4-year-old 108.6-kg American Miniature horse mare (Horse 2) was referred to the PUVTH with Horse 1 for weakness progressing to recumbency. Similar to Horse 1, Horse 2 was reported first to have shown incoordination and weakness 24 hours before referral and was intermittently recumbent (but able to rise voluntarily) for approximately 12 hours before referral. Otherwise, the history was similar to that of Horse 1. On examination at the PUVTH, Horse 2 was standing and able to walk off the trailer with assistance, but the horse was noted to be tetraparetic and ataxic (grade 4/5 gait deficit in all 4 limbs). The horse was bright, alert, and responsive but anorexic with a BCS of 5/9. Rectal temperature was 38.4°C (reference range, 37.2°C-38.6°C), heart rate was 100 beats/min (reference range, 28-40 beats/min), and respiratory rate was 52 breaths/min (reference range, 8-20 breaths/min). Approximately 100 embedded and engorged ticks (D. variabilis) were identified as Dermacentor variabilis by a parasitologist. Abnormal results of hematologic and serum biochemical analyses included mature neutrophilia (8.3 K/μL; reference range, 3.0-7.0 K/μL), hyperglycemia (142 mg/dL; reference range, 73-124 mg/dL), hypocalcemia (9.3 mg/dL; reference range, 10.7-13.4 mg/dL), and an increase in creatine kinase activity (2864 IU/L; reference range, 88-453 IU/L).

For both horses, plasma fibrinogen concentration and serum triglyceride concentrations were normal. Ultrasound-guided atlantoaxial cerebrospinal fluid (CSF) was obtained and cytology results were normal in both horses. The CSF WBC count was 2/μL and 5/μL (reference range, 0-6/μL), and the total protein concentration was 20 and 49 mg/dL (reference range, 5-100 mg/dL) for Horses 1 and 2, respectively. Equine herpesvirus-1 (EHV-1) nasal swab quantitative polymerase chain reaction (qPCR) and EHV-1 whole blood qPCR were negative. Botulism testing was not performed. Initial treatment of both horses included manual tick removal, IV fluid support (2.2 mL/kg/h; PLASMA-LYTE A Injection pH 7.4, Zoetis, Inc, Parsippany, New Jersey) because the horses were not eating or drinking, a single dose of trivalent equine botulinum antitoxin (2.5 mL/kg IV; Lake Immunogenics, Inc, Ab Select - BOTABC [Botulism A, B, & C Toxins] Equine H1 Plasma, Ontario, New York) considering the possibility of botulism, ceftiofur crystalline free acid (6.6 mg/kg IM q72h; Excede, Zoetis, Inc) for pneumonia prophylaxis, and flunixin meglumine (1.1 mg/kg IV q12h; Banamine Injectable Solution, Merck Animal Health, Madison, New Jersey) for potential central nervous system (CNS) inflammation and muscle pain associated with recumbency. Horse 1 also was treated for bilateral corneal ulcers that likely were a consequence of recumbency-associated trauma. Triple antibiotic ophthalmic ointment (PerriGo Neo-Polycin [bacitracin, neomycin, polymyxin B], Minneapolis, Minnesota) was applied to both eyes q6h. Horse 1 also was manually repositioned on a foam mattress alternating between right and left lateral recumbency q6-8h to minimize muscle damage and prevent decubital ulcer formation.

On the morning of Day 2, anorexia persisted in both horses, and dextrose was added to the IV fluids in an effort to blunt lipid catabolism and prevent hypertriglyceridemia. To provide approximately 20% of the daily resting energy requirement of approximately 3000 kcal/d for each horse, 1.5% dextrose (3.4 kcal/g) was administered at twice the maintenance fluid rate (4.4 mL/kg/h). Additional ticks were discovered on both horses and immediately removed. Topical 45% permethrin (Spot On Protection for Horses, Farnam Companies, Inc, Phoenix, Arizona) was applied on multiple body sites in both horses. For Horse 1, the foam mattress was replaced with a waterbed (New World Manufacturing, Clovernud, California) to further protect against muscle necrosis and decubital ulcer formation, in the event that long-term management of recumbency was needed (eg, botulism). Warm water was used to fill the waterbed so that the horse's dependent tuber coxa

FIGURE 1  Embedded and engorged Dermacentor variabilis ticks concentrated at the base of the tail in a 3-year-old American Miniature horse (Horse 1)
was approximately 12” off the ground (Figure 2). By the evening of Day 2, both horses showed improvement of 1 grade in their neurologic gait deficits; Horse 1 was able to stand with assistance, and Horse 2 had clinically relevant improvement in strength. Tongue and tail muscle tone was subjectively improved in both horses. Whole blood glucose concentrations were obtained q4-6h and remained within the reference range.

On Day 3, Horse 1 was able to stand voluntarily and had only mild weakness. The waterbed was removed from the stall and replaced with deep wood shavings. Both horses continued to improve and were neurologically normal with no apparent gait deficits by Day 4. Reevaluation serum biochemical analyses on Day 4 for Horse 1 identified further increased aspartate aminotransferase activity (4258 IU/L; reference range, 206-810 IU/L) and creatine kinase activity (4489 IU/L; reference range, 88-453 IU/L) compared to results at presentation. For Horse 2, reevaluation serum biochemical analyses on Day 4 identified further increased aspartate aminotransferase activity (1146 IU/L; reference range, 206-810 IU/L) compared to presentation, and slightly decreased (albeit higher than the reference range) creatine kinase activity (1284 IU/L; reference range, 88-453 IU/L) compared to presentation. Hyperbilirubinemia was present in both horses (total bilirubin concentration 5.00 and 5.50 mg/dL for Horses 1 and 2, respectively; reference range, 0.10-2.60 mg/dL). Antimicrobial and anti-inflammatory drugs were discontinued in both horses by Day 4. The corneal ulcers of Horse 1 were resolved by Day 6. Horse 1 continued to have a poor appetite, whereas Horse 2’s appetite returned to normal by Day 5. Intravenous fluids were discontinued in Horses 1 and 2 on Days 9 and 4, respectively.

Reevaluation hematologic and serum biochemical analyses on Day 8 for Horse 1 identified hypertriglyceridemia (359 mg/dL; reference range, 11-65 mg/dL), increased alkaline phosphatase activity (400 IU/L; reference range, 109-331 IU/L), increased gamma-glutamyl transferase activity (2207 IU/L; reference range, 206-810 IU/L), and increased total bilirubin concentration (3.50 mg/dL; reference range, 0.10-2.60 mg/dL). Given these indicators of negative energy balance likely leading to hepatic lipidosis, fluid therapy at 4.4 mL/kg/h containing 2.5% dextrose was continued. These test results normalized and appetite returned to normal by Day 9; therefore, fluid therapy was discontinued. Horse 1 was discharged from the hospital on Day 15.

For Horse 2, reevaluation hematologic and serum biochemical analyses performed on Day 7 showed expected increases in muscle enzyme activity (aspartate aminotransferase, 1146 IU/L; reference range, 206-810 IU/L; and creatine kinase, 1284 IU/L; reference range, 88-435 IU/L). Horse 2 was discharged on Day 10.

Both horses were discharged with instructions to remove them from the wooded area at home and to implement tick control measures. Both horses were reported to be normal 1 week and 8 months after discharge from the hospital.

2 | DISCUSSION

Tick paralysis most frequently is reported in dogs, but can cause disease in humans, cats, cattle, sheep, and horses.3-8 Tick paralysis in dogs occurs when an adult female tick attaches to the host and produces salivary neurotoxins that then are introduced into the circulatory system of the host. These neurotoxins act on presynaptic membranes at the neuromuscular junction and prevent the release of acetylcholine, most commonly resulting in ascending flaccid motor paralysis.4,9,10 The primary tick species implicated in cases of tick paralysis of dogs and cats in North America are Dermacentor andersoni (the Rocky Mountain Wood tick) and D. variabilis (the American Dog tick), whereas tick paralysis in Australia most commonly is caused by Ixodes holocyclus.4 In small animals, this disease typically is characterized by rapidly progressive symmetrical lower motor neuron (LMN) tetraparesis to tetraplegia with decreased spinal reflexes. Additional clinical abnormalities can include facial paresis or paralysis, dysphonia, dysphagia, regurgitation, and in severe cases, progression to recumbency and eventual respiratory muscle failure.4,11,12 Sensory function remains normal. Other abnormalities only reported in the Australian form of tick paralysis can include cardio-pulmonary dysfunction such as left-sided congestive heart failure, pulmonary hypertension, cardiac arrhythmias, and long Q-T syndrome, which are secondary to autonomic dysfunction and sympathetic overdrive.11,13,14 Tick paralysis in small animals in Australia typically causes more severe and prolonged clinical disease that can progress even after

FIGURE 2  Implementation of a waterbed for a 3-year-old recumbent American Miniature horse presumptively diagnosed with tick paralysis (Horse 1)
ticks are removed compared to that caused by *Dermacentor* spp. in North America. Tick paralysis generally occurs in the spring and summer months when ticks are actively seeking hosts, which is consistent with the cases described here. A single tick can cause clinical signs of tick paralysis in dogs, and increasing numbers of ticks are associated with increased severity of clinical signs.15

Cases of tick paralysis in large animals have also been reported, but only in Australia. The typical presentation of large animals with tick paralysis is similar to that in small animals and consists of paresis progressing to recumbency over hours to days after tick attachment, as well as abnormal phonation and cranial nerve dysfunction.16 In a retrospective study of 103 horses in Australia with presumptive tick paralysis secondary to *I. holocyclus*, 88% of the horses were recumbent and unable to stand on presentation.17 Recumbency for >120 hours increased the odds of nonsurvival, which is not surprising given that recumbency in large animals can exacerbate morbidity secondary to decubital ulceration, intertrigo, corneal ulceration, and rhabdomyolysis.17-20 Seventy-six percent of horses were <1 year of age with over half <6 months of age, and 39% were Miniature horses or ponies. Other studies also have found that affected animals tend to be young and have relatively low body weight (eg, calves, foals, and sheep), suggesting that a high tick burden to body mass ratio might be important in whether or not clinical signs develop.3,6,8 However, no association has been identified between the number of ticks (range, 1-100 ticks) found on a horse and survival.17 This observation suggests that whereas body size might be important, other factors such as immune status (eg, naive immunity in younger animals) and tick virulence factors are also likely important in determining susceptibility to disease. In fact, only 1 to 2 ticks were identified on 63% of horses with presumptive tick paralysis.17

Here, we report the first 2 cases of presumptive tick paralysis in horses outside of Australia. Both horses initially showed generalized ataxia progressing to profound weakness and recumbency. Although ataxia typically occurs with upper motor neuron (UMN) weakness secondary to CNS disease, these horses had profound weakness with intact postural reactions supporting diffuse LMN disease. Both horses had intact spinal reflexes (consistent with UMN dysfunction), but decreased tongue and tail muscle tone with normal mentation also was suggestive of LMN weakness. Overall, profound weakness with normal proprioception is most consistent with LMN weakness despite the fact that other classic signs of neuromuscular dysfunction such as muscle trembling while standing or neurogenic muscle atrophy were not apparent in these horses. Although clinical signs of tick paralysis caused by both *Dermacentor* spp. and *Ixodes* spp. in small animals are caused by presynaptic neuromuscular dysfunction and likely a consequence of the same mechanism in the cases presented here, the neuroanatomical site of toxin activity of *Dermacentor* spp. neurotoxins in horses is unknown. It is possible that UMN, LMN, neuromuscular junction, or some combination of these might be targeted, resulting in a different constellation of clinical signs in affected horses.

Given that both horses presented with nearly identical and acute clinical signs within the same time period, toxocosis was suspected. Detection of numerous embedded and engorged ticks made tick paralysis likely, although botulism could not be ruled out as a cause of decreased muscle tone, weakness, and recumbency. The horses had been fed with round bale grass hay, which is a known risk factor for botulism.19 Given the insensitivity of available antemortem diagnostic tests for botulism and the importance of rapid treatment, trivalent equine botulinum antitoxin was given to both horses within hours of presentation.21 However, the rapid improvement in neurologic status observed in these 2 horses made botulism unlikely because recovery requires regeneration of new motor end plates, which can take up to 3 weeks.22 Neurotoxicity associated with ingestion of locoweed, yellow star thistle (*Centaurea solstitialis*), moldy corn, or Bermuda or rye grass (“grass staggerers”) was not considered because of the absence of forebrain signs. Although equine motor neuron disease causes LMN dysfunction, the history in these horses (ie, access to pasture) did not support vitamin E deficiency, and classic signs of LMN weakness were not observed. Additionally, some of the clinical signs were consistent with equine herpes myeloencephalitis (EHM) and were present in more than 1 animal, making it important to test for EHV-1. Normal CSF cytology and negative EHV-1 PCR ruled out EHM and allowed the horses to remain in the main hospital without quarantine. Although testing for West Nile Virus was not performed given the unlikely possibility that both horses would develop signs simultaneously, doing so would have been appropriate given the unknown vaccination history and compatible clinical signs.23 Finally, diagnosis of tick paralysis was confirmed by the observation of rapid improvement after removal of the ticks in the absence of other disease-specific treatments.

Hematologic and serum biochemical results in small animals with tick paralysis are typically normal.4,15 The laboratory abnormalities observed in the 2 horses described here were likely a result of anorexia, stress, and recumbency. Hypertriglyceridemia and subsequent hepatic lipidosis are common complications of prolonged anorexia in over-conditioned horses and in certain breeds, including the American Miniature horse.

Treatment consisted of manual tick removal, supportive care, and topical administration of an insecticide. Although hyperimmune tick antiserum derived from dogs often is administered to horses with tick paralysis in Australia, it is specific for *Ixodes* spp. and is not commercially available in North America. Even if *Dermacentor* spp. antiserum is developed in the future, it might be unnecessary given the shorter clinical course of disease after removal of *Dermacentor* spp. ticks as compared to *Ixodes* spp. ticks.17

Both horses reported here showed rapid improvement upon tick removal and survived. Neither horse had residual neurologic deficits. Given the lack of published reports on tick paralysis in horses in North America, it is likely that horses are relatively resistant to the development of clinical signs. Because the prognosis for tick paralysis associated with *Dermacentor* spp. generally is better than that associated with *Ixodes* spp. in small animals, it is likely that prognosis also is favorable in horses, even with a heavy infestation. The survival rate for horses with tick paralysis caused by *I. holocyclus* was found to be 74% in a large retrospective study, and if host factors that play a role in the pathophysiology of tick paralysis are shared among mammalian species, it is reasonable to expect an even better prognosis for tick paralysis associated with *Dermacentor* spp.17
To summarize, tick paralysis in horses has not been recognized previously in North America. However, a thorough physical examination with a focus on the integument to identify the presence of ticks should be performed in horses presented with acute-onset ataxia or tetraparesis progressing to recumbency. Further research is needed to elucidate the mechanism of action and neuroanatomical target or targets of the Dermacentor spp. neurotoxins in horses with tick paralysis. Additional reporting of presumptive tick paralysis in horses would better clarify the clinical course and confirm the assumption that prognosis is favorable in affected horses.

ACKNOWLEDGMENTS

The authors thank Dr. Gillian Haanen and Dr. Alexis Powers for their contribution to case management. This work was presented at the 2019 American College of Veterinary Internal Medicine (ACVIM) Forum in Phoenix, Arizona.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Sandra D. Taylor @ https://orcid.org/0000-0002-8807-5554

REFERENCES


Antigen array for serological diagnosis and novel allergen identification in severe equine asthma

S. J. White¹,²,³*, M. Moore-Colyer¹, E. Marti⁴, D. Hannant⁵, V. Gerber⁴, L. Cöüetil⁶, E. A. Richard⁷,⁸ & M. Alcocer²

Severe equine asthma (sEA), which closely resembles human asthma, is a debilitating and performance-limiting allergic respiratory disorder which affects 14% of horses in the Northern Hemisphere and is associated with increased allergen-specific immunoglobulin E (IgE) against a range of environmental proteins. A comprehensive microarray platform was developed to enable the simultaneous detection of allergen-specific equine IgE in serum against a wide range of putative allergenic proteins. The microarray revealed a plethora of novel pollen, bacteria, mould and arthropod proteins significant in the aetiology of sEA. Moreover, the analyses revealed an association between sEA-affected horses and IgE antibodies specific for proteins derived from latex, which has traditionally been ubiquitous to the horse’s environment in the form of riding surfaces and race tracks. Further work is required to establish the involvement of latex proteins in sEA as a potential risk factor. This work demonstrates a novel and rapid approach to sEA diagnosis, providing a platform for tailored management and the development of allergen-specific immunotherapy.

Severe equine asthma (sEA) is a performance-limiting, debilitating condition which is prevalent in 14% of horses in the Northern Hemisphere¹. The pathogenesis of this condition remains controversial with many contradictory reports²,³; but several studies have indicated the role of immunoglobulin E (IgE) through in vitro histamine release assays⁴-⁶, and allergen-specific IgE (sIgE) analyses of bronchoalveolar lavage fluid (BALF) and sera⁵,⁶. Specific IgE assays suggest that Aspergillus fumigatus (Asp f (extract), rAsp f 8, Asp f 1/a), Alternaria alternate, Tyrophagus putrescentiae, Saccharopolyspora rectivirgula, Aspergillus terreus, Eurotium amstelodami, Geotrichum candidum and Wallemia sebi may be implicated in the aetiology of sEA²,⁴,⁸-¹¹. More recently, White et al. (2017) identified 40 potential allergens of interest, from several genera, including fungi, bacteria, pollen and arthropod¹².

sEA diagnosis is presently conducted on clinical history and readily identified clinical signs¹³, which have been shown to correlate with sEA severity¹⁴, with ancillary diagnostic tests such as BALF cytology, lung function testing, haematology, and immunological testing used to improve diagnostic accuracy¹⁵. While several studies have addressed the potential benefits derived from in vitro allergen assessment in diagnosis of sEA, commercial application has been hampered due to a lack of statistical approaches for clear disease classification, and the limited range of allergens tested to date.

More recently, White et al., (2019) developed microarray methods to enable IgE profiling in sEA-affected horses, elucidating previously unidentified causal allergens and demonstrating a strong correlation between BALF and sera specific IgE profiles¹⁶. The aim of the present study was to use sera from a large group of horses from France, Switzerland, USA and Canada, exposed to a wide range of potential allergens in the normal equine

¹Royal Agricultural University, Cirencester, Gloucestershire, GL7 6JS, UK. ²School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK. ³Nottingham Trent University, Brackenhurst Campus, Southwell, Nottinghamshire, NG25 0QF, UK. ⁴Department of Clinical Research and Veterinary Public Health, University of Bern, Bremgartenstr, Postfach, 3001, Bern, Switzerland. ⁵School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK. ⁶Veterinary Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, IN, 47907, USA. ⁷LABEO Frank Duncombe, 1 route de Rosel, 14053, Caen, Cedex 4, France. ⁸Normandie Univ, UniCaen, BIOTARGEN, 3 rue Nelson Mandela, 14280, Saint-Contest, France. *email: samuel.white@ntu.ac.uk
environment and determine if a combination of microarray and mathematical modelling could be used to elucidate previously unidentified allergens involved in the aetiology of sEA, as a potential diagnostic test for sEA, and to evaluate the influence of samples from mixed environments without matched controls. To achieve this, we primarily used specific allergen molecules, thus identifying genuine sensitisation and minimising cross-reactivity, potentially enabling precise allergen selection for future immunotherapy.

Materials and Methods
Equine sera samples. Horses from Canada, France and the US were classified according to clinical assessment, including physical examination, tracheal mucus, pulmonary function test, reversible airway obstruction after medical/environmental change and BALF cytology, demonstrating moderate to severe neutrophilia (>25% cells), as previously described56. Control horses had no record of lung disease, no previous history of laboured breathing, coughing or nasal discharge, no tracheal mucus, and <10% BALF neutrophils. Swiss samples were those published in Verdon et al.17, sEA was classified using the horse owner assessed respiratory signs index (HOARS) ≥3 and partial pressure of arterial oxygen <90 mm Hg, and Insect Bite Hypersensitivity (IBH) classified via IBH scoring57. Blood was collected from the jugular vein in VACUETTE Serum Clot Activator Tubes, centrifuged at 2000 × g for 10 minutes, serum removed and stored at −80 °C. This study was approved by the Royal Agricultural University Ethical Review Group. All experiments were performed in accordance with the relevant guidelines and regulations.

In the first part of the study, a sub-group of the total of 138 sports horses, consisting of n = 35 environmentally matched samples from France (5 sEA; 6 control), USA (6 sEA; 6 control) and Canada (6 sEA; 6 control) were analysed. These were modelled to enable reliable comparison of samples with matched controls collected from horses in the same environment, thus accounting for any antigenic stimuli associated IgE responses. In order to test the robustness and clinical relevance of the test, in the second phase of the study, microarray analysis was carried out on a larger group, including the aforementioned horses and those from differing environments without matched controls (n = 138), consisting of sEA n = 33, IBH/sEA n = 23, IBH n = 24 and control n = 58 from France, Switzerland, USA and Canada. Horses suffering with IBH, a classic equine hypersensitivity, were included to further assess the discriminatory power and clinical relevance of this approach. This group (n = 138) was used to build and test the mathematical predictive model and identify relevant allergens.

IgE sera determination by protein microarray. The comprehensive complex microarray comprised of extracts (n = 153) and pure proteins (n = 231) from a wide range of fungi, bacteria, pollen, arthropods and others associated with the equine environment. The extracts and pure proteins were obtained from commercial suppliers, produced in-house and donated to our group. Fungi and bacteria strains were purchased from Deutsches Sammlung von Mikroorganismen und Zellkulturen, grown in liquid media, and extracts produced via sonication. Samples were normalised to 0.5 mg/ml protein and printed onto NOCYTE® NOVA Nitrocellulose Film Slides (GRACE Bio-Labs, Oregon, USA) using an Ultra Marathon II by Arrayjet, (Roslin, Scotland) to a final spot density of 12,288 spots/slide, with an approximate spot size of 200 μm diameter and replicated twice into two blocks on each pad. For alignment purposes Cy3/Cy5 were included, and for quality control purposes a number of sham antigens were spotted (e.g. PBS, Equ c 3, Ara h 1-NT, Man e, Gal d 1–4). Slides were blocked in 3% BSA (w/v) in PBS inside a Corning 5 slide holder (product # 40082) using a mini hybridization oven (Appligene, USA) at 37 °C. Antigens were spotted (e.g. PBS, Equ c 3, Ara h 1-NT, Man e, Gal d 1–4). Slides were blocked in 3% BSA (w/v) in PBS inside a Corning 5 slide holder (product # 40082) using a mini hybridization oven (Appligene, USA) at 37 °C. Antigens were spotted (e.g. PBS, Equ c 3, Ara h 1-NT, Man e, Gal d 1–4).

Slides were washed with Milli-Q water and dried via centrifugation at 300 × g for 10min at room temperature. Slides were then washed three times for 2 min in PBS containing 0.05% (w/v) Tween-20, followed by five times 1 min washes with Milli-Q water, and dried via centrifugation (MSE Mistral 3000i, Sanyo, UK) 300 × g for 10 min at room temperature.

Slides were fitted with Proplate slide modules (Grand Bio-Labs, product # 204862) and washed three times (60s second dwell time) with PBST (0.2%). Sera samples were diluted 1:2 with 4% BSA in 0.4% PBST, and 100 μl of prepared sample was added to each well, excluding well 4, which was a control filled with 100 μl of the dilution solution (1:2) in 2% BSA in 0.2% PBST (final dilution). The Proplate was fitted with an adhesive seal strip and incubated for 16 hours at 4 °C on the Stuart mini see-saw rocker (SSM4) at 13 oscillations/minute. The Proplate was washed three times with PBST (0.05%) using the BioTek plate washer and incubated for 2 hours at 37 °C in a ThermoHybrid (HyPro 20) at AVS 3 with 100 μl per well of anti-horse IgG (BioRad, #MCA5982GA) 1:400 in 1% BSA in 0.2% PBST. They were washed a further 3 times with PBST (0.05%) and incubated for one hour at 37 °C in the ThermoHybrid with 100 μl per well of DyLight 649 conjugated anti-mouse IgG (Rockland, Product #610-443-040) 1:400 in 1% BSA in 0.2% PBST. Slides were then washed three times with PBST (0.05%) followed by three washes with Milli-Q water and dried via centrifugation at 300 × g for 10 min (Mistral 3000i, rotor 43124-708).

Data analysis. Processed slides were scanned in a GenePix 4000B (Molecular Devices, USA) with the PMT settings 440 and 310 at 635 and 532 nm and saved as TIFF files. Images were processed in GenePix Pro software v6.0.1.27 (Axon Instruments) and saved as comma-delimited text files. Digital fluorescence units (DFUs) were calculated for each spot by subtracting local background from the median fluorescence value of the spot. One pad on each microarray was used as a control, containing reagents and no serum, the results of which were subtracted from all other pads to account for any auto-fluorescence or non-specific binding. Clinically healthy and IBH horses were used as control.

PLS toolbox (version 5.8.3, Eigenvector Research Inc., USA) running on a MATLAB platform (MathWorks, Cambridge, UK) was used to carry out principal component analysis and partial least squares discriminant analysis (PLS-DA) was used as a classifier which enabled construction of the predictive mathematical models. A variable influence on the projection (VIP) score of each variable was calculated as a weighted sum of the squared correlations between the original
variable and the PLS-DA components. This is a measure of the contribution that a specific variable has on the model\textsuperscript{19}. In order to test the mathematical model produced, multiple rounds of cross validation (CV) were performed using different partitions, and the validation results were amalgamated through the rounds giving an estimate of the model’s predictive performance\textsuperscript{19}.

### Results

**Environmentally matched group.** The initial calibration of the PLS-DA classification method using the small subset of environmentally matched samples (n = 35) was highly encouraging, with CV values confirming good prediction (Table 1). In an effort to reduce the background noise and improve robustness of the mathematical model, a second round of modelling was conducted using the main VIPs (n = 129) identified in the calibration step (Fig. 1). This improved both sensitivity and specificity of the (CV) mathematical model (Table 1).

**Environmentally mixed group.** The mathematical calibration model with the reduced number of sEA horses and matched samples has shown that it is feasible to discriminate sEA from control animals. Whether other allergic diseases and samples without matched controls would interfere with the detection and classification method has been tested using a cohort of 138 horses (34 sEA, 23 IBH/EA, 23 IBH and 58 controls). The PLS-DA calibration modelling involving this new cohort (n = 138) confirmed the good prediction for sEA obtained with non-matched samples, particularly after the second round of mathematical modelling using the sEA VIP selection (Table 2). A wide range of VIPs were identified as significant for class prediction (see Supplementary Data), predominant variables included Hev b 11, Hev b 6.02, Hev b 5.0101, rAsp f 8 and rHel as 7.

#### Table 1. Partial least squares discriminant analysis statistics of the calibrated and cross validated data from the environmentally matched group of horses (n = 35) from the first (before VIP selection) and second (after VIP selection) rounds of modelling. CAL = calibration; CV = cross validation.

<table>
<thead>
<tr>
<th></th>
<th>Before VIP selection</th>
<th>After VIP selection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAL</td>
<td>CV</td>
</tr>
<tr>
<td>Specificity</td>
<td>1.00</td>
<td>0.70</td>
</tr>
<tr>
<td>Sensitivity Error</td>
<td>1.00</td>
<td>RMSEC = 0.092</td>
</tr>
</tbody>
</table>

#### Figure 1. Variable influences on the projection calculated by PLS-DA software from the environmentally matched group of horses (n = 35) after VIP selection. A threshold of α > 1 was used to identify those VIPs significant in class prediction.
Previously, using Partial Least Squares Regression we demonstrated that human IgE microarray analysis with extracts and pure proteins correlate well with standard laboratory methods such as ELISA, UniCAP and immunoblot test. Similarly, there was good agreement between equine IgE microarray and ELISA results in this study (see Supplementary Data). Mathematical modelling of profiling data for disease classification and allergen identification is well utilised in human allergology, but has had little application in the veterinary sector. Based on these principles, we utilised latest technological developments and mathematical modelling to explore sEA. This enabled the widest scale sEA-associated allergen profiling to date.

This study utilised whole protein extracts to maximise allergen coverage, while maintaining specificity by including purified proteins where allergens were known. This was essential because of the limited numbers of potential sEA allergens screened by others to date. Further work would benefit from purifying proteins of the identified whole extracts of interest, thus providing well-defined reagents for component resolved diagnostics enabling increased specificity and sensitivity, particularly aimed at the use of specific immunotherapy.

Many of the allergens identified in the initial model (Fig. 1) have previously been implicated in human allergic asthma, but not previously assessed in the horse. Erwinia, Geotrichum candidum and Eurotium amstelodami have been associated with asthma and occupational respiratory diseases in farmers. Moreover, Juniperus virginiana and Corylus avellane pollen are often noted as inciting hay fever and asthma. Particularly noteworthy allergens include Aspergillus versicolor, A. niger and A. fumigatus all from the most significant genus associated with the aetiology of human asthma and sEA. Similarly, Dermatophagoides farinae is associated with human asthma as well as sEA, and Pen i 1 is cross-reactive to many arthropods. Furthermore, several latex allergens are significant for class prediction, a group previously untested in the horse.

The classification results (CV sensitivity and specificity) from the second model (Fig. 2) were however lower than the first small subset of horses. The discrepancies between the two subgroups most likely results from the second group being collected from varying environments as described by Eder et al.

Table 2. Partial least squares discriminant analysis statistics of the calibrated and cross validated data from the environmentally mixed group of horses (n = 138) showing different classification values after sEA VIP selection. CAL = calibration; CV = cross validation.

<table>
<thead>
<tr>
<th></th>
<th>sEA</th>
<th>Control</th>
<th>IBH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAL</td>
<td>CV</td>
<td>CAL</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.865</td>
<td>0.865</td>
<td>0.787</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.765</td>
<td>0.735</td>
<td>0.931</td>
</tr>
<tr>
<td>Error</td>
<td>RMSEC = 0.315</td>
<td>RMSECV = 0.360</td>
<td>RMSEC = 0.366</td>
</tr>
</tbody>
</table>

Figure 2. Variable influences on the projection (VIP) scores calculated by PLS-DA from the environmentally mixed group of horses (n = 138) after VIP selection. A threshold of $\alpha > 1$ was used to identify those VIPs significant in class prediction.
allergen-specific serum IgE levels. The second group presented here did not have matched controls to account for environmental-associated IgE production and possessed a strong IBH response bias which may have weakened the mathematical predictive model. The most influential VIPs for class separation were those from natural rubber latex (Hevea brasiliensis, Hev b), these included Hev b 11; Hev b 6.02; Hev b 5.0101; Hev b 3.0101 and Hev b-extract (see Supplementary Data). To the authors knowledge, this is the first time Hev b allergens have been assessed in relation to sEA. As shown in Fig. 2, a smaller level of IgE-binding to latex allergens was detected in the sera of IBH positive horses and controls used in this study (latex means: 1445, 735, 803 for sEA, IBH and control respectively with P < 0.0001 when compared to sEA), however latex allergens alone were not able to discriminate the sEA group. As shown in Table 2 this discrimination is much improved with the other VIPs, particularly Aspergillus (Asp f 8).

Work in human asthma patients has revealed a higher frequency of Hev b allergies in affected individuals39. A major source of respirable Hev b allergens in the horse's environment is from artificial riding surfaces. Although the use of recycled tyres was banned in many parts of Europe in 2007, in the UK it is permitted under current Environment Agency waste regulations (Waste Exemption: U8 use of waste for a specific purpose), and many arena surfaces throughout the world contain components of natural rubber. These surfaces have high levels of respirable dust, which has previously been associated with chronic bronchitis in riding instructors29–31. Respiration of Hev b particles have also been shown to induce inflammation and oxidative stress in the lungs of humans32. Furthermore, particles such as Hev b, have been shown to exhibit an adjuvant effect by increasing the primary response during sensitisation when present either before, during or after allergen exposure33. Diaz-Sanchez et al., (1999) demonstrated particulate inhalation during allergen exposure could induce a mucosal IgE response under conditions in which the allergen alone could not42. Similarly, experimental animal models in strains of mice not prone to developing IgE responses, demonstrated that particulate allergens may enhance sensitisation34. Given the adjuvant and sensitising effects of latex, these airborne particles could contribute to the increase in both latex sensitisation and asthma through direct and indirect mechanisms36,37, which may explain the association between sEA and Hev b-specific IgE demonstrated here. Moreover, these results are in agreement with previous work identifying the urban environment, which is high in respirable natural rubber latex37, as a risk factor in sEA. In humans, regular exposure to latex particles in the work environment can lead to occupational asthma, commonly known as latex-induced asthma. The prevalence of latex sensitisation in occupationally-unexposed groups is significantly lower (<1%) than those regularly exposed (>18%)39. The main allergen associated with occupational latex-allergy (Hev b 6.02)40 was the second most influential VIP in our study group with sEA-affectted horses, along with other major Hev b-allergens used for occupational latex-allergy diagnosis (Hev b 11; Hev b 5.0101)41. The results of this study would suggest there may be an association between sEA and increased latex-specific IgE. Further equine specific work is required to establish the exposure levels of latex in the horse's daily environment, demonstrating the benefit of latex avoidance, latex inhalation reactivity tests, epidemiological studies and further hypersensitivity confirmation through basophil activation tests. At present, exposure should be considered a potential risk to the respiratory health of the horse.

Several fungal allergens were found to significantly influence class prediction, these included Aspergillus fumigatus (Asp f 8), Mucor circinelloides f. lusitanicus (Muc ci), Geotrichum candidum (Geo c) and Eurotium amstelodami (Eur a) (see Supplementary Data). The Asp f 8 results confirm those of Eder et al., (2000) and Künzle et al., (2007) whom also found significantly more IgE against this recombinant mould allergen in sEA-affectted horses10,42. Tahon et al., (2009) also reported significantly higher positive intradermal reactions to Asp f 8 in sEA-affectted horses43. Mucor circinelloides f. lusitanicus (Muc ci) results further confirm previous research demonstrating Mucor allergen extract sensitisation is associated with sEA-affectted horses via in vitro basophil assay1. Similarly, increased levels of specific IgE against E. amstelodami and G. candidum have been identified in the bronchoalveolar lavage fluid of sEA affected horses via western blot47. Several arthropods were significant for class separation, including the tropomyosin of Helix aspersa (Hel as 7 and Periplaneta Americana (Per a 7), the proteases from Blattella germanica (Bla g 2) and Dermatophagoides farinae (Der f 1), the complex mixture of Bliomia Tropicalis (Blo 1), and Dermatophagoides pteronyssinus (Der p 2). The array results therefore ratified recent reports on the involvement of Acarus siro, Dermatophagoides, farinae/pteronyssinus, Tyrophagus putrescentia in sEA and their association with high concentrations of specific IgE against mites, particularly T. putrescentia44. Bla g 2 is associated with the development of asthma in humans and increased sIgE against Bla g has previously been reported in sEA-affectted horses45,46. Tropomyosin results (Hel as 1 and Per a 7) are to be expected, as Tropomyosin are major allergenic components accounting for cross-reactivity with mites and other arthropods46. Furthermore, the high VIP scores demonstrated for Culicoides proteins (Cul nu 2, CO145, Cul o 2) could have resulted from the sEA/IBH horses, even though these were matched with IBH controls, or from multiple hypersensitivities, as sEA horses are at increased risk of IBH45 which is associated with airway hyperreactivity. The only bacteria considered significant for class separation was Thermoactinomyces vulgaris, which has long been associated with sEA and increased levels of IgE in affected horses47,48. Interestingly, our study showed 28 pollens were significant for class separation, including Betula verrucosa (Bet v 2 0101), Meratculis annua (Mer a 1), Eupatorium capillifolium (Eup c), Quercus robur (Que r) and Helianthus annuus (Hel a). To the authors knowledge, this is the first study to show an association between sEA in horses and a hypersensitivity to pollens. When utilising a panel of 131 allergens, Einarsson et al., (2018) demonstrated that horses are most likely to be sensitised to Fag e 2, Cyn d 1 and Aln g 1, similarly here we found Fag e was significant for class prediction (Fig. 2).

As expected, the environmentally matched (MA) group has several VIPs in common with the environmental mixed (MI) group. Most notably Hev b 11, Hev b 6.02, rAsp f 8, Eur s and Hev b 5.0101. Moreover, many similarities are apparent, such as Der f and tropomyosin Pen i 1 in MA compared with Der f 1 and tropomyosin Hel as 7 in MA. The MI group was equally reliant on a range of aspergillus species (Asp v, Asp n, rAsp f 8), whereas the MA group primarily relied on rAsp f 8. Bovine milk proteins are important for class prediction in both models (MA - Bos d 4, Bos d 9; MI - Bos d LF), the significance of this warrants further research, these
molecules commonly cross react between species and have shared common allergenic components with other allergens, such as Glycine max. Use of a PLS-DA model enabled the classification of sEA-affected horses using IgE as a biomarker, which has previously not been possible with the utilised statistical methods due to overlap between affected and non-affected groups. Such models have been employed in the human sector to enable diagnosis of asthma patients using metabolomics with great success, and proved to be just as effective with sEA. Furthermore, the identification of specific IgE-auto-reactivity through VIP identification contributes to an understanding of the pathogenesis of the disease. The ability to discriminate sEA-affected horses from other IgE-mediated conditions demonstrates the robustness of the test. Further research expanding the repertoire of allergens tested in the form of pure proteins would increase the diagnostic accuracy of the mathematical model as well as beneﬁtting identiﬁcation of genuine sensitisation and enabling therapeutic and diagnostic development. This advanced bioinformatics enabled the largest scale allergen proﬁling of sEA to date, signiﬁcantly contributing to aetiological understanding of this complex disease.

In conclusion, the microarray platform demonstrated here may be utilised as an auxiliary diagnosis for sEA, informing accurate allergen-avoidance regimes based on its sensitisation proﬁles; while simultaneously elucidating important factors associated with the aetiology and pathogenesis of this complex disease. Moreover, it enables further diagnostic developments and the creation of speciﬁc immunotherapy treatments. This serological investigation of 138 horses living in varying environments identiﬁed that sEA is associated with a large sensitisation proﬁle, and predominantly involves latex, fungi, mite and pollen proteins; demonstrating similar proﬁles to that found with allergic asthma in the human. These results indicate that exposure to latex may be detrimental to the respiratory health of the horse. Further research is required to establish the levels of latex exposure in the equine environment and its in vivo effects. Sensitivity and speciﬁcity values conﬁrmed the high discriminatory power of the technique in combination with mathematical modelling. The microarray platform demonstrated here will enhance the health, welfare and performance of sEA affected horses. This has been achieved on a number of levels through (a) the development of a novel serological diagnostic test, (b) improved understanding of disease pathogenesis, and (c) identiﬁcation of novel allergenic candidates.

Data availability
The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 24 April 2019; Accepted: 8 October 2019;
Published online: 23 October 2019

References
Thi collection and diagnosis. S.J.W. and M.A. performed data analysis and processing. S.J.W. prepared the

Author contributions
Respiratory Tissue Bank for the supply of Canadian samples.

Acknowledgements
This work was supported by the Morris Animal Foundation (Grant No.: D16EQ-039), the Swiss National Science
Foundation (Grant No.: 310030-160196/1), Stiftung Forschung für das Pferd, the Royal Agricultural University,
the Fred and Marjorie Sainsbury Charitable Trust and HAYGAIN. The authors would like to thank the Equine
Respiratory Tissue Bank for the supply of Canadian samples.

Author contributions
collection and diagnosis. S.J.W. and M.A. performed data analysis and processing. S.J.W. prepared the first draft of
the manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.
Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-019-51820-7.

Correspondence and requests for materials should be addressed to S.J.W.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019
Development of a comprehensive protein microarray for immunoglobulin E profiling in horses with severe asthma

Samuel White1,2,3 | Meriel Moore-Colyer1 | Eliane Marti4 | Laurent Coüetil5 | Duncan Hannant6 | Eric A. Richard7,8 | Marcos Alcocer2

1School of Equine Management and Science, Royal Agricultural University, Gloucestershire, United Kingdom
2School of Biosciences, University of Nottingham, Loughborough, United Kingdom
3Animal and Equine Science, Nottingham Trent University, Nottinghamshire, United Kingdom
4Department of Clinical Research and Veterinary Public Health, University of Bern, Bern, Switzerland
5Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana, USA
6School of Veterinary Medicine and Science, University of Nottingham, Loughborough, United Kingdom
7LABEO Frank Duncombe, Caen Cedex, France
8Normandie University, UniCaen, BIOTARGEN, Saint-Contest, France

Correspondence
Samuel White, Animal and Equine Science, Nottingham Trent University, Brackenhurst Lane, Southwell, Nottingham, NG25 0QF, United Kingdom.
Email: samuel.white@ntu.ac.uk

Funding information
Fred And Marjorie Sainsbury Charitable Trust; HAYGAIN; The Royal Agricultural University

Abstract

Background: Severe asthma in horses, known as severe equine asthma (SEA), is a prevalent, performance-limiting disease associated with increased allergen-specific immunoglobulin E (IgE) against a range of environmental aeroallergens.

Objective: To develop a protein microarray platform to profile IgE against a range of proven and novel environmental proteins in SEA-affected horses.

Animals: Six SEA-affected and 6 clinically healthy Warmblood performance horses.

Methods: Developed a protein microarray (n = 384) using protein extracts and purified proteins from a large number of families including pollen, bacteria, fungi, and arthropods associated with the horses, environment. Conditions were optimized and assessed for printing, incubation, immunolabeling, biological fluid source, concentration techniques, reproducibility, and specificity.

Results: This method identified a number of novel allergens, while also identifying an association between SEA and pollen sensitization. Immunolabeling methods confirmed the accuracy of a commercially available mouse anti-horse IgE 3H10 source (R² = 0.91). Biological fluid source evaluation indicated that sera and bronchoalveolar lavage fluid (BALF) yielded the same specific IgE profile (average R² = 0.75). Amicon centrifugal filters were found to be the most efficient technique for concentrating BALF for IgE analysis at 40-fold. Overnight incubation maintained the same sensitization profile while increasing sensitivity. Reproducibility was demonstrated (R² = 0.97), as was specificity using protein inhibition assays. Arthropods, fungi, and pollens showed the greatest discrimination for SEA.

Conclusions and Clinical Importance: We have established that protein microarrays can be used for large-scale IgE mapping of allergens associated with the environment of horses. This technology provides a sound platform for specific diagnosis, management, and treatment of SEA.

KEYWORDS
allergen, horse, IgE, protein microarray, severe equine asthma

Abbreviations: BALF, bronchoalveolar lavage; BSA, bovine serum albumin; IgE, immunoglobulin E; PBS, phosphate buffered saline; PBST, phosphate buffered saline with Tween 20; RAST, radioallergosorbent test; SEA, severe equine asthma; sIgE, specific immunoglobulin E.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2019 The Authors. Journal of Veterinary Internal Medicine published by Wiley Periodicals, Inc. on behalf of the American College of Veterinary Internal Medicine.
Severe asthma in horses, known as severe equine asthma (SEA), is a performance-limiting, allergic response to inhaled allergens in genetically predisposed horses that affects approximately 14% of the equine population in the United Kingdom.1-3 Allergen exposure in affected horses results in small airway inflammation, mucus hypersecretion, and bronchoconstriction, altering pulmonary resistance, dynamic compliance, and pleural pressure.4-6 The predominant source of these aeroallergens is the organic dust portion of forage and bedding, which contains fungi, bacteria, pollen, and arthropods.7-11 Removal from the aeroallergen-rich stabling environment results in some degree of remission,12 but owner compliance is limited because of seasonality, competition schedule, health issues, and nutritional demands. Treatments with corticosteroids and bronchodilators provide short-term relief, but such therapeutic approaches have been associated with undesirable adverse effects, and their use is prohibited under Fédération Equestre Internationale and Jockey Club rules.13,14 Allergen avoidance is the cornerstone of prevention and effective treatment, but the efficacy of the latter approach relies on identification of causal allergens.15

Currently, the main obstacles to diagnostic and therapeutic developments include major limitations in the number of allergens screened to establish causal agents and lack of a clinically applicable in vitro test.

The pathogenesis of this condition remains unclear, but several studies have implicated immunoglobulin E (IgE) by in vitro histamine release assays,16-18 natural hay and straw challenges,19 intradermal testing,20 and specific IgE (sIgE) analysis of bronchoalveolar lavage fluid (BALF) and sera.21,22 The sIgE assays suggest that Aspergillus fumigatus, Alternaria alternata, rAsp f 8, Phyllographus putrescentiae, Saccharopolyspora rectivirgula, Asp f 1/a, Aspergillus terreus, Eurotium amstelodami, Geotrichum candidum, and Wallenia sebi are implicated in the etiology of SEA.8,21,23-25 Although many recombinant proteins are available,26 advances in causal allergen identification have been limited because of the practicality of testing with classical methods, such as ELISA, which are time-consuming, expensive, and require large quantities of samples and reagents.27

In recent years, protein microarrays have been gaining popularity in allergy diagnostic testing because of their ability to assess the interaction of thousands of proteins with specific immunoglobulin isotypes using techniques such as fluorescence on a miniaturized scale, a technique known as microarray profiling.28 This technique circumvents the aforementioned limitations associated with techniques such as ELISA, enabling multiallergen testing to assess complex sensitization profiles. Furthermore, with specific allergens, these tests show similar sensitivity to standard laboratory methods, including ELISA, UniCAP, radioallergo-sorbent test (RAST), ImmunoCAP, and immunoblot testing.29-31 Previously published sensitivity and specificity values using protein microarrays have indicated the high discriminatory power of the protein extracts and pure recombinant Culicoides proteins associated with insect bite hypersensitivity in the horse.22

Our aim was to develop a widespread allergen profiling technique using microarray methods that would enable rapid and accurate IgE profiling of SEA-affected horses. Furthermore, we wanted to analyze the correlation between BALF and sera-specific IgE profiles, a crucial consideration with respect to diagnostic sample requirements. Profiling data allows for advances in diagnostic testing and treatment.

2 MATERIALS AND METHODS

2.1 BALF and sera samples

Clinical assessment including physical examination, pulmonary function tests, and BALF cytology were used to define the inclusion and exclusion criteria for the selection of 6 horses with SEA and 6 control horses.5 Bronchoalveolar lavage fluid was collected as previously described,32 filtered through a 100-μL syringe filter (Biocomma, Shenzhen, China), and decanted into 10-μL aliquots in 15-μL centrifuge tubes with the addition of Thermo Scientific Pierce Mini-Protease Inhibitor Tablets—EDTA free (product # 13437766). The mixture was gently agitated and incubated at 4°C for 10 minutes before addition of 2.5 mL of glycerol (Fisher Scientific, Leicestershire, United Kingdom) and stored at −80°C until analysis. To concentrate, BALF was thawed, maintained at 4°C, and filtered using a Sartorius Stedim 0.45-μm filter syringe (product # 17598). The BALF samples then were concentrated in an Amicon Ultra-15 centrifugal filter (product # UFC905024) and used immediately. Blood was collected, and sera were prepared as previously described,33 before storage at −80°C until analysis, at which time samples were thawed at room temperature and placed on ice.

2.2 Proteins, printing and hybridization

To maximize utility, the microarray was designed to be as comprehensive as possible by including extracts and pure proteins from a wide range of protein families derived predominantly from fungi, bacteria, pollen, and arthropods. The extracts and pure proteins were obtained from commercial suppliers, produced in house, and from donations. Because of the limited commercial availability of some bacterial and fungal protein extracts, it was necessary to produce them in house. Lysophilized purified samples of the desired strain were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (https://www.dsmz.de) and grown in 150 mL of liquid media according to the supplier’s recommendation (250-mL Erlenmeyer flask). Once grown, the media were centrifuged in 50-mL tubes at 400g for 10 minutes, and the supernatant was carefully removed before washing the individual pellets in 5 mL of phosphate buffered saline (PBS). The tubes were centrifuged at 400g for 10 minutes, the supernatant was removed, and 1 mL of lysis buffer solution was added to each tube (PBS, 0.5% TritonX-100 wt/vol and Thermo Scientific EDTA free protease inhibitor mini-tablet). The resuspended pellets were pooled into a single 50-mL centrifuge tube, placed on ice, and sonicated using an MSE Soniprep 150 (15 seconds sonication with 30-second cooling periods in between for 10 cycles). Subsequently, the solution was filtered through a Nalgene 0.45-μm syringe filter (product # 190-25-45), and the protein content quantified using a Pierce Bicinchoninic Acid Protein Assay Kit (product # 23225). The remaining solution underwent lyophilization, was resuspended in MilliQ water with 10% glycerol (filtered through a 0.02 μm syringe filter), and was normalized to 1 mg/mL protein and stored at −80°C.
Bronchoalveolar lavage concentration work initially was conducted using slides described previously and consisting of extracts (n = 240) and pure proteins (n = 120) from a range of protein families, including fungi, pollens, and arthropods, to establish the optimal BALF concentration to be utilized in subsequent development work.22 In house extracts not present in the initial array setup were tested by printing normalized samples (1 mg/mL protein) onto 16-pad FAST slides (Whatman Schleicher & Schuell, Dassel, Germany) using a QArraylite arrayer (Genetix, United Kingdom). After sample selection, a new set of 384 proteins (see Supporting Information Appendix) was printed in a professional setting using a Marathon microarrayer (ArrayJet, Roslin, Scotland) printer as previously described22 with an approximate spot size of 200 μm diameter and replicated with even spacing 2 times across each of the individual 16 pads into 2 identical blocks to final spot density of 12,288 spots/slide. For alignment and quality control, spots of Cy3, Cy5, and PBS were printed onto each slide. Once printed, slides were blocked for 3 hours at 37°C in 3% bovine serum albumin (BSA; wt/vol) in PBS inside a Corning 5 slide holder (product # 40082) using a mini hybridization oven (Appligene, USA), washed 3 times for 2 minutes in PBS containing 0.05% (wt/vol) Tween-20, followed by five 1-minute washes with MilliQ water, and dried by centrifugation (MSE Mistral 3000i, Sanyo, United Kingdom) at 100g for 10 minutes at room temperature.

Slides were fitted with Proplate slide modules (Grace Bio-Labs, product # 204862) and washed 3 times (60-second dwell time) with PBS with Tween 20 (PBST; Tween at 0.2%). Samples (BALF/sera) were diluted in a ratio of 1:2 with 4% BSA in PBST (Tween at 0.4% wt/vol) containing Thermo Scientific Pierce Mini-Protease Inhibitor Tablets—EDTA free (product # 13437766; 1 tablet in 5 mL), which previously had been passed through a Whatman 13-mm, 0.45-μm filter syringe (product # 6784-1304). One hundred microliters of the prepared sample was added to each well, excluding well 4, which was used as control and filled with 100 μL of the dilution solution (1:2) in PBS. The Proplate was fitted with an adhesive seal strip and incubated for 16 hours at 4°C on the Stuart mini see-saw rocker (SM4) at 13 oscillations/minute. Slides were washed 3 times with PBST (Tween at 0.05%) using the BioTek plate washer and incubated for 2 hours at 37°C in a ThermoHybaid (HyPro 20) at vibration setting 3 with 100 μL per well of mouse anti-horse IgE (BioRad, product # MCA5982GA) in a ratio of 1:400 in 1% BSA in PBST (Tween at 0.2% w/v), washed 3 more times with PBST (Tween at 0.05%) and incubated for 1 hour at 37°C in the ThermoHybaid with 100 μL per well of DyLight 649 conjugated anti-mouse IgG1 (Rockland, product # 610-443-040) in a ratio of 1:400 in 1% BSA in PBST (Tween at 0.2% wt/vol). The slide then was washed 3 times in PBST (Tween at 0.05%) followed by 3 washes with Milli-Q water, and dried by centrifugation at 300×g for 10 minutes (Mistral 3000i, rotor 43124-708).

2.3 | Data analysis

Processed slides were scanned in a GenePix 4000B (Molecular devices, USA) with the photomultiplier tube settings at 440 and 310 at 635 and 532 nm, respectively, and saved as TIF files. Images were processed in GenePix Pro software v6.0.1.27 (Axon Instruments) and saved as comma-delimited text files. Fluorescence values were calculated for each spot by subtracting local background from the median fluorescence value of the spot. One pad per slide contained all reagents with addition of PBS instead of serum for control purposes; these results were deducted from samples on the same slide to account for any protein autofluorescence and nonspecific binding. Further analysis and data presentation were carried out using Microsoft Excel. Average fluorescence values for each protein were compared between SEA and control groups using a conventional Z-test in Excel (Microsoft, USA). The Benjamini-Hochberg method was used to account for false discovery at a rate of 0.05. Benjamini-Hochberg corrected values were considered significant at P < .05. Linear regression (coefficient of determination) of IgE fluorescence values for all proteins (n = 384) was used to establish the relationship between BALF and sera, reproducibility of results, and various mouse anti-horse IgE sources. Bronchoalveolar lavage fluid concentration techniques and concentrations were tested by 1-way analysis of variance. Tukey's honestly significant difference test for multiple comparisons was performed if significant differences were found (P < .05).

3 | RESULTS

3.1 | Optimizing sera incubation conditions

3.1.1 | BALF concentration techniques

Bronchoalveolar lavage fluid concentration employing Amicon and PD10/lyophilizing methods were compared using a BALF pool from 6 horses (n = 3 SEA and 3 control). Total IgE for each protein group was used to compare concentration methods (Figure 1A) and indicated no significant difference (P > .05) between concentration techniques. The Amicon concentration method was used to evaluate optimal BALF concentration by total IgE fluorescence for each protein group, indicating that a plateau was reached at 40-fold concentration (Figure 1B). Therefore, all subsequent BALF concentrations were carried out by Amicon filtration to a 40-fold final concentration.

3.1.2 | Incubation time

Two conditions were tested for optimal sera incubation times using a sera pool from 6 horses (n = 3 SEA and 3 control): 3 hours at 37°C as previously used for equine sera22 and overnight (16 hours) at 4°C, which previously has been shown to be more sensitive in studies performed in humans.24 As shown in Figure 2, the IgE profile between the 2 incubation times was significantly correlated (R² = 0.76). However, when the serum was incubated for 16 hours at 4°C, it was more sensitive with 28.1% of proteins showing positive reactions, compared to the 4 hours incubation, which showed 16.4% of proteins with positive reactions (data not shown). Therefore, subsequent serological incubations were conducted overnight at 4°C to increase sensitivity.

3.1.3 | Comparison of specific IgE in BALF and sera

Bronchoalveolar lavage samples concentrated by Amicon (40-fold) were compared with sera from 6 horses (n = 3 SEA; n = 3 control),
and correlation coefficients were calculated for each separate protein group. Strong correlations were found between BALF and sera (Table 1). Thus, all subsequent incubations were conducted with serum because it is far easier to obtain, less invasive, more economical, and stable to transport. Horse 5, a clinically healthy horse, showed poor BALF/sera correlations across all protein groups, which was thought to be a result of the horse recently changing to a different barn on the same yard and, therefore, localized IgE production in the lung because of environmental allergen correlated poorly with serological IgE.23,35

3.2 | Reproducibility

3.2.1 | Printing lot variation

The effect of printing lot on reproducibility of the protein microarray was assessed using 2 microarray slides printed on the same day. Sera from 3 SEA and 3 control horses was hybridized on the 2 slides simultaneously. Fluorescence results from replicate arrays were evaluated using linear regression and indicated that fluorescence results from the array were repeatable between printing lots ($R^2 = 0.97$).
3.2.2 | Comparison between monoclonal mouse anti-horse 3H10 sources

Two mouse anti-horse IgE monoclonal antibodies (derived from the 3H10 clone) were compared using linear regression of the fluorescence results using a sera pool from 6 horses (n = 3 severe equine asthma; n = 3 control), which included the original 3H10 from a previous study\(^3\) and the commercially available BioRad 3H10 (product # MCA5982GA).

As shown in Figure 3, the fluorescence results from the array had a correlation coefficient of \(R^2 = 0.91\), indicating a significantly similar IgE profiles.

3.3 | Specificity—protein inhibition assay

To test the IgE specificity of IgE-protein binding, a protein inhibition assay was performed to assess cross-reactivity, in which pooled sera were spiked with several proteins in serial dilution and the effect on related and neighboring proteins evaluated. A protein inhibition assay enables the confirmation of specificity of an antibody against the target protein and usually is conducted using several proteins to confirm both the antibody’s specificity to the target protein and to assess potential cross-reactivity.\(^3\) Two different protein inhibition groups were used, each containing 2 different proteins. Group 1 consisted of *Blattella germanica* (Bl g 1) and *Rumex crispus* (Rum cr), and group 2 consisted of *Penicillium notatum* (Pen ch) and *Acinetobacter gerneri* (Aci g). Interestingly, decreased fluorescence from proteins other than those targeted also was observed, indicating either nonspecific inhibition or some similarity among the allergenic components of the proteins. Group 1 showed no nonspecific binding in the bacteria, arthropod, or fungi groups, but cross-reactivity was seen among grass pollens, most notably *Cynodon dactylon*, *Rc r i s p u s*, *Zea mays*, and *Anthoxanthum odoratum*.

Group 2 demonstrated no nonspecific binding in the bacteria, arthropod, and pollen groups, but cross-reactivity was seen among *Aspergillus niger*, *Aspergillus versicolor*, *Penicillium expansum*, *P notatum*, *Aspergillus nidulans* and *A fumigatus* (Figure 4).

3.4 | Allergen comparison

Whether or not the prototype array was able to identify novel allergens associated with SEA was assessed using Z-tests with Benjamini-Hochberg adjusted \(P\)-values among 6 SEA and 6 control sera samples. The results shown in Table 2 confirmed the ability to conduct IgE
TABLE 2  Z-test results with Benjamini-Hochberg corrected P-values showing all statistically significant allergen between the severe equine asthma (n = 6) and control group (n = 6; P = .05)

<table>
<thead>
<tr>
<th>Name</th>
<th>Benjamini-Hochberg P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dermatophagoides farinace</em></td>
<td>Der f 2</td>
</tr>
<tr>
<td><em>Blattella germanica</em></td>
<td>Bla g 5</td>
</tr>
<tr>
<td><em>Aspergillus restrictus</em></td>
<td>Asp r 1</td>
</tr>
<tr>
<td><em>Linum usitatissimum</em></td>
<td>Lin us [pollen]</td>
</tr>
<tr>
<td><em>Dermatophagoides pteronyssinus</em></td>
<td>Der p 7</td>
</tr>
<tr>
<td><em>B germanica</em></td>
<td>Bla g 5</td>
</tr>
<tr>
<td><em>Hevea brasiliensis</em></td>
<td>H ev b 11</td>
</tr>
<tr>
<td><em>Triticum polonicum</em></td>
<td>Tri tp</td>
</tr>
<tr>
<td><em>H brasiliensis</em></td>
<td>H ev b 5.0101</td>
</tr>
<tr>
<td><em>Penicillium notatum</em></td>
<td>Pen ch</td>
</tr>
<tr>
<td><em>Actinidia chinensis</em></td>
<td>Act c 5</td>
</tr>
<tr>
<td><em>Malassezia pachydermatis</em></td>
<td>Mala p</td>
</tr>
<tr>
<td><em>Actinidia delicosa</em></td>
<td>Act d 11</td>
</tr>
<tr>
<td><em>Olea europaea</em></td>
<td>Ole e 2</td>
</tr>
<tr>
<td><em>Anthoxanthum odoratum</em></td>
<td>Ant o [pollen]</td>
</tr>
<tr>
<td><em>H brasiliensis</em></td>
<td>H ev b 6.02</td>
</tr>
<tr>
<td><em>Parietaria judaica</em></td>
<td>Par j 1</td>
</tr>
<tr>
<td><em>Alternaria alternata</em></td>
<td>Alt a 1</td>
</tr>
<tr>
<td><em>Triticum turgidum ssp. durum</em></td>
<td>Tri td</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>Asp f</td>
</tr>
</tbody>
</table>

4 | DISCUSSION

We previously have determined the sensitivity and specificity of microarrays in the diagnosis of insect bite hypersensitivity, confirming the high discriminatory power of complex extracts and pure recombinant *Culicoides* proteins associated with the allergy. Based on these principles, an array was constructed to enable multiallergen testing and assess the complex sensitization of profiles associated with SEA in a single assay, based on proteins in the environment of the horse. The use of protein extracts was essential because very few proteins have been assessed in relation to SEA to date. Therefore, in the early stages of development, we used a range of extracts to maximize coverage in combination with pure proteins, where available or where the allergen has been previously associated, thus simultaneously maintaining specificity. Furthermore, we observed comparable accuracy between natural extracts and recombinant allergens, whereas some studies have indicated that the use of the recombinant component alone may be insufficient for some allergens. Although the eventual goal will be to move toward component-resolved diagnostics utilizing individual allergen molecules for increased sensitivity and minimizing cross-reactivity, the genus and species must be identified to enable the production of pure proteins. Component-resolved diagnostics offers markedly increased accuracy over routine diagnostic tests (eg, skin prick and specific IgE determination), and enables the accurate selection of allergens to be used for allergen immunotherapy. Moreover, the identification of sensitization to pure proteins will assist in development of diagnostic tests and treatments. This microarray approach has several advantages. It allows substantial allergen profiling with minimal sample, collection of less invasive readily obtained in vivo samples, permits automation, and enables the generation of mathematical predictive models to assist in clinical allergy diagnosis.

Protein microarray methods primarily consist of 2 steps: first, the printing of proteins onto the nitrocellulose slides, and second, the profiling of equine IgE. Printing methods are well established; therefore, the latter was optimized to enable analysis of SEA BALF and sera samples. Although it has been suggested that developing technology means sensitivity is such that many immunoglobulin isotypes in BALF can be assessed unconcentrated to the nanogram or microgram, it is often not possible to detect allergen-specific IgE in BALF because of the low concentration of the isotype present. Therefore, concentration techniques often must be employed to assess BALF IgE. Lyophilization and centrifugal filtration methods have been successfully utilized to concentrate BALF for immunoglobulin analysis, but certain techniques, such as ammonium sulfate precipitation, can result in denaturation of the liable epitopes. Similarly, lyophilizing BALF samples without desalting results in high concentrations of sodium chloride, which can denature proteins. The most commonly utilized BALF concentration technique is the centrifugal filter (Amicon Ultra-15 or Centricon-10) to a 10-fold concentration of immunoglobulin, but even this technique results in a 20% loss of specific IgE and IgG.

On collection of BALF for immunoglobulin analysis, protease inhibitors must be added to prevent proteolytic cleavage of proteins, which would otherwise leave the immunoglobulin unviable. Similarly, immunoglobulin in BALF is markedly affected by freeze–thaw cycles, and the inclusion of a cryopreservative in the form of glycerol has been shown to be effective. Therefore, glycerol was added as a cryoprotectant to a final concentration of 20% to help stabilize the proteins and prevent formation of ice crystals during freezing that destroy protein structure. Unfortunately, samples containing cryopreservative tend to thaw during lyophilization, meaning it was not possible to achieve a 40-fold concentration using this technique. To avoid this problem and remove sodium chloride, samples underwent a buffer exchange using a PD-10 column before lyophilization. Bronchoalveolar lavage concentrations and techniques were trialed, varying hybridization temperatures and times were assessed, and BALF/sera analyzed. The highest binding capacity was observed with overnight incubation at 4°C. When using BALF, the highest binding was seen using the Amicon filter and the PD-10 elution column/lyophilizing at a 40-fold final concentration. The Amicon filter technique was quicker, easier, decreased the risk of contamination, and had previously been utilized, and, therefore, this technique was...
selected. Interestingly, during optimization, a biased response toward pollens was observed. Plants are polyploids and show a large number of gene duplications; hence, high cross-reactivity among species generally is observed. It is our experience that pollen response in humans and other animals is commonly amplified.51

Because of the significant correlation of BALF and sera (average $R^2 = 0.75$), sera were used in subsequent analysis because of the ease of use. Previous studies comparing the specific IgE profiles of BALF and sera using ELISA techniques have been limited and contradictory to date. Previous authors concluded that although BALF may be valuable for analysis, sera was of little clinical relevance.21,23 Here, we demonstrated the ability to profile unconcentrated sera instead of BALF to assess potential allergens. This approach has several advantages because serum is far easier to collect, store, and prepare for analysis in comparison to BALF. Collection also is less stressful for the horse. Moreover, it holds further potential in the use of diagnostic microarrays because serum is far more stable to ship for analysis.52

Repeatability is an essential factor in the development of new diagnostic tests. Therefore, the effects of printing lot and mouse anti-horse IgE 3H10 sources were assessed. These results confirmed reproducibility among printing lots (average $R^2 = 0.97$). The original mouse anti-horse IgE 3H10 used was that from a previous study.40

The commercial availability of antibodies is essential in diagnostic tests; therefore, the 3H10 clone40 was compared with the commercially available BioRad 3H10 (product # MCA5982GA), confirming reproducibility with the commercially available clone ($R^2 = 0.90$).

Specificity is an important aspect of protein microarrays, which was confirmed by a protein inhibition assay. In this assay, some cross-reactivity was seen, predominantly with grass pollens, as well as with Aspergillus and Penicillium. A previous study reported that pollens from grasses (Poaceae) often had high immunological cross-reactivity, potentially indicating common antigenic/allergenic component(s).53 Furthermore, cross-reactivity was identified between the genus Penicillium and Aspergillus, which is expected because taxonomically, the genera Penicillium and Aspergillus have many similarities, because both produce and contain galactomannans with similar galactofuranosyl and immunogenic side chains. Cross-reactivity in the fungi group was seen only with whole protein extracts, emphasizing the importance of including pure proteins. Analysis of human sera in a variety of assays has indicated that A fumigatus contains determinants in common with Cladosporium, Candida, Alternaria, Trichophyton, and Epidermophyton,54 but cross-reactivity was not identified here.

Several allergens of interest identified in our study were consistent with those previously identified as SEA-associated by ELISA, Western blot, and RAST methods (A fumigatus, A alternata, E amstelodami, and G candidum). Ours was the largest panel of proteins tested with a controlled SEA group to date and thus identified new and relevant allergens. Several SEA-associated allergens identified in our study previously have been associated with allergic asthma in humans (eg, Dermatophagoides farina, B germanica, Aspergillus restrictus, Dermatophagoides pteronyssinus). The novel SEA-associated allergens identified in our study strongly implicate fungi and mite as the main reactants, as well as identifying previously unrecognized reaction with pollens. This observation confirms the future potential of specific IgE as a biomarker for the serological diagnosis of SEA.

Our results have established a reliable protein microarray for large-scale IgE profiling of environmental proteins of horses, confirming identified SEA-associated allergens and elucidating a range of previously unidentified allergens. The technique is sufficiently sensitive and specific to differentiate between sensitized allergens in SEA and in control horses. Furthermore, the developed serological assay enables accurate identification of an individual horse’s sensitization profile. This information provides a reliable, fast, and repeatable method for screening a wide variety of potential allergens found in the stable environment in a miniaturized and affordable format, while offering a platform to support management and treatment of this debilitating respiratory disorder in horses.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approval by the Royal Agricultural University’s Animal Welfare Committee.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Samuel White https://orcid.org/0000-0002-3675-7545
Laurent Couëtil https://orcid.org/0000-0002-1516-6666

REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Dr. Couetil chaired the organizing committee for the Dorothy R. Havemeyer Foundation Workshop: Equine Asthma; Current Understanding and Future Directions that was help in Custer, SD in May 2019. The workshop was supported in part by PVM.
Havemeyer Foundation Workshop

EQUINE ASTHMA: CURRENT UNDERSTANDING AND FUTURE DIRECTIONS

MAY 22-25, 2019

CUSTER, SOUTH DAKOTA, USA

Cover Art Reproduction Provided by Artist Kathy Sigel
SPECIAL THANKS TO OUR GENEROUS SPONSORS
<table>
<thead>
<tr>
<th>CONTENTS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Listed by presenting author</td>
<td></td>
</tr>
<tr>
<td>Bienzle, Dorothee</td>
<td>61</td>
</tr>
<tr>
<td>Bond, Stephanie</td>
<td>49</td>
</tr>
<tr>
<td>Bullone, Michela</td>
<td>39</td>
</tr>
<tr>
<td>Cardwell, Jackie</td>
<td>42</td>
</tr>
<tr>
<td>Christmann, Undine</td>
<td>27</td>
</tr>
<tr>
<td>Couetil, Laurent</td>
<td>36</td>
</tr>
<tr>
<td>du Preez, Surita</td>
<td>28</td>
</tr>
<tr>
<td>Franklin, Sam</td>
<td>29</td>
</tr>
<tr>
<td>Frodeilla, Christa</td>
<td>69</td>
</tr>
<tr>
<td>Gerber, Vinzenz</td>
<td>64 &amp; 70</td>
</tr>
<tr>
<td>Hansen, Sanni</td>
<td>30</td>
</tr>
<tr>
<td>Ivester, Kathleen</td>
<td>58</td>
</tr>
<tr>
<td>Lavoie, Jean-Pierre</td>
<td>15 &amp; 67</td>
</tr>
<tr>
<td>Leclere, Mathilde</td>
<td>72</td>
</tr>
<tr>
<td>Lee, Gary</td>
<td>74</td>
</tr>
<tr>
<td>Leguillette, Renaud</td>
<td>52</td>
</tr>
<tr>
<td>Mainguy-Seers, Sophie</td>
<td>48</td>
</tr>
<tr>
<td>Martin, James</td>
<td>14</td>
</tr>
<tr>
<td>Mazan, Melissa</td>
<td>19</td>
</tr>
<tr>
<td>Morán, Gabriel</td>
<td>60</td>
</tr>
<tr>
<td>Niedźwiedź, Artur</td>
<td>80</td>
</tr>
<tr>
<td>Olave, Carla</td>
<td>31</td>
</tr>
<tr>
<td>Pusterla, Nicola</td>
<td>54</td>
</tr>
<tr>
<td>Rahmel, Daniela</td>
<td>47</td>
</tr>
<tr>
<td>Richard, Eric</td>
<td>83</td>
</tr>
<tr>
<td>Secombe, Cristy</td>
<td>32</td>
</tr>
<tr>
<td>Sheats, Katie</td>
<td>73</td>
</tr>
<tr>
<td>Swiderski, Cyprianna</td>
<td>17</td>
</tr>
<tr>
<td>Uberti, Ben</td>
<td>71</td>
</tr>
<tr>
<td>Westermann, Cornélie</td>
<td>46</td>
</tr>
</tbody>
</table>
The effort to clarify the phenotype and terminology used to characterize horses with chronic inflammatory airway disease started in 2000 with a workshop in East Lansing, Michigan. Several workshops were subsequently held with similar goals in mind with the latest hosted in Cabourg, France in 2014. In the last couple of years, the terminology has further evolved with the term Equine Asthma now being recommended to describe horses with chronic respiratory signs ranging in severity from severe to mild that were previously referred to recurrent airway obstruction and inflammatory airway disease, respectively.

The goal of the 2019 Equine Asthma Workshop is to attract researchers and clinicians from different disciplines and countries who are actively investigating airway inflammation. We will discuss the latest contributions to the understanding of asthma in horses and other species. The workshop format will facilitate productive discussions that will inform potential future revisions of the 2016 ACVIM consensus statement and provide future research directions.

We are extremely grateful to Gene Pranzo, President of the Havemeyer Foundation, for his support of this workshop on airway disease in horses and his continuous dedication and leadership of the foundation aimed at improving the health and welfare of horses. We are also thankful for the additional sponsorship provided by Boehringer Ingelheim Animal Health, Haygain, Nortev, Trudell Medical International, and Zoetis. Their contributions allowed us to provide travel grants to graduate students and organize high quality activities for the workshop participants.

Havemeyer Workshop Organizers:

Laurent Couetil, Purdue University, USA
Jackie Cardwell, Royal Veterinary College, UK
Renaud Leguillette, University of Calgary, Canada
Melissa Mazan, Tufts University, USA
Eric Richard, LABÉO, France
PROGRAM

Equine Asthma: Current understanding and future directions
Custer State Park, Custer, South Dakota, USA – May 22-25, 2019

Wednesday, May 22\textsuperscript{nd}
- 6:00 – 9:00 PM Welcome reception, Blue Bell Lodge, Custer State Park

Thursday, May 23\textsuperscript{rd}: Equine vs. human asthma: Definitions and diagnosis

SESSION I
(Chairperson: L. Couetil)

- 8:00 – 8:10 AM: Introductory remarks
  Gene Pranzo & Laurent Couetil

- 8:10 – 8:40 AM: Clinical and molecular phenotypes of human asthma
  Jim Martin

- 8:40 – 9:10 AM: Phenotypes and endotypes of equine asthma: is the 2016 ACVIM consensus statement definition of equine asthma still valid?
  Jean-Pierre Lavoie

- 9:10 – 9:30 AM: Summer-pasture RAO phenotype and triggers
  Cyprianna Swiderski

- 9:30 – 9:50 AM: Recommended minimum database for diagnosis of equine asthma by equine practitioners in the field vs. criteria used for research?
  Melissa Mazan

- 9:50-10:00 AM: Break

- 10:00 – 11:15 AM: Research abstracts (n = 6; 12 min/abstract)
  - Effect of stabling on lipid markers in surfactant and plasma from young healthy horses – Undine Christmann
  - Exhaled breath condensate hydrogen peroxide, pH and leukotriene-B4 are associated with lower airway inflammation and airway cytology – Surita du Preez
  - Comparison of left and right bronchoalveolar lavage cytology – Samantha Franklin
Correlation between allergen specific IgE test, cytokines and cytology in horse with Mild Equine Asthma – Sanni Hansen

Effect of forage type on respirable dust exposure and airway cytology in thoroughbred racehorses – Carla Olave

Bronchoconstriction and bronchodilation in horses can be verified using electrical impedance tomography – Cristy Secombe

11:15 – noon: Round table discussion
12:00 – 1:00 PM: Lunch

SESSION II
(Chairperson: J. Cardwell)

1:00 – 1:20 PM: Health effects of equine asthma
Laurent Couetil

1:20 – 1:40 PM: Tissue remodeling in EA and functional consequences
Michela Bullone

1:40 – 2:00 PM: Equine asthma from the UK practitioner’s perspective: Perception and practices
Jackie Cardwell

2:00 – 2:50 PM: Research abstracts (n = 4; 12 min/abstract)
Reducing exposure to dust in equine asthma: the effect of water nebulization on air quality in horse stables – Cornélie Westermann

The third generation steroid ciclesonide shows short plasma half-life and fast elimination after single and repeated inhalation in horses – Daniela Rahmel

The combination of azithromycin and fluticasone decreases airway neutrophilia in severe equine asthma – Sophie Mainguy-Seers

Effects of nebulized dexamethasone on the respiratory microbiota and mycobiota in horses with mild equine asthma – Stephanie Bond

3:30 PM Social activities
- 3:30 pm Departure for the State Game Lodge Resort
- 4-6 pm Buffalo jeep tour
- 6:00 pm Departure for Custer
- 6:30 – 9 pm Reception at Kleemann’s house in downtown Custer
Friday, May 24th: Etiology & pathophysiology of equine asthma

SESSION III

(Chairperson: E. Richard)

- **8:00 – 8:20 AM:** Role of microbiome in equine asthma
  
  *Renaud Leguillette*

- **8:20 – 8:40 AM:** Role of viruses in equine asthma
  
  *Nicolas Pusterla*

- **8:40 – 9:00 AM:** Role of non-infectious exposures in equine asthma
  
  *Katy Ivester*

- **9:00 – 9:20 AM:** Is the neutrophil a key player in equine asthma?
  
  *Gabriel Moran*

- **9:20 – 9:40 AM:** Insights into equine asthma pathophysiology from transcriptomics
  
  *Dorothee Bienzle*

- **9:40 – 10:00 AM:** Genetic risk factors of equine asthma
  
  *Vinzenz Gerber*

- **10:00 – 10:15 AM:** Break

- **10:15 – 10:35 AM:** The Equine Tissue Bank: an update
  
  *Jean-Pierre Lavoie*

- **10:35 – 11:50 AM:** Research abstracts (n = 6; 12 min/abstract)
  
  - The lung transcriptome of horses with pasture-associated severe equine asthma identifies a Th17-High Th2-Low phenotype – *Christa Frodella*
  
  - An Integrated Analysis of Lung Tissue microRNA and mRNA Expression Profiles from Horses with Severe Equine Asthma – *Vinzenz Gerber*
  
  - Metabolomics of bronchoalveolar lavage fluid samples in horses with naturally-occuring asthma and experimentally-induced airway inflammation – *Ben Uberti*
  
  - Beta-glucan receptor dectin-1 in equine asthma – *Mathilde Leclère*
  
  - Evaluating Myristoylated Alanine Rich C Kinase Substrate (MARCKS) family proteins as potential therapeutic targets in equine asthma syndrome – *Katie Sheats*
  
  - Investigating the Salivary Scavenger and Agglutinin protein in Equine Asthma – *Gary Lee*
11:50 – 12:30 PM: Round table discussion

12:30 – 1:30 PM: Lunch

SESSION IV
(Chairperson: R. Leguillette)

1:30 – 3:30 PM: ROTATING ROUND TABLE DISCUSSIONS

- 4 tables @ 30 min/rotation

4:00 PM: Social activities
- 4-6:30 PM: visit to Mount Rushmore
- 7-9:30 PM: Banquet at Sylvan Lake

Saturday, May 25th: Reaching a consensus on Equine Asthma

SESSION V
(Chairperson: M. Mazan)

08:00 – 08:20 AM: Are pertinent biomarkers of equine asthma already available to practitioners/researchers?
   
   Artur Niedźwiedź

08:20 – 08:40 AM: How do we standardize immunologic laboratory testing
   
   Eric Richard

08:40 – 10:00 AM: Summary of Table discussion topics (20 min/topic)

10:00 – 10:15 AM: Break

10:15 – 12:00 PM: Round table discussion – reach a consensus regarding table topics #1-4 & Future direction for equine asthma research

Noon: End of Meeting
Thursday, May 23rd: Equine vs. human asthma: Definitions and diagnosis

SESSION I

(Chairperson: L. Couetil)
Clinical and molecular phenotypes of asthma

James G Martin, MD, DSc

Meakins Christie Laboratories, McGill University Health Center Research Institute

Asthma phenotypes have been recognized for many decades but were collapsed into a unified hypothesis of asthma as an allergic disease in 1989. It has taken more than 20 years to consider asthma again in its various forms with an emerging emphasis on endotypes, an intrinsically more interesting approach to understanding asthma pathobiology. Cluster analyses have been performed on asthma cohorts and have revealed groups with different ages of onset, lung function, concordance or lack thereof between measures of airway inflammation by sputum analysis and symptoms. These studies have confirmed the existence of apparently different phenotypes but shed little light on mechanism. The application of the analysis of gene expression on airway epithelial cells and sputum cells from well-characterized groups of asthmatics has led to the appreciation of asthma associated with T helper 2 cytokines and non-T2 asthma. The former is the more allergic subset with higher IgE, peripheral and sputum eosinophilia. Non-T2 asthma has fewer of these features and is less responsive to inhaled corticosteroids. T cells that express interleukin-17 have been linked to severe neutrophilic asthma. These so-called Th17 cells have been shown in animal models to be associated with steroid-unresponsiveness. The Th1 cytokine interferon-γ likewise has been found to be expressed in the airways of severe asthmatics.

In recent years there has emerged another lymphoid cell that participates in host responses to mucosal injury. These innate lymphoid cells are lineage negative, lacking the usual lymphocyte surface markers. They express similar panels of cytokines to the T helper subsets and are labelled innate lymphoid cell (ILC) 1, 2 and 3. They are rapidly activated by epithelial signals such as thymic stromal lymphopoietin (TSLP), interleukins 25 and 33, molecules termed alarmins. The secretion of IL-5, IL-13 may lead to a pattern of inflammation previously interpreted as Th2. These cells are less steroid sensitive. Additionally they prime cells such as dendritic cells and therefore may have a role in adaptive immunity as well as innate immune responses. The synthesis of amphiregulin, an epidermal growth factor receptor ligand by ILC2s is postulated to promote mucosal integrity. This growth factor is also recognized to be synthesized by Th2 cells. One could anticipate viral infection of epithelial cells or damage by irritants giving rise to inflammation mediated by ILCs. Their roles are not fully explored.

Transcriptomic analysis of sputum has revealed three patterns of inflammation and gene signatures consistent with both Th2 and ILC2 driven inflammation and oxidative stress. Application of this knowledge to the clinic will follow.
Phenotypes and endotypes of equine asthma: is the 2016 ACVIM consensus statement definition of equine asthma still valid?

Jean-Pierre Lavoie, DMV, DACVIM

Faculty of Veterinary Medicine, University of Montreal, Quebec, Canada

The purpose of the 2007 ACVIM Consensus Statement on Inflammatory Airway Disease (IAD) was to review the current knowledge and opinions concerning this condition and to help practitioners differentiate IAD from heaves (or recurrent airway obstruction; RAO). The Consensus was revised in 2016 and discussed the use of Equine asthma to describe these conditions. It recognized that asthmatic horses of all severities have common clinical presentations (such as chronic cough, excess mucus, poor performance) but also a wide heterogeneity in terms of triggering factors, severity, and pathologic characteristics.

A phenotype is the observable physical properties of an organism, including measurable laboratory findings, which is the results of the expression of the genes in response to the environment. Identifying distinct phenotypes are of interest if they facilitate the diagnosis, the prognosis or allow to the implementation of targeted therapy. While currently loosely defined, the equine asthma phenotypes discussed in the 2016 Consensus statements were based on clinical presentation (Severe versus Mild/Moderate), triggering factors (Barn/hay or Pasture), endoscopy findings (mucus) and bronchoalveolar cytology.

On a clinical standpoint, further dividing equine asthma as distinct "Mild" and "Moderate" phenotypes, may help recognize that equine asthma is an underdiagnosed cause of exercise intolerance in high performance horses. Horses with a cough or increased respiratory rate at rest or following exercise will commonly undergo further diagnostics to confirm asthma, or anti-asthma treatments implemented. However, this is generally not the case when no clinical signs suggesting of an airway disease is present. The term "Mild equine asthma" could be used to describe the condition affecting these horses while "Moderate equine asthma" would be used when clinical signs of airway disease (such as cough) are present, but without the periods of labored breathing at rest seen in "Severe equine asthma". In addition to the inflammatory airway cell phenotypes (neutrophils, mast cells, eosinophils...) recognized in the 2007 and 2016 consensus statements, future phenotypes may include early onset or late onset, or specific remodeling features affecting the airways.

The development of new portable and sensitive devices for measuring the lung function of horses, or the discovery of blood biomarkers for equine asthma may help not only to facilitate the diagnosis of mild and moderate forms of equine asthma in clinical practice, but also to possibly identify new phenotypes for these conditions.
The term "endotype" is used to describe a subtype of disease defined by a molecular mechanism, genetic variation or by treatment response. To date, different inflammatory pathways have been proposed as contributing to equine asthma, which may eventually lead to novel therapies. The discrepancies between results of the different studies may be an indication of different endotypes in equine asthma, although future studies on large cohorts of horses from multiple sites would be required before specific endotypes be recognized. Multicenter tissue banking could facilitate these studies.

In summary, the 2016 ACVIM Consensus Statement recognized the currently known distinctive features of equine asthma. Further defining "Mild" and "Moderate" equine asthma based on the presence or absence of easily identified clinical signs may promote the investigation of the subclinical (Mild) phenotype. The identification of novel phenotypes and endotypes may lead to "Precision medicine" where treatments most likely to help equine patients would be selected.
Summer Pasture Associated Severe Equine Asthma: Phenotype and Triggers

Cyprianna Swiderski, DVM, PhD, DACVIM
College of Veterinary Medicine, Mississippi State University, MS, USA

Pasture-associated severe equine asthma (equine pasture asthma, EPA) is characterized by episodes of reversible airway obstruction in horses grazing pasture during the summer in hot humid climates (1-3). Affected horses demonstrate chronic neutrophilic airway inflammation, persistent airway hyper-responsiveness extending throughout the season of remission, and airways remodeling (1,4,5). The authors’ experience is restricted to EPA as first described in horses residing in Louisiana, and diagnosed in states with subtropical climates (Mississippi, Alabama, and Florida) (6). Veterinarians in regions of adjoining states and distant states (Oregon) describe similar signs in horses grazing pastures during hot humid conditions. EPA is described in the United Kingdom where it differed in its association with hot dry weather or exposure to dust from harvest/burning of crops (7,8). EPA demonstrates adult onset (X= 12 +/- 6 years; range 1-29 years) without sex predilection (3). Asthma exacerbations generally begin in summer (July), persisting until temperature and humidity decrease (October/November) (9). Fewer horses experience asthma in the spring (9). A history of prior seasonal cough and/or exercise intolerance may be identified. Improvement within hours to days of isolation from pasture particulates in a stall environment is a key diagnostic feature of EPA in the southeastern USA (6); some severe cases necessitate isolation in a climate-controlled environment. In the author’s experience, without adequate environmental management, disease severity is progressive and responsiveness to parenteral corticosteroids decreases.

Though agent(s) that elicit EPA exacerbation are not identified, disease association with pasture housing coupled with improvement following adequate isolation from pasture implicate seasonal pasture-associated particulates (2,6). Costa reported increases in grass but not tree pollens were significantly associated with EPA exacerbation using a pollen station ~90 miles from affected horses (9). Subtropical grasses differ substantially from grasses in temperate and continental climates (10). Specifically, in subtropical regions the Chloridoid grass Cynodon dactylon (Bermuda grass) and the Panicoid grasses Paspalum notatum (Bahia grass) and Sorghum halepense (Johnson grass) predominate, not the Pooid grasses (orchard, timothy, fescue, rye, bluegrass, bent, and sweet vernal) characteristic of temperate regions. Pollen from subtropical grass subfamilies is important to rhinitis and human asthma in subtropical zones of Australia, Asia, India, Africa, and America (11-20).

Pollination seasons of Bahia and Bermuda grass (spring through September/October) align to the season of EPA exacerbation (21,22). Pollen season for Johnson grass is temperature dependent, flowering from May to July, with higher temperatures moving flowering later into Autumn (23). Bermuda grass pollen is one of the most allergenic of grass pollens, yet in humans from subtropical regions, triggering of asthma and allergic rhinitis in late summer is considered a strong implicating factor for Bahia pollen as the sensitizing allergen (11,24). Anecdotal associations between EPA in the UK and exposure to oil seed rape are noteworthy because antigens of this pollen cross react with Bermuda grass pollen and components of oil
seed rape induce airway hyper-responsiveness and occupational asthma in humans (7,25-28).
Grass pollen is associated with TH2 responses and IgE-mediated hyper-sensitivity reactions. However, chronic exposure to the TH2 sensitizing antigen OVA, addition of LPS to OVA sensitization, exposure to complex antigen combinations, as well as re-exposure of tolerized individuals to antigen each generate TH17 responses accompanied by neutrophilic airway inflammation that typifies pasture asthma (29-31). While intact pollen is too large to reach the respirable zone of humans in order to elicit asthma, moist conditions associated with EPA exacerbations can shatter pollen and disseminate respirable particles (32). Well known among these particles are the group 13 family of pollen allergens which are polygalacturonases that are also expressed by fungi and have the ability to induce allergic sensitization and precipitate asthma attacks by inhalation (33,34).

Supporting relevance of fungi in triggering EPA exacerbations is the near identical clinical picture presented by horses with EPA and barn dust-associated severe asthma, wherein a role for fungal triggering is substantiated in the latter (35,36). Chronic neutrophilic airway inflammation characterizing both forms of severe equine asthma also aligns to TH17-mediated neutrophilic inflammation in fungal asthma models (37). Costa identified fungal spores of the genus Nigrospora, and Curvularia, as well as basidiospores, as temporally associated with exacerbations of pasture asthma (9). Associations to Botrytis, Drechslera/Helminthosporium, and Cercospora were less significant. Species including Alternaria, Cladosporium, Epicoccum, Erysiphe/Oidium, Fusarium, Periconia, Peronospora, Pithomyces, Polythrinium, Stemphylium, Torula, as well as rusts, smuts and ascospores were not temporally associated with EPA exacerbations. These findings agree with the correlations between EPA exacerbation and high dew point temperature (9). Specifically, Cladosporium, Alternaria, Epicoccum, and Drechslera spp increase during warm, dry conditions, whereas precipitation is required to release many ascospores (38). In contrast, basidiospores are more directly affected by relative humidity, peaking during the early morning congruent with associations between EPA exacerbations and increased dew point temperature (9,39,40). These characteristics influence the major sensitizing fungi for asthma in different locales, and could influence associations of pasture asthma in the UK with hot dry conditions rather than hot humid conditions precipitating pasture asthma in the southeastern US (7,41).

More than 100 species of fungi exist in biotropic relationships with Bermuda, Bahia, and Johnson grasses (42). Genera of note for their association with human asthma includie Curvularia, Helminthosporium, Alternaria, Puccinia, Epicoccum, and Fusarium (43). Complicating recognition of the role of fungi in triggering EPA are the more than 5.1 million unique fungal species, with comparatively few that can be identified morphologically or even grown in a laboratory (44,45). Basidiospores alone originate from roughly 30,000 species of fungi that cannot be easily distinguished (46). Fungi also have sexual and asexual stages with the potential for differing antigenic complexity in each stage. Finally, identifying fungi by their genomic signatures is complicated by a lack of high quality genome sequences for many fungal species.

EPA, a chronic and progressive form of severe equine asthma of undetermined cause, is most effectively managed by segregation from pasture during warm seasons. Horses are extensively ridden and maintained on pasture during this time, presenting a conundrum that is ultimately fatal for most horses and highlighting a critical need to define the etiologic agents responsible for EPA.
Recommended minimum database for diagnosis of equine asthma by equine practitioners in the field vs. criteria used for research

Melissa R. Mazan, DVM, DACVIM

Cummings School of Veterinary Medicine, Tufts University, Grafton, MA, USA

Both veterinary practitioners and researchers often muse about the armamentarium available to physicians – if only we had the chest CT, the advanced lung function testing, the biomarkers – then we would be able to have a better diagnosis. A quick search of the literature, however, shows us that our counterparts face many of the same diagnostic dilemmas that we do, albeit often with higher bills! While pulmonologists have drawn up multiple guidelines to help in the diagnosis of asthma in humans with its multiple phenotypes and endotypes, physician-diagnosed asthma criteria often fail to be consistent with the official guidelines rendering the results of large epidemiologic studies or clinical trials fraught with the perils of resting findings on nebulous datasets. Various forms of spirometry or simple pulmonary function testing is readily available in human medicine, few non-pulmonologists avail themselves of objective data, and instead rest on reported symptoms such as difficulty breathing on exertion, cough, or positive response to bronchodilation (Koumbourlis 2019, von Bulow 2017). Moreover, the heterogeneity in published algorithms for diagnosis of asthma – more than 66 in the literature at last count – make even an algorithm-based diagnosis unsure. (Al Salleck Eur Resp J 2017;49) Thus, the conclusion that symptom-based diagnosis is associated with a significant risk of over-diagnosis has been reached for asthma in humans. (Von Bulow 2017) The current push in human medicine to refine both the phenotypes and endotypes for multiple different subtypes of asthmas aims to elucidate the underlying causes and thus treatments that may be very different. We are still searching for the criteria that will help us in this in equine medicine. If there are indeed mechanistically different groups of horses within the categories of mild, moderate, and severe equine asthma that are associated with genetic differences or cellular or molecular biomarkers, then perhaps we will gain better understanding of treatment successes and failures and will be able more logically to choose clinical therapies and predict responses. The aim of this paper is to suggest a minimum database that allow us to diagnose equine asthma and help us to get a little closer to creating subgroups of both phenotype and endotype.

The difficult case for the clinician and the researcher alike is not the horse with heaves – severe asthma – because the history and clinical exam alone can often suffice to diagnose, there is a visible relief in respiratory embarrassment with administration of bronchodilator (although it can take some time in horses with diaphragmatic exhaustion. (Hoffman 2007, Mazan 2004) and there is a recognizable trigger. The difficult horse is the one with moderate/severe asthma in remission and the horse with mild asthma/IAD. As was recently pointed out, the biggest difference that we note in the clinical diagnosis of horses with mild asthma (IAD) v. severe asthma (RAO) is the presence of an increased respiratory effort at rest, which is due to the underlying pathophysiology of bronchoconstriction, increased mucus, and bronchiolar inflammation. The need, then, is to detect the horse that is NOT this severe (Bond 2018). The Global Initiative for Asthma in humans (GINA, https://ginasthma.org) calls for spirometry as a
minimum database – but is this reasonable in horses? Interestingly, although we in equine medicine rely, sometimes almost exclusively, on analysis of respiratory secretions, the GINA initiative relies on 1. history, and 2. evidence of variable expiratory airflow limitation, with the caveat that if no airflow limitations exist on initial examination, bronchoprovocation may be necessary.

In our veterinary armamentarium, we have at hand history, clinical signs, lung function testing, radiographs, endoscopy, analysis of airway secretions, blood biomarkers and clinical pathology which can be used in a minimum database in order to classify horses into clinically useful categories that have a pathophysiologic basis that can simultaneously allow us to diagnose, treat, and translate clinical cases into field research.

**History:** A tentative diagnosis of equine asthma in its most severe form, otherwise known as heaves, can often be made on history alone, with the key component being the recognition of episodes of reversible respiratory embarrassment precipitated by exposure to specific triggers – namely, moldy hay in the northeast, and pasture allergens and particulates in the south. History in subclinical or mild cases is seldom of such definitive use; this does not mean that it is unimportant. Such questions as parentage (Marti 1991), type of feed and how it is fed (Ivester 2018 and earlier), and heat and pollen counts at the time of diagnosis (Bullone 2016) may be important risk factors for equine asthma. While it has been proposed that coughing and poor performance may serve to define a phenotype of moderate v. mild equine asthma (Bullone 2017), these signs are not sufficiently sensitive (Wasko 2011, Cardwell 2014) and would misclassify a subset of horses. alert the clinician that IAD is likely, but the absence of these signs does not rule out disease. The connections between EA and viral or bacterial disease are not linear, but it is becoming increasingly clear that the connection exists (Fortier 2013, Houtsma 2015, Cardwell 2014), thus a thorough history should include probing for past infectious respiratory disease. One of the best described questionnaire analysis tools for classification of horses based on history is the HOARSI index (Jost 2007), developed as a means of distinguishing among normal v IAD or RAO phenotypes. However, clinical signs and indices are insufficiently sensitive to distinguish horses with IAD (mild EA) from normal horses or horses with RAO (severe equine asthma) in remission. (Laumen 2010).

Proposed minimum database for both practitioners in the field and for research: A common history tool should be developed that addresses the main concerns of parentage if known, current and lifetime exposures to particulates and allergens including feeds and feeding practices, barn environment, vaccinations, travel history, and recent illnesses.

**Clinical scoring/clinical presentation:** Multiple scoring systems have been shown to be useful for distinguishing the horse with sEA in remission or healthy horses from horses with RAO in exacerbation, but, similar to questionnaire indices, these scoring systems do not help in the more difficult problem of distinguishing horses with mild EA from healthy or RAO in remission. (Tilley 2012) Indeed, 19 years ago, Robinson et al (EVJ 2000) found that even in horses with historical severe EA, clinical score failed to reflect low-grade airway obstruction, and suggested that without easily used, field-accessible testing equipment, lower airway disease would go underdiagnosed. Recently, the adapted 23-point scoring system (Lavoie 2019, Hoffman 1991) has been shown to be the most useful in discriminating mild from severe cases, but it is unlikely
to distinguish normal from subclinical disease. Thus, while clinical scoring is essential to a good examination and careful research, and can potentially be useful in measuring response to treatment in the individual, it is insufficient in making the phenotypic distinction between mildly affected horses and healthy horses.

Proposed minimum database for both practitioners in the field and for research: The 23-point modified clinical score appears to best stratify horses with obstruction ranging from mild to severe. An app suitable for smartphone use would enhance the adoption of a common scoring tool.

Lung function testing: In human asthma, the gold standard is the detection of variability in pulmonary function using spirometry or other methods of lung function testing. (GINA) Although lung function testing has been considered for many years to be available only to a few specialized centers (Couetil 2016); the recent development of portable lung function testing modalities such as OpenPleth (Hoffman 2007) has resulted in more extensive use. While the classic esophageal balloon/pneumotachometer method is effective in demonstrating increased maximal pleural pressures and allows for calculation of pulmonary resistance and elastance as well as dynamic compliance in sEA, it is not sufficient for demonstrating abnormal function in mildly affected horses in which baseline lung function is rarely abnormal (Bedenice 2008) and histamine or other bronchoprovocation or bronchodilation must be used in order in order to detect low-grade obstruction. Unfortunately, in some studies, even histamine bronchoprovocation has not been sufficient to distinguish between normal horses and horses with mild asthma, (Tilley 2012) and a lack of concordance between HBP and BAL cytology has been noted in several studies. (Wichtel 2016, Cullimore 2018). While lung function testing and HBP have shown moderate to strong correlations with BAL cytology in some studies (Couetil 2001, Bedenice 2008, Hoffman 1998, Houtsma 2015, Hare 1999, Richard 2009, others have not (Wichtel 2016, Cullimore 2018). Methods of performing HBP are equally important: studies in human asthmatics have shown that it is the total dose of histamine that is most important rather than the duration of exposure. (Cockcroft 2005) A more precise method of dosing may be important to establish. In human athletes, indirect stimuli, such as cold air, hypertonic solutions such as mannitol, exercise, and AMP are all considered more accurate and useful in predicting asthma than are direct stimuli such as methylcholine or histamine, this is an area that requires exploration in equine pulmonology. (Sue-Chu 2010) While hay challenge is useful for research in sEA, it is inappropriate in a clinical case, especially in a horse that is expected to do athletic work. (Couetil 2016) In moderate to severe EA, variability in airflow should be demonstrated not through bronchoprovocation but through bronchodilation using either systemic (Buscopan) or inhaled (albuterol, ipratropium bromide) to assess reversibility; it is possible that a 24-hour period of bronchodilation is necessary for maximum effect in horses with diaphragmatic fatigue. (Mazan 2004).

Proposed minimum database for both practitioners in the field and for research: In research, lung function should be assessed and airflow variability/changes in airway caliber should be assessed with either bronchoprovocation or bronchodilation. More research is necessary to determine if field assessment of lung function after bronchoprovocation or bronchodilation is sufficient to determine change with the 23-point scoring system. It is essential that a robust, easily used system for testing lung function in the field be developed.

Airway secretions – bronchoalveolar lavage: Unlike in human pulmonology, examination of airway secretions is a primary method of diagnosis in equine asthma, be it mild, moderate or
severe. Although a standard volume of between 250-500 mls of saline using a 2-m long endoscope or 3-m BAL tube, (Couetil 2016) this practice is not always followed, and it is important to note the amount of fluid used in order to judge the cytology appropriately, remembering that the amount of fluid infused will affect the cell percentages. The relationship between BAL cytology and performance is still not clear. Certainly, poor performance has been associated with what have been determined to be abnormal cell types or percentages (Couetil 2016). There has been much discussion as to what is normal on BAL cytology; it likely depends on a combination of technique, environment and population. Even the ‘stringent’ definition proposed by Couetil et al (2016) of <5% PMNs, 2% mast, 1% eos, would be considered elevated in some high-performance populations (Dauvillier 2019, Hermange 2019). Although an earlier study found no evidence of a clear phenotype (Nolen-Walston 2013) in mast cell v. neutrophilic inflammation with respect to pulmonary gas exchange during exercise, recently, an increase in mast cells by 1% was shown to decrease speed figures by 2.9 points, and an increase in PMNs by 1% decreased speed figures by 1.4 points, although well-performing horses had an upper limit of 1.6% mast cells and 6.2% PMNs. (Ivester 2018) The way that cells are counted in BAL cytologies is also important, especially for rare cells. In our laboratory we count a minimum of 500 cells at 400x for common cells such as macrophages and lymphocytes or neutrophils in mild EA, whereas for rare cells such as mast cells we count 1000 cells. Other techniques, such as using a 5-field differential for mast cells, are only useful if the cell density is high. (Fernandez 2013)

The conundrum of whether to assess airway fluid from both lungs rather than blind sampling, or to pool samples, has also occupied attention from researchers. One group found that, depending on whether the ‘loose’ or ‘stringent’ categorization was used, 8-37% of horses would have been categorized as control v. IAD if only one lung were used. (Depecker 2014) As it is the rare practitioner who has a bronchoscope in the field, it is unlikely that even pooled samples (Hermange 2019), which may be a better representation of overall lung inflammation, will be taken other than in referral centers or practices. The problem is most important for rare cells. More attention will need to be paid in future to morphology and perhaps typing of cells. The existence of neutrophil extracellular traps (NETosis) in horses with sEA (Cote 2014, Vargas 2017) present an additional method to determine response to treatment, and recently the presence of degenerate neutrophils has been shown to raise suspicion for bacterial infection Jocelyn EVJ 2018. The question of macrophage morphology (du Preez 2019, McKane 1993) as an indicator of inflammation is also an area that will profit from further investigation. Recently, as well, the paucigranulocytic phenotype has been described (Bullone 2017) in which horses with clear signs of sEA have low PMN percentages in the BAL. This is thought to be due to mucus plugging of small airways that essentially sequesters neutrophils. Although a recent publication showed a rather shocking 81% of high-performing European horses with IAD had fungal elements in the BAL (Dauvillier 2018), other catchment areas have fewer than 15% of horses with IAD with fungal elements in the BAL (Mazan, unpublished data). Clearly, these findings will vary with the population.

Proposed minimum database for both practitioners in the field and for research: For the BAL, at least 250 mls of saline should be used, and there is a preference for counting at least 500 cells to adequately represent rarer cells. For research purposes where rare cells are of interest (e.g. mast cells or eosinophils), sampling of both lungs appears preferable. Better categorization of cells through morphological descriptions including apparent neutrophil extracellular traps and notations of fungal or polarizing elements should be done. Characterization of mucus on
cytology (hypercellular, etc) may help to elucidate the paucigranulocytic phenotype. BAL in the field will usually be done blindly with a specialty tube.

**Airway secretions - tracheal wash:** The debate continues to swirl around the utility of tracheal wash v bronchoalveolar lavage, with Malikides (2003) finding a 37% disagreement in young racehorses, Derksen et al (1989) determining that there was no correlation between BAL and TA, and Holcombe (2006) finding no relationship between tracheal neutrophil counts and racing performance; thus, the tracheal aspirate has been considered inappropriate for diagnosis of mild EA. (Couetil 2016). Recently, however, Rossi et al (2018) looked at a comparison of TW and BAL in 145 horses, along with evidence of mucus and endoscopy and found that fewer than 18% would have been classified differently, eventually concluding that there IS no gold standard – except for mast cells, which are rare in the trachea, and thus demand that a BAL be performed. As in some areas, such as the authors, more than 50% of horses with IAD have predominantly mast cell inflammation (Mazan, unpublished data), this becomes an important problem.

*Proposed minimum database for both practitioners in the field and for research:* Tracheal wash may be most practical for some practitioners in the field and has the added benefit of allowing for bacterial culture. The inability to assess mast cells adequately continues to limit this modality. In research settings, both tracheal aspirate and BAL are preferable.

**Endoscopy:** Many clinical diagnoses are made on the basis of endoscopic visualization of mucus, with strong support from the finding that tracheal mucus quite nicely correlated with racing performance or lack thereof. (Holcombe 2006) The recent consensus statement considers that the demonstration through tracheobronchial endoscopy of mucus grade 2/5 in racehorses or 3/5 for sport/pleasure horses is sufficient to diagnose IAD and in support of this recommendation, Rossi (2018) found that visible mucus in the trachea is indeed likely to predict inflammation. There are varying degrees of certainty about mucus in the trachea predicting inflammation. (Kolbinger 2011, Richard 2010, Gerber 2003, Dauvillier 2018, Cardwell 2011). Nonetheless, other studies have shown that mucus is insufficient to parse out mild v unaffected cases. (Rettmer 2015). Endoscopy has also been shown to be useful in detecting an increase in upper airway abnormalities in horses with mild EA (IAD), with Courouce-Malblanc (2010) raising the chicken-and-egg question of the relationship between IAD and DDSp (2010), and more recently, Wysocka et al (2018) found that more horses with IAD had dynamic pharyngeal abnormalities. It may be that the answer will rest in whether any of these modalities can help to define a phenotype rather than simply further describing an already understood phenotype – in that if the presence or absence of a phenotypic feature ruled in or out a subset of disease. At this point, there is a lack of clarity with upper airway endoscopy other than for mucus.

*Proposed minimum database for both practitioners in the field and for research:* Upper airway endoscopy should be performed to rule out upper airway cause of obstruction as a primary cause of signs or that might confound lung function testing. Assessment of tracheal mucus should be performed.

**Bronchial biopsies/brushings:** Endobronchial biopsies offer an excellent method of sampling larger airways, although deeper layers cannot be accessed. The brass ring – being able to distinguish normal from remission or mild EA – remains elusive, however, as correlates were evident between histopathology and impulse oscillometry and showed a difference between
horses in remission at pasture and those that remained stabled and treated with glucocorticoids, but did not show any difference between remission and controls (Bullone 2014)

Proposed minimum database for both practitioners in the field and for research: At this time, brushings/biopsies are not considered part of a minimum database

Radiography/ultrasound: Imaging is considered an important ancillary diagnostic in humans, and Wisner (1993) showed a strong correlation between histopath for what was probably mild EA (IAD) and radiographs, but radiographs have not been shown to be sensitive or specific in horses with EA (Mazan 2005) and were thought not to be a least necessary part of a staging scheme for EA (Simoes 2019), and CT is currently not feasible in large animals. While endobronchial ultrasound shows promise for the elucidation of airway smooth muscle thickening in severe equine asthma (heaves) the ultimate goal of being able to detect low-grade disease in erstwhile healthy horses, or to distinguish normal from asthma in remission remains elusive. (Bullone 2015)

Proposed minimum database for both practitioners in the field and for research: at this time, imaging is not considered part of the minimum database.

Other testing/biomarkers: Unexpectedly, a subset of horses in one study was found to be shedding virus – but did not have any clinical signs of infectious disease. (Houtsma 2015) Serum amyloid A has been shown to be useful in ruling out infectious disease in comparing horses with EA versus those with infectious disease (Viner 2017), thus it is possible that this biomarker should be used as an exclusion criterion. Other biomarkers, such as serum cytokines or metabolites from condensed breath measurements may eventually assist in diagnosis and categorization into subtypes, but the variability in biomarkers at this time is too great to allow for useful diagnostics.

Proposed minimum database for both practitioners in the field and for research: At this time, it is highly recommended that researchers bank any serum, BAL fluid, biopsies, or cytology slides for future analysis. The tissues bank should be expanded and further accessed by researchers.
Thursday, May 23rd: Equine vs. human asthma: Definitions and diagnosis

SESSION I

(Chairperson: L. Couetil)

Research Abstracts
The purpose of this study was to evaluate the effect of stabling on lipid markers in surfactant and plasma from young healthy horses.

The objectives were to assess alterations in clinical and laboratory parameters and in lipidomic markers as a result of exposure to barn environment.

Methods: At baseline 20 young healthy horses were housed on pasture. Subsequently, 10 of the horses were moved to a barn and exposed to dusty hay and the other 10 horses remained on pasture. Horses were assessed at baseline and after 2 weeks and 4 weeks in their respective environment. The assessment included physical examination, airway endoscopy, venous blood and bronchoalveolar lavage fluid (BALF) collections. Complete blood cell counts and BALF cytologies were analyzed. Phospholipid content was determined in crude surfactant pellets (CSP) and lipidomic analysis was performed on plasma and CSP. Statistical analysis included ANOVA with Tukey-Kramer post-hoc test (statistical significance p<0.05).

Results: Clinical and basic laboratory parameters did not differ significantly between or within groups of horses. CSP phospholipid concentration did not differ significantly between groups of horses but decreased significantly from baseline to the 2 week sample in horses exposed to hay (p=0.0001) and from baseline to the 4 week sample in both groups of horses (p=0.02, p=0.0009). No significant differences were noted between or within groups for cyclic phosphatidic acid levels in plasma or CSP. Diacylglycerol levels did not differ significantly between groups of horses but decreased significantly from baseline to the 2 week sample in horses exposed to hay (p=0.04).

In conclusion, the environmental exposure used in this study did not induce airway inflammation and did not result in alterations in lipidomic markers in young, healthy horses. An effect of sample time on surfactant phospholipid content (both groups) and diacylglycerol (horses exposed to hay) was noted.

Declarations
- Ethical Animal Research: protocol and procedures used in this study were approved by the IACUC at the Gluck Equine Research Center, University of Kentucky.
- Source of funding: Internal funding from the Gluck Equine Research Center.
- Disclose any potential conflict of interest: none.
Exhaled breath condensate hydrogen peroxide, pH and leukotriene-B$_4$ are associated with lower airway inflammation and airway cytology


School of Animal and Veterinary Sciences, Graham Centre for Agricultural Innovation, School of Agricultural and Wine Sciences, Quantitative Consulting Unit, Charles Sturt University, Wagga Wagga, New South Wales, Australia; School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy, South Australia, Australia.

**Purpose of the study:** Exhaled breath condensate (EBC) analysis is a non-invasive method to assess the lower respiratory tract. In human subjects, EBC hydrogen peroxide (H$_2$O$_2$), pH and leukotriene B$_4$ (LTB$_4$) are useful for detection and monitoring of inflammatory lung diseases, including asthma. The purpose of the study was to determine if EBC could be a non-invasive alternative or adjunct method to evaluate lower airway health in horses.

**Objectives:** To determine associations between EBC biomarkers and cytological definitions of mild equine asthma (EA) whilst controlling for sampling and environmental variables.

**Methods:** Clinical, endoscopic and airway cytological findings from 47 horses were compared with EBC pH and concentrations of H$_2$O$_2$ and LTB$_4$ by univariate and multivariable analyses. Dichotomous (presence / absence of airway inflammation) and continuous outcome variables (differential cell counts in bronchoalveolar lavage fluid, BALF) were evaluated and potential effects of environmental and methodological factors were included. Non-normally distributed data were log-transformed prior to analysis. ROC analysis and cross-tabulation with BALF cytology as gold standard, were used to determine optimal cut-points and assess performance of EBC biomarkers, without consideration of confounding covariates.

**Results:** EBC pH and H$_2$O$_2$ concentrations were higher in horses with mild EA (0.11 units and 0.78 pmol/100L EB, P=0.04 and P=0.03, respectively). EBC pH and H$_2$O$_2$ concentrations were positively associated with % neutrophils in BALF, increasing by 0.23 units and 0.78 pmol/100L EB for each 1.72% increase, respectively (P=0.01 and P=0.03). Mast cell % in BALF was negatively associated with EBC pH (0.2 unit decrease for each 1.72% increase, P=0.04), and BALF eosinophil percentage was positively associated with EBC LTB$_4$ (0.52 pg/100L EB increase for each 1.72% increase, P=0.03). Some analytes were significantly influenced by ambient temperature (LTB$_4$), relative humidity (pH, LTB$_4$) and assay methodology (vial type, deaerated pH and batch, H$_2$O$_2$). EBC pH had a sensitivity and specificity of 78% and 67% respectively, for identification of mild EA and performed equal or better for identification of neutrophilic and eosinophilic subtypes, though sample size was limiting. EBC H$_2$O$_2$ was unable to predict mild EA without consideration of environmental and methodological factors.

**Conclusions:** EBC pH and H$_2$O$_2$ concentrations are altered by airway inflammation, suggesting a role for these biomarkers in the diagnosis and monitoring of EA. Environmental and methodological factors can influence these biomarkers and should be considered in the interpretation of results. EBC pH has demonstrated promise as a simple, non-invasive screening tool for detection of EA.
Comparison of left and right bronchoalveolar lavage cytology

Franklin SH¹, Tennant KV² and Allen KJ²

¹School of Animal & Veterinary Science, University of Adelaide, AUSTRALIA; ²Bristol Veterinary School, University of Bristol, UK

Purpose of the study: Previous studies have shown that an increase in neutrophils, eosinophils or mast cells in bronchoalveolar lavage (BAL) can be detrimental to performance. Few studies have investigated regional variations in BAL cytology and have reached different conclusions. For the development of a consensus statement for equine asthma, further data comparing left (L) and right (R) lung cytology would be of value.

Objectives: To compare L and R BAL cytology obtained in horses with naturally occurring lower airway disease.

Methods: Left and right BAL (300ml each) were performed, using a transendoscopic method in 65 sedated horses (58 TB, 7 mixed breeds) referred for investigation of poor performance. Cytospin preparations were made and slides were stained with modified Wright’s stain. Slides were examined to assess cell numbers, types and morphology before performing a manual differential count of 200 total nucleated cells.

Results: There was a moderate correlation in neutrophil proportions between L/R lung (R=0.558, p<0.001) but no correlation in mast cells (R=0.233, p=0.061). The median L/R difference in neutrophil proportions was 5% (range: 0-62%) and mast cell proportions was 2% (range: 0-9%). The neutrophil proportions were higher in the left lung in 37 (57%) horses and the right lung in 21 (32%) horses. Mast cell proportions were higher in the left lung in 21 (32%) horses and the right lung in 26 (40%) horses. When >5% neutrophils was used as a ‘cut off’ for airway inflammation 20 (31%) horses were categorised differently between L and R samples compared with 11 (17%) when a ‘cut off’ of >10% neutrophils was used. For mast cells, 26 (40%) horses were categorised differently between L and R samples when >2% mast cells was used as a ‘cut off’ for airway inflammation compared with 7 (11%) when a cut off of >5% mast cells was used. Three (5%) horses had >1% eosinophils in the L sample of which one horse also had >1% eosinophils in the R sample. No horse had >5% eosinophils.

Conclusions: Regional lung cytology variations are common in horses with naturally occurring lower airway disease. If single BAL samples are obtained, BAL ‘cut off’ recommendations should not be interpreted as absolute and cytology should be interpreted together with other clinical and diagnostic findings.

Declarations:
- Ethical Animal Research: Informed consent for procedure and contribution to clinical research was obtained.
- Sources of Funding: None
- Conflict of Interest: None
Correlation between allergen specific IgE test, cytokines and cytology in horse with Mild Equine Asthma

_Hansen S_¹, Birch K¹, Skovgaard K², and Fjeldborg J¹

¹University of Copenhagen, Faculty of Health and Medical Sciences, Department of Large Animal Sciences, Hoejbakkegaard Allé 5, DK-2630 Taastrup
²DTU Bioengineering, Department of Biotechnology and Biomedicine, Translational Immunology, Kemitorvet, DK-2800 Lyngby

**Objective:** To search for correlation between the allergen-specific IgE test, bronchoalveolar lavage (BAL) cytology and BAL messenger ribonucleic acid (mRNA) cytokine expression in a group of horses presented with mild equine asthma (MEA).

**Method used:** BAL fluid and serum was collected from 62 horses with a history of lower airway problems. The expression of interleukin (IL)-4, IL-5, IL-8, IL-10, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, toll-like receptor (TLR)-4, IL-1RN, IL-1β, MMP-8, TLR-9, chemokine ligand (CCL)-5 and cluster of differentiation (CD)-14 in BAL fluid were measured using qPCR. Cell population in BAL fluid was established by cytology. Allergen specific IgE was measured in serum and BAL fluid using an allergen-specific IgE ELISA test.

**Results:** BAL neutrophils correlated with the mRNA expression of IL-8 (r=0.517, p<0.001), IL-10 (r=0.418, p=0.001), TLR-4 (r=0.673, p<0.001), IL-1RN (r=0.616, p<0.001), IL-1β (r=0.563, p<0.001) and MMP-8 (r=0.750, p<0.001). BAL mast cells correlated with the expression of CD14 (r=-0.267, p=0.041) and with allergen-specific IgE for pollen (r=0.315, p<0.001) and insects (r=0.370, p=0.003). Correlation was found for BAL eosinophils and BAL mRNA expression of TGF-β (r=-0.324, p=0.022) and BAL mRNA expression of IL-4 (r=0.386, p=0.006). Furthermore a significant correlation between BAL eosinophils and allergen-specific IgE for storage mites in BAL (r=0.942, p<0.001) and allergen-specific IgE for pollen2 in BAL (r=0.848, p<0.001) was found. Correlation between BAL mRNA expression of IL-4 and storage mites and pollen2 measured in BAL fluid (r=0.298, p=0.021, r=0.273, p=0.035) was found.

**Conclusions:** BAL eosinophils did correlate with IL-4 and allergen-specific IgE for storage mites and pollen2. Further studies on horses with eosinophilic MEA are needed to further validate these preliminary results.

**Declarations**
- Ethical Animal Research: Ethical approval was given by the University of Copenhagen, Large Animal Teaching Hospital, Ethical Committee and by the Danish Animal Experiments Inspectorate.
- Sources of funding: Danish Horse Levy Foundation.
- Disclose any potential conflict of interest: None of the authors declare conflicts of interest.
Effect of forage type on respirable dust exposure and airway cytology in thoroughbred racehorses.

Olave CJ¹, Ivester KM¹, Park JH², Couetil LL¹

¹Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, Lynn Hall, 625 Harrison Street, West Lafayette, IN 47907.
²School of Health Sciences, College of Health and Human Sciences, Purdue University, 550 Stadium Mall Drive, West Lafayette, IN 47907.

Purpose of the study: Mild equine asthma is associated with dust exposure and is detrimental to racehorses’ performance. The purpose of the study was to evaluate the effect of low-dust forages on airway health in racehorses.

Objectives: To compare respirable dust exposure and bronchoalveolar lavage fluid (BALF) cytology between racehorses fed dry hay, steamed hay, and haylage.

Methods: Thoroughbred racehorses (n=44) were randomly assigned to be fed hay, steamed hay or haylage for six weeks. A BAL was performed at baseline and after three and six weeks. Differential cell counts were performed on cytospin preparations. Respirable dust was measured gravimetrically at the horse’s breathing zone on two occasions. Mixed models were constructed to examine the effect of forage assignment upon BALF cytology and respirable dust. Adjusted P-value < 0.05 was considered significant.

Results: All measurements were accomplished in 28 horses (hay = 5, steamed hay = 12, and haylage = 11). Respirable dust exposure of horses fed hay (0.078 ± 0.037 mg/m³) was higher than those fed haylage (0.053 ± 0.016 mg/m³; p=0.0049) but not different from steamed hay (0.056 ± 0.018 mg/m³). By week 3, horses eating haylage had a significantly lower proportion of BALF neutrophils compared with the hay group (p=0.038). BALF neutrophils were 3-fold higher for each 0.1 mg/m³ increase in respirable dust exposure (p<0.001). Horses eating haylage exhibited a significant decrease in mast cell proportions from baseline to week3 (p=0.008) but not by week6.

Conclusion: Haylage may benefit airway health due to decreased respirable dust exposure.

Declarations:
- The Purdue University Animal Care and Use Committee and the Indiana Horse Racing Commission approved all procedures.
- Funding: Grayson-Jockey Club Research Foundation
- Authors declare no conflicts of interest.
Bronchoconstriction and bronchodilation in horses can be verified using electrical impedance tomography (EIT)

Secombe C1, Adler A2, Raisis A1, Hosgood G1, Mosing M1

1College of Veterinary Medicine, Murdoch University, Perth, Australia
2Carlton University, Ottawa, Canada

Purpose of the study: Electrical impedance tomography (EIT) measures impedance changes in the lungs as measured by electrodes mounted on a belt placed around the thorax. The study aimed to determine if drug-induced bronchoconstriction followed by bronchodilation in horses could be detected by novel EIT variables.

Methods: Bronchoconstriction was induced using stepwise histamine bronchoprovocation quantified by a 50% increase in open plethysmography variables in six healthy horses sedated with detomidine. EIT variables were recorded before the facemask was applied (BL) and at the histamine concentration causing bronchoconstriction (Cmax = three horses at histamine 8 and 16 mg/ml, respectively) and three (S1) and 10 (S2) minutes after start of salbutamol administration. Global peak inspiratory (Flowinsp) and expiratory flow (Flowexp), slope of the global flow-volume curves (FVslope), steepest FVslope over all pixels in the lung field (FVslopemax), abdominal intercept on the FVslope (FVintercept), and total impedance change (surrogate for tidal volume; VT_EIT) were analysed for a difference at Cmax from BL. Cmax was indexed against BL (Cmax/BL-1) and tested against the null hypothesis of 0 using a one-sample t-test.

Results: Flowinsp, Flowexp and FVslopemax significantly increased at Cmax (p=0.024, p=0.033 and p=0.033, respectively) and returned to BL at S1 and S2. The means for FVslope and FVintercept increased and decreased at Cmax, respectively, but were not significant due to high standard deviations. Both variables returned to BL at S1 and S2. VT_EIT did not change.

Conclusion: Histamine-induced bronchoconstriction and subsequent bronchodilation can be verified using EIT variables, offering clinical application for pulmonary function testing in horses with equine asthma.

Declarations:
- Ethical Animal Research: This study was approved by Murdoch University ethics committee R2895/17
- Sources of Funding (which should be described in the comments box after ‘competing interests’ in the online system): Institutional funding
- Competing Interests. The authors declare that there were no conflicts of interest.
Thursday, May 23rd: Equine vs. human asthma: Definitions and diagnosis

SESSION II

(Chairperson: J. Cardwell)
The syndrome of equine asthma encompasses mild to severe forms of chronic airway inflammation. The severe form also known as heaves, affects approximately 14-17% of horses in countries with Northern, cool climate.1,2 The milder form affects 68-77 % of pleasure horses based on tracheal wash cytology (neutrophils > 20%) and up to 80% of racehorses based on bronchoalveolar lavage cytology.3,4

**Severe equine asthma:**

Horses affected with severe equine asthma experience exacerbation of clinical signs when exposed to organic dust originating from hay and bedding, in particular molds present in poor quality hay. As a result, clinical signs tend to be worse during the winter when horses are housed indoors for extended periods of time.5 Some horses exhibit disease flare-ups while at pasture during summer months (summer-associated equine asthma).6 These horses improve clinically during winter or after being housed indoor. A small percentage of horses appear to suffer from both classic and summer-associated equine asthma. Horses with severe asthma tend to be mature (>7 years) to old animals and a genetic predisposition has been identified in some families.7,8

The main clinical sign characteristic of severe equine asthma is increased respiratory effort (“dyspnea”) that can rapidly improve following bronchodilator administration. Although the decrease in respiratory effort following bronchodilator administration can be detected within minutes of drug administration using lung function testing, clinical improvement is not apparent to clinicians.9 Acute exacerbation is associated with increased pulmonary artery and right-heart vascular pressures as well as increased pulmonary artery diameter on ultrasound.10 Blood pressure return to baseline during clinical remission however, cardiac ultrasound abnormalities such as right ventricular wall thickness remained increased.10 Surprisingly, severe equine asthma is rarely fatal unless complications develop such as cor pulmonale.11 Affected horses are more likely to be euthanized because owners get discouraged with the expense associated with chronic therapy and maintaining a low-dust environment.7

Coughing and nasal discharge are non-specific signs of respiratory disease commonly reported in horse with severe equine asthma.2,12 Horses with a history of both coughing and mucoid nasal discharge are at increased risk of developing severe equine asthma.13 Thoracic auscultation may reveal increased breath sounds bilaterally, extended area of auscultation, and abnormal breath sounds (i.e. crackles, wheezes). However, the thick chest wall of horses makes auscultation an insensitive indicator of pulmonary disease, with abnormal findings obtained in less than 50% of horses with RAO.12

Exercise intolerance or decrease performance is usually marked especially, during periods of disease exacerbation.2
Strict management changes or medical therapy will result in rapid improvement in clinical signs however, if exposure to triggering factors is not addressed improvement will be short lived or incomplete.\textsuperscript{14,15}

**Mild/moderate equine asthma:**

This form of mild respiratory disease is mainly subclinical with horses showing non-specific signs such as intermittent coughing and poor performance.\textsuperscript{16} However, mild asthma should not be ruled out in horses that do not cough because coughing is reported in only 38\% of horses with mild asthma.\textsuperscript{17} Coughing is associated with increased bronchoalveolar lavage (BAL) neutrophils.\textsuperscript{18}

Poor performance and reduced willingness to perform are associated with increased tracheal mucus scores in racehorses and show-horses, respectively.\textsuperscript{16} In racehorses, poor performance has been associated with increased neutrophils and mast cells in bronchoalveolar lavage fluid (BALF).\textsuperscript{4}

There is an association between nasal discharge and increased tracheal mucus in racehorses.\textsuperscript{19} However, the association between tracheal mucus and BAL cytology has not been reported yet.

References:


Tissue remodeling in equine asthma and functional consequences

Michela Bullone, DVM, PhD

Asthma and COPD Laboratory, San Luigi Gonzaga University Hospital, Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy.

The term “remodeling” defines a process resulting in a tissue that is structurally and architecturally altered compared to its healthy counterpart. In asthma, structural alterations are represented by quantitative or qualitative changes of the bronchial wall components or their surrounding tissues, whilst architectural alterations refer to the skewed relationships among such structures.

Airway remodeling has been studied only in horses affected by severe equine asthma (SEA), while no data is available for mild equine asthma. Recently, an increased expression of metalloproteinases and their tissue inhibitors has been reported in a group of horses with mild respiratory clinical signs and bronchoalveolar lavage (BAL) cytology compatible with mild equine asthma [1]. However, the possibility that the horses studied were horses with SEA in remission of the disease was not excluded, preventing our ability to conclude on the presence of airway/lung remodeling in mild equine asthma.

Almost all airway components undergo remodeling in SEA, both at the peripheral (airways <2 mm in diameter) and central levels. During SEA remission, peripheral airways are characterized by increased airway smooth muscle mass as well as collagen and elastic fiber deposition in the lamina propria compared to healthy airways [2; 3]. Mucostasis, mucus cell hyperplasia, peribronchiolar metaplasia, and interstitial fibrosis were more frequently detected in horses with SEA in remission compared to controls [4]. On the other hand, a study using histomorphometric techniques found no differences in the number of mucus cells per mm of lamina reticularis or in the volume of stored mucosubstance in bronchial epithelial cells [5]. Central airway remodeling during disease remission is less pronounced. Indeed, whether airway submucosal structures (smooth muscle and lamina propria) remains increased in thickness during SEA remission compared to control remain to be established [6; 7; 8]; mucus cells only tend to be increased [9].

Functionally, SEA remission is associated with a normalized lung function in spite of significant structural alterations of the peripheral airways. In these conditions, the respiratory resistance correlates with the amount of collagen within the lamina propria of peripheral airways [3]. Together, these data indicate that peripheral airways only minimally contribute to the clinical presentation of asthma exacerbations but, in absence of bronchospasm, their stiffness is the major determinant of respiratory resistance in asthmatic horses. The functional implications of peripheral remodeling become more
important during disease exacerbations, when most of them are further accentuated [4; 10] and the mechanics of breathing are altered.

There is no doubt that the major determinant of airway obstruction during SEA exacerbations is smooth muscle contraction and that central airways play a major role [11]. By definition, the forced produced by a muscle is proportional to its cross-sectional area. Given the increased smooth muscle mass (and cross-sectional area) during SEA exacerbations [7], asthmatic muscle is “stronger”. In this way, it can contrast the thickened lamina propria that lies between the smooth muscle and the epithelial layer, and further reduce the airway lumen. Increased mucus secretions in to the airway lumen also contribute to airway occlusion. These same mechanisms operate in peripheral airways, where the effects on lung function are somehow blunted by the fact that their overall contribution to pulmonary resistance is low, due to their large cumulative cross-sectional area. At this level, the more relevant functional effects of remodeling are the loss of lung elasticity and airway-parenchymal tethering. Adequate small airway patency is guaranteed by their intimal connection to the lung parenchyma by elastic and connective fibers. When the lung inflates during inspiration, small airways are stretched and passively dilate. Remodeling of elastic fibers and of the extracellular matrix within and around the airways and in the alveolar septa alters this mechanism, preventing the smallest airways to remain open. The effect is even worse during expiration, when the lungs physiologically recoil and the airway diameter physiologically narrow. With a significantly impaired expiratory airflow, part of the air that reaches the alveoli remains trapped there (air-trapping). This lead horses with SEA in exacerbation to breath at increasing lung volumes (functional residual capacity) in the attempt to maintain airway patency, which causes lung hyperinflation and enlarged fields of lung auscultation.

In summary, airway and lung tissue remodeling contribute to the altered lung function observed in SEA exacerbations. Central airway smooth muscle activation and remodeling mostly account for the increased airway resistance and clinical presentation of the disease, whilst peripheral airway remodeling mainly cause reduced lung compliance.

References


Inflammatory Airway Disease (mild equine asthma): UK racing veterinarians’ views and practices.

Tierney Kinnison & Jacqueline M Cardwell

Department of Pathobiology & Population Sciences, Royal Veterinary College, London, United Kingdom

Correspondence: Dr Jackie Cardwell jcardwell@rvc.ac.uk

Introduction

Anecdotal observations have suggested a continuing divide between the international consensus on defining, diagnosing and managing inflammatory airway disease (IAD) and what is standard or feasible for UK racing veterinarians. This risks an important equine population, and significant disease process within that population, being poorly served by research. The aim of this study was to investigate the current concerns, views and practices of UK racing veterinarians and the challenges they face in diagnosing and managing airway inflammation in racehorses.

Methods

Qualitative data were gathered through semi-structured focus group discussions. A discussion schedule was designed to capture current practices and opinions relating to the diagnosis and treatment of IAD or lower airway inflammation, as well as familiarity with and views on the most recent IAD consensus statement. Four UK veterinary practices, two primarily serving the flat racing community and two primarily serving the National Hunt (jump racing) community, in different geographical regions of the UK, were purposively selected to participate. Focus group discussions were conducted at the practice premises, moderated by author TK, who is not a veterinarian. Discussions were audio-recorded and transcribed verbatim, and transcripts were analysed using an inductive, thematic analysis. Themes were developed at the semantic level by author TK, using an iterative approach including discussion with author JC. This was followed by ‘member checking’ (participant validation) of findings. Ethical approval for the study was obtained from the Royal Veterinary College Social Science Research Ethical Review Board (Reference number: SR2017-1224).

Results

In total, 25 participants contributed to 4 focus groups (Focus Group [FG]1 n=6; FG2 n=3; FG3 n=11; FG4 n=5). All were veterinarians, with the exception of one laboratory team member, and four were female. Discussions lasted between 46 and 74 minutes.

Three key themes were developed in preliminary analysis of focus group data: (i) the consensus definition of IAD is not applicable in this context (ii) the clinical approach is strongly trainer-influenced (iii) veterinarians work with an unsatisfactory jigsaw of diagnostic information.

(i) The consensus-defined IAD is not applicable in this context: The unanimous view across all four groups was that the consensus-type IAD is largely not seen in UK racehorses, which are instead affected predominantly with excess endoscopically-visible tracheal mucus attributed to acute inflammation largely driven by bacterial infections. It
was also unfeasible to fulfil two key aspects of the consensus case definition: waiting for chronicity of clinical signs (>3 weeks duration), and bronchoalveolar lavage sampling, neither of which would be acceptable to trainers.

(ii) **The clinical approach is strongly trainer-influenced**: The trainer selects horses for respiratory assessment, often because of racing schedules rather than clinical signs, and the approach to investigation and treatment is strongly influenced by trainer expectations. This varies with trainer personality, experience and approach to training, as well as stage of the racing season, signalment of the affected animal and yard ‘herd’ health, and is in turn driven by commercial pressures of the racing industry.

(iii) **An unsatisfactory jigsaw of diagnostic information**: UK racing veterinarians often need to rely on experience and instinct, rather than on research-based evidence. Visual assessment of tracheal mucus and of tracheal wash samples is valued as much, if not more than, cytological assessment, and aetiology is often defined by response to treatment rather than culture and sensitivity or other laboratory investigations. While emphasising the benefits of this flexible, individualistic approach, participants expressed frustration with the sometimes unsatisfactory jigsaw of diagnostic information available to them.

**Conclusions**
The UK racehorse population and its veterinarians are currently not well supported by equine respiratory research-based evidence. Research furthering our understanding of the causes, diagnosis and management of airway inflammation in young UK racehorses is still required.
Thursday, May 23rd: Equine vs. human asthma: Definitions and diagnosis

SESSION II
(Chairperson: J. Cardwell)

RESEARCH ABSTRACTS
Reducing exposure to dust in equine asthma: the effect of water nebulization on air quality in horse stables

Westermann C.M.¹, Heijstek M.¹, Damhuis L², Siegers E.W.¹, van Schothorst I.J.³, van Eerdenburg F.J.C.M.¹, van den Broek J.¹, and Wouters I.M.³

¹Faculty of Veterinary Medicine, Utrecht University, Yalelaan 114, 3584CM Utrecht, The Netherlands.
²Tierklinik Lüsche, Essener Str. 39a, 49456 Bakum, Germany
³Institute for Risk Assessment Sciences (IRAS), Utrecht University, Yalelaan 2, 3584CM Utrecht, The Netherlands.
C.M.Westermann@uu.nl

Purpose of the study: Exposure of stabled horses to airborne irritants can cause equine asthma. Nebulization devices should reduce airborne dust.

Objectives: To determine the effect of nebulization of water on inhalable dust and fungal concentrations in equine stables, under lowdust (LD, shavings and silage) and highdust (HD, straw and dry hay) regimes.

Methods used: Four units (2 HD and 2 LD) of six boxes were used of which two were equipped with an nebulization installation. Eight-hour averaged inhalable dust samples were collected at one central location (CL) and on the head of two horses within each unit (HH). Airborne fungal samples were collected by biosampler impingment at CL. After one week the HD/LD units were alternated. Samples were analyzed for dust and fungi. Statistical analysis was performed using a mixed effect model applying Akaike’s Information Criterion for model reduction and 95% profile likelihood confidence intervals.

Results: 180 dust samples and 60 fungi samples were analyzed. HD regime resulted in more fungi (13.82 times higher CI95% 9.14-22.48) and dust (1.37 times higher CI95% 1.30-1.45) at CL as well as more dust at HH (3.76 times higher CI95% 2.23-5.00) than LD regime. The nebulizer generally did not affect air quality except for a small rise in CL dust concentrations (1.10 times more dust, CI95% 1.04-1.16).

Conclusions: The installation of a water nebulizer in horse stables, under the conditions used in this study, doesn’t contribute to a healthier stable air quality. The enormous effect of dust free bedding and feeding is confirmed.

Declarations:
- Ethical Animal Research: This research was performed following appropriate review and approval by the Animal Welfare Body of Utrecht University.
- Sources of funding: Partly funding was obtained by the company that manufactured the nebulizing machine. Publication was agreed upon regardless of the outcome of the research.
- There is no conflict of interest.
The third generation steroid ciclesonide shows short plasma half-life and fast elimination after single and repeated inhalation in horses

Rahmel DK*, Szkuta O**, Barker S***, Lösel S****

*Boehringer Ingelheim Vetmedica GmbH, Binger Str. 173, 55216 Ingelheim, Germany; Email: Daniela.Rahmel@boehringer-ingelheim.com
**Avogadro LS, Parc de Génibrat, 31470 Fontenilles, France
***Covance Laboratories Limited, Otley Road, Harrogate, North Yorkshire, HG3 1PY, UK
****ACC GmbH Analytical Clinical Concepts, Schöntalweg 9, 63849 Leidersbach, Germany

Purpose: To investigate the pharmacokinetics of the steroid ciclesonide (CIC) and its active metabolite desisobutyryl-ciclesonide (des-CIC) in plasma and urine following inhalation of ciclesonide in horses.

Objectives: To evaluate the pharmacokinetics of CIC and des-CIC after single inhalations of different doses to assess dose linearity and to evaluate the pharmacokinetics and urinary elimination after repeated daily treatment.

Methods: Twelve healthy male and female horses received single inhalative doses of 4116 and subsequently 5488 µg/horse. Afterwards, horses were treated for 5 days twice daily with 2744 µg/horse followed by 5 days once daily with 4116 µg/horse. After single and repeated treatment, several blood samples were collected up to 24 hours. Urine samples were taken 24 and 48 hours after the last dose. CIC and des-CIC concentrations in plasma and urine were determined using validated methods.

Results: Ciclesonide was absorbed after inhalation with a median T_max of about 5 min and rapidly converted to its active metabolite. Dose-linearity after single inhalation of 2744, 4116, and 5488 µg/horse was observed, with a half-life of 3-5 hours for CIC and 4-5 hours for des-CIC. CIC and des-CIC were below the limit of quantification at 24 and 48 hours in urine after the last dose of the 10-day dosing period. No significant differences between males and females regarding AUC and C_max were found.

Conclusions: Ciclesonide shows dose-linear exposure in horses after inhalation with a short plasma half-life. Neither CIC, nor its active metabolite were detected in urine 24 and 48 hours after treatment.

Declarations:

- Ethical Animal Research: This study was performed following appropriate review and approval by institutional animal care and use committee responsible for oversight.
- Sources of funding: this study was completely funded by Boehringer Ingelheim Vetmedica GmbH
- Disclose any potential conflict of interest: Daniela Rahmel is an employee of Boehringer Ingelheim Vetmedica GmbH funding the study
The combination of azithromycin and fluticasone decreases airway neutrophilia in severe equine asthma

Mainguy-Seers S.a, Boivin, R.a, Hélie, P.b, Martin J.G. c, Lavoie J.-P.a

sophie.mainguy-seers@umontreal.ca

a. Université de Montréal, Faculty of Veterinary Medicine, Department of Clinical Sciences, 3200 rue Sicotte, St-Hyacinthe, J2S 2M2, QC, Canada.
b. Université de Montréal, Faculty of Veterinary Medicine, Department of Pathology and Microbiology, 3200 rue Sicotte, St-Hyacinthe, J2S 2M2, QC, Canada.
c. Meakins Christie Laboratories, McGill University Health Center Research Institute, 1001 Boulevard Décarie, Montréal, H4A 3J1, QC, Canada.

Purpose of the study: Airway neutrophilia is poorly controlled with conventional therapy in severe equine asthma (SEA) when horses are kept in an offending environment. In human asthmatics, macrolides decrease the exacerbation rate and improve the quality of life, but their impact on neutrophilic inflammation and lung function are debated and their effects on airway remodeling have not been investigated in naturally occurring asthma.

Objectives: To determine whether azithromycin potentiates the therapeutic effects of inhaled corticosteroids in SEA.

Methods: Severe asthmatic horses were administered inhaled fluticasone (2500 ug twice daily) alone (n=6) or combined with oral azithromycin (10 mg/kg, q48h; n=6) for 5 months. Lung function, bronchoalveolar lavage fluid cytology and endobronchial biopsies were sequentially obtained (at baseline, after two weeks of therapy (T2), T4, T6, T8, T12, T16, T20). Data were analyzed with two-way ANOVA and Dunnett’s for multiple comparisons.

Results: Pulmonary resistances and elastances similarly improved in both groups of horses with therapy. There were group and time effects on airway inflammation with the combination of azithromycin and fluticasone significantly reducing neutrophilia from T4 to T20 while fluticasone alone decreased it temporarily at T8 and T12. The endobronchial biopsy scores improved overtime (p = 0.03) in both groups and were significantly decreased at T6 and T20 only in the group receiving fluticasone and azithromycin.

Conclusion: This study suggests that adding a macrolide to inhaled corticosteroid can reduce neutrophilic inflammation in SEA, without substantially potentiating the improvement in lung function and central airway remodeling.

Declarations:
- Ethical Animal Research: All experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal (Protocol # 16-Rech-1324).
- Research funding source: Canadian Institutes of Health Research (# PJT-148807).
- Authors disclose no conflict of interest.
Effects of nebulized dexamethasone on the respiratory microbiota and mycobiota in horses with mild equine asthma

Stephanie L Bond, Matthew Workentine, Jana Hundt, Persephone McCrae*, Angela Galezowski, Renaud Léguillette

Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada; * UCVM Class of 2019

Objectives: To characterize the upper and lower respiratory tract microbiota and mycobiota associated with health and mild equine asthma (MEA); to investigate the effects of nebulised dexamethasone in a sustained dusty environment.

Methods: prospective, randomized, controlled, blinded clinical trial (n=20). PCR amplification of the 16S (microbiome) and ITS2 (mycobiome) genes and subsequent sequencing was performed on DNA extracted from nasal swab and transendoscopic tracheal wash samples at two timepoints.

Results: In the upper respiratory tract dexamethasone treatment resulted in a significant drop in diversity and a significant change in the abundance of eight genera (five increased, three decreased). Treatment with nebulized saline had no significant effect on diversity but did alter the abundance of three genera. Dexamethasone-specific effects were tested (interaction) with two genera (Alysiella and Bordetella) showing a differential effect between treatments. Beta-diversity analysis showed that timepoint (effect of dusty environment) was more prominent than treatment effects.

The mycobiota was dominated by timepoint effects; when the interaction was tested no significant genera were detected. Saline treatment resulted in a loss of diversity but not dexamethasone treatment. Treatment with saline altered the abundance of 12 genera; dexamethasone treatment altered nine genera. Notably, the genus Alternaria, a known opportunistic pathogen and allergen in humans, was significantly increased in both treatment groups.

Conclusions: Nebulized dexamethasone treatment affected the upper respiratory tract microbiota, but not the mycobiota, which was overwhelmed by the effect of a sustained dusty environment. This highlights the importance of environmental modification as part of the treatment strategy for MEA.

Declarations:

- This study was conducted in accordance with the recommendations of the Canadian Council of Animal Care. The research protocol was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC17-0097).

- This study was supported by the Calgary Chair in Equine Sports Medicine.

- The authors report no conflict of interest.
Friday, May 24th: Etiology & pathophysiology of equine asthma

SESSION III

(Chairperson: E. Richard)
The microbiome in equine asthma

Renaud Leguillette, DVM, PhD, DACVIM
College of Veterinary Medicine, University of Calgary, Calgary, AB, Canada

The respiratory system is an interface between the outer environment and the inner body. Lower airways have historically been seen as a sterile milieu, thanks to the anatomical configuration, local surface immunity and mucus production and clearance systems. However, with the development of high sensitivity and high throughput technologies, the microbiota of the respiratory system has been described in healthy subjects in many species, including horses. Further investigation of the relationship between infectious agents, lower respiratory tract microbiota and the development of mild equine asthma is warranted. We and others have reported descriptive results about the microbiota of horses with mild equine asthma, but the causality between bacterial flora and the disease is far from being understood.

Studies on the microbiome use DNA extraction followed by high throughput amplification and sequencing of the 16S amplicon. The sequences are then filtered and aligned against a taxonomy database to identify and organize operational taxonomic units (OTUs). Descriptive analysis of the phyla, OTUs and bacterial species are then performed, followed by statistical analysis at the community level (within and between samples; alpha and beta diversity respectively) and at the individual level (OTU diversity analysis). Statistical analysis can be used to compare between groups: healthy horses versus those with mild asthma, upper versus lower respiratory tract.

The lower airways have a decreased richness (alpha diversity, corresponding to the number and proportion of each bacterial species) when compared to the upper airways in healthy horses. However, a very large majority of the same OTUs are present in both the upper and the lower airways, showing an overlap and some continuity in the bacterial population between the two anatomical environments in healthy horses. Furthermore, treatment with corticosteroids did not affect the composition of the bacterial flora in the upper airways. The role of the upper airways microbiota in mild equine asthma is unknown, but two studies did not find any difference in beta diversity of the upper airways between healthy horses and those with mild equine asthma.

The relationship between bacteria and the lower respiratory tract of the equine host seems to be dynamic. As an example, a change in the environmental respirable particulates has an effect on the lower respiratory tract flora in horses. Furthermore, treatment with systemic or nebulized dexamethasone induces some changes in the microbiota of both healthy and mild asthma horses. Systemic dexamethasone administration decreased the evenness of the flora and increased the abundance of 9 OTUs. There is an agreement between studies that the lower airways microbiota between healthy and mild equine asthma horses are clearly different. Interestingly, Streptococcus is one of the 6 OTUs which differed with disease status, and was the OTU with the greatest increase in relative abundance in mild equine asthma.

The effect of the environment on the composition of the lower airways’ microbiota is also a common finding between studies. However, a study found that treatment with corticosteroids had more effect on the composition of the bacterial flora than changes in the environment.
The microbiome studies are recent in equine medicine and are limited to being descriptive. The challenge for the scientific community will be to answer the causality dilemma of the chicken or the egg regarding the role of the airway microbiota in mild equine asthma.
Role of viruses in equine asthma
Nicola Pusterla, DVM, PhD

Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California, USA

Human asthma
Asthma development is most probably caused by the interaction of multiple factors, including, genetics, allergen exposure, microbiome and invading pathogens. Human rhinovirus, human respiratory syncytial virus, human metapneumovirus, human parainfluenza virus, human enterovirus and human coronavirus are strongly associated with asthma exacerbations (1,2). The association between human rhinovirus-induced wheezing and the development of childhood asthma/wheezing has been confirmed in a recent meta-analysis (3). The risk for asthma by age 6 years has been shown to increase (odds ratio 9.8) if children have been wheezing with rhinovirus during the first 3 years of life (4). Further, many prospective long-term follow-up studies have shown that human respiratory syncytial virus-induced bronchiolitis is associated with later development of asthma (5). However, the pathogenic role of respiratory viruses as triggers for the development and/or exacerbation in asthmatic human patients has not been fully characterized. Changes in the immune response to viral infections in genetically predisposed individuals are very likely to be the main factor involved in the association between viral infection and asthma (6).

Equine asthma
The pathogenesis of equine asthma remains incompletely defined. However, similar to human asthma, a multifactorial process is suspected. Conditions associated with exercise, feeding and housing practices, location, seasonality, infection of the upper and lower airways and genetic influences have been linked to equine asthma (7,8). A variety of viral (equine influenza virus (EIV), equine herpesviruses (EHV) equine rhinitis viruses (ERVs)) and bacterial (Streptococcus equi subspecies zooepidemicus, Actinobacillus spp., Pasteurella spp.) etiological agents have been linked to mild to moderate equine asthma (9-13). It remains to be determined if these agents are triggers for the development of equine asthma or are secondary colonizers of already compromised airways.

Evidence for viruses in equine asthma (Table 1)
Viral respiratory infections are one of the most common health problem in horses throughout the world. These infections are often self-limiting and a full recovery can be expected in most horses. Young performance horses, such as racing horses, have an increased risk of respiratory viral infections. This relates to age susceptibility, commingling, stress and suboptimal biosecurity protocols (14-16).

Amongst respiratory viruses, only EIV and ERVs have an affinity to the lower respiratory tract, leading to airway hyperresponsiveness. Clinical signs associated with EIV are usually more severe than those seen with mild to moderate equine asthma. Further, no association has been determined between mild to moderate equine asthma and infections with EIV, EHV-1 and EHV-4 (17-19). This is in sharp contrast to the detection of ERVs (ERAV and ERBV), known to cause subclinical or mild clinical disease (17,18). In a recent study, horses with mild to moderate equine asthma were significantly more likely to have a positive titer as well as higher log-transformed titers to ERAV when compared to control horses (17). In another study, the detection of ERBV by qPCR was significantly associated with coughing in Standardbred racehorses in training (18). Subclinical respiratory viral activity in horses with poor performance has been associated with EHV-2 and EHV-5 infection (17,18). In a recent study, the detection of
EHV-2 by qPCR in nasal secretions was significantly associated with mild to moderate equine asthma (17). In another study, the detection of EHV-2 by qPCR was significantly associated with coughing and excessive tracheal mucus in Standardbred racing horses (18). These results are in sharp contrast to two recent studies performed on 66 Swedish Standardbred trotters, which were followed for 13 months via qPCR analysis of nasal secretions and serology (19,20). Despite occurrence of poor performance and subclinical viral activity in the Swedish Standardbred trotters, the authors were unable to detect associations between EHV-2/-5 and clinical respiratory disease and/or poor performance. These conflicting results reflect the ongoing challenges in establishing causality between mild to moderate equine asthma and gamma herpesviruses, known to be ubiquitous in both healthy and clinically affected horses. In conclusion, associations between specific viruses detected via antigen or antibody detection and clinical signs of mild to moderate equine asthma may suggest that viruses may play a role in triggering or exacerbating asthma. However, because some viruses are ubiquitous both in healthy and clinically affected horses or are often associated with subclinical disease, establishing causality is challenging and in need for further research.

References
TABLE 1. Association of respiratory viruses with mild to moderate equine asthma based on antigen and/or antibody detection.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Year</th>
<th>Country</th>
<th>Population</th>
<th>Sample type</th>
<th>Outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAV</td>
<td>2015</td>
<td>Sweden</td>
<td>STBD trotters</td>
<td>NS</td>
<td>No detection by qPCR</td>
<td>19</td>
</tr>
<tr>
<td>EIV</td>
<td>2015</td>
<td>Sweden</td>
<td>STBD trotters</td>
<td>NS</td>
<td>No detection by qPCR</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>USA</td>
<td>Adult horses</td>
<td>BAL fluid</td>
<td>No detection by qPCR</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2016</td>
<td>France</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>No detection by qPCR</td>
<td>18</td>
</tr>
<tr>
<td>ERAV</td>
<td>2015</td>
<td>Sweden</td>
<td>STBD trotters</td>
<td>NS</td>
<td>No association with PP</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>USA</td>
<td>Adult horses</td>
<td>BAL fluid</td>
<td>High seroprevalence and titers</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2016</td>
<td>France</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>No detection by qPCR</td>
<td>18</td>
</tr>
<tr>
<td>ERBV</td>
<td>2015</td>
<td>Sweden</td>
<td>STBD trotters</td>
<td>NS</td>
<td>No association with equine asthma</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>USA</td>
<td>Adult horses</td>
<td>BAL fluid</td>
<td>No association with equine asthma</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2016</td>
<td>France</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>Detection by qPCR in horses with cough</td>
<td>18</td>
</tr>
<tr>
<td>EHV-1/- 4</td>
<td>2015</td>
<td>Sweden</td>
<td>STBD trotters</td>
<td>NS</td>
<td>No association with PP</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>USA</td>
<td>Adult horses</td>
<td>BAL fluid</td>
<td>No detection by qPCR</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2016</td>
<td>French</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>No association with equine asthma</td>
<td>18</td>
</tr>
<tr>
<td>EHV-2</td>
<td>2015</td>
<td>Sweden</td>
<td>STBD trotters</td>
<td>NS</td>
<td>No association with PP</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>USA</td>
<td>Adult horses</td>
<td>NS</td>
<td>Detection associated with equine asthma</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2016</td>
<td>France</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>No association with equine asthma</td>
<td>18</td>
</tr>
<tr>
<td>EHV-5</td>
<td>2015</td>
<td>Sweden</td>
<td>STBD trotters</td>
<td>NS</td>
<td>No association with PP</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>USA</td>
<td>Adult horses</td>
<td>BAL fluid</td>
<td>No association with equine asthma</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2016</td>
<td>France</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>No association with equine asthma</td>
<td>18</td>
</tr>
<tr>
<td>ECoV</td>
<td>2016</td>
<td>France</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>No detection by qPCR</td>
<td>18</td>
</tr>
<tr>
<td>EAdV-1</td>
<td>2016</td>
<td>France</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>No association with equine asthma</td>
<td>18</td>
</tr>
<tr>
<td>EAdV-2</td>
<td>2016</td>
<td>France</td>
<td>STBD trotters</td>
<td>NS, TW</td>
<td>No detection by qPCR</td>
<td>18</td>
</tr>
</tbody>
</table>

BAL: bronchoalveolar lavage; EAV: equine arteritis virus; EAdV: equine adenovirus; NS: nasal secretions; PP: poor performance; STBD: Standardbred; TW: tracheal wash
A growing body of research demonstrates the link between organic dust exposure and equine asthma. Introduction of horses to high dust environments not only induces profound bronchoalveolar lavage fluid (BALF) neutrophilia and airway obstruction in horses susceptible to severe asthma, but also significant neutrophilic airway inflammation in previously healthy horses.[1; 2; 3] Outside of the experimental exposure setting, higher dust exposure has also been associated with increased risk of tracheal mucus accumulation in racing Thoroughbreds.[4]

Barn dust is a complex mixture, rich in potential sources of allergens as well as immunomodulators such as endotoxin and β-glucan.[5; 6; 7; 8; 9] In addition to individual horse factors such as age and susceptibility, this complexity may partially account for the heterogeneity of asthma phenotypes. Respirable particulates, nominally less than 4 μm in diameter, have been linked to eosinophilic inflammation in young Thoroughbreds entering race training[10] and neutrophilic inflammation in actively racing Thoroughbreds.[11] Increasing respirable endotoxin exposures have been shown to provide an apparent protective effect against neutrophilic inflammation at low doses[11], while high doses of endotoxin augment the inflammatory response to particulates,[12] suggesting a non-linear response to inhaled endotoxin in the horse. Mast cell inflammation has been found to be common in both young, untrained Thoroughbreds[10] and those that are actively racing,[11] but unrelated to respirable dust or respirable endotoxin exposures.[11] Instead, BALF mast cell proportions are related with respirable β-glucan exposures. Conversely, inhalable dust exposures have not been found to affect BALF inflammatory cell proportions. Thus, inhalable particulates, those nominally less than 100 μm in diameter, appear to be less relevant than respirable particulates in equine respiratory health.

Setting exposure recommendations will require better understanding of the dose-response to inhaled non-infectious agents across wider ranges of age, breed, and discipline through study designs that include both exposure and respiratory health outcome measures and utilize appropriate statistical tools to relate them. Advanced characterization of respiratory health, such as investigation of alveolar macrophage function and BALF cytokine profiles, coupled with extensive exposure assessment is likely to offer valuable insight into equine asthma pathophysiology and identify new targets for intervention. Miniaturization of optical particle counters has rendered real-time breathing zone exposure measurements on the horse both affordable and technically feasible. Finally, the equine airway is arguably most susceptible to particle penetration during athletic exertion due to large tidal volumes and extension of the head and neck, yet the exposures that horses sustain during exercise are largely unexplored. Such measures of exposure are complicated by the air speed and turbulence generated at the breathing zone during such activity and will require specialized sampling strategies.


Is the neutrophil a key player in equine asthma?

Moran G, DVM, PhD

Department of Pharmacology, Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile. gmoran@uach.cl

Neutrophils are key actors in host defense, migrating toward sites of inflammation and infection, where they act as early responder cells toward external insults. However, neutrophils can also mediate tissue damage in various non-infectious inflammatory processes. Airway inflammation is one of the primary characteristics of an asthma-affected horse’s response to aeroallergens, neutrophilic bronchiolitis being the primary lesion. Several authors suggest that equine asthma is the study model of neutrophilic asthma in humans. The mechanism by which airway inflammation develops in asthma-affected horses is a multifaceted and dynamic process. Current knowledge suggests that the inflammatory component of this disease results from a combination of elements from both the innate and adaptive immune responses. Generally, airway inflammation involves activation of pathogen-specific inflammatory cells, modulation of gene transcription factors, and release of inflammatory mediators. Within the airways, these cells likely contribute to bronchoconstriction, mucus hypersecretion, and pulmonary remodeling by release of pro-inflammatory mediators, including the cytokines interleukin (IL)-8 and IL-17, neutrophil elastase, reactive oxygen species (ROS), and neutrophil extracellular traps (NETs). The destructive nature of ROS may contribute to increased inflammation, apoptosis, or necrosis by modifying nucleotide chains and disrupting DNA stability. Horses that suffer from asthma also have a decreased pulmonary antioxidant capacity, which may render them more susceptible to oxidative challenge. Research on oxidative stress in horses with asthma has been conducted, and some authors showed that neutrophilia induced by exposure to organic dust is associated with increases in elastase and decreases in ascorbic acid concentrations in BALF. The pathogenic role of NETs has been described for many infectious and non-infectious human diseases, including respiratory cases with a massive influx of neutrophils into the airways. Excessive NET release is particularly deleterious in lung diseases because NETs can expand easily in the pulmonary alveolar space and cause lung injury. NETs and their associated molecules can also directly induce epithelial and endothelial cell death.

The mechanisms that regulate neutrophil functions in the tissues are complex and incompletely understood, and must be regulated with exquisite precision and timing. Timely apoptosis of neutrophils is central to the resolution of inflammation; dying neutrophils are known to stimulate their own efferocytosis, inducing macrophagic transition from a pro-inflammatory to an anti-inflammatory profile. Thus, dysregulated apoptosis and mechanisms of inflammation may play an important role in the pathogenesis of equine asthma. The persistence of apoptosis-resistant neutrophils in the airways of horses with asthma may also impede timely neutrophil clearance and delay the resolution of airway inflammation. The discovery and development of compounds that can help regulate ROS, NET formation, cytokine release and clearance would be highly beneficial in the design of therapies for this disease in horses.
Insights into equine asthma pathophysiology from transcriptomics


Department of Pathobiology, University of Guelph
Guelph, Ontario, Canada

Asthma is a highly heterogeneous condition of the lung. Akin to the lining of the gastrointestinal tract, the lining of the airways is also in contact with external substances throughout life. Ingested substances generally pass through the gastrointestinal tract unidirectionally, and a careful balance between processing of digested food materials, nutrient absorption and limiting immunoreactivity is maintained during homeostasis, with well-known severe consequences of deviations in this balance. The airways function differently in that only gaseous substances normally pass into the distal alveoli, and are exhaled in the reverse direction. Inhaled particulates also have to be expelled in reverse direction toward the nasopharynx by largely mechanical means, or taken up by alveolar macrophages for disposition with minimal inflammatory evocation.(1) Hence, a complex and selective epithelial barrier with differing functions characterizes both organs.

The epithelium lining the airways has unique composition, morphology and function throughout the lung, and is intimately connected to subepithelial structures such as the basement membrane, mucous glands, smooth muscle, fibroblasts, endothelium and immune cells.(2) The epithelium forms a barrier between inhaled components and the subepithelial constituents, and also has to balance efficient transfer of gases with controlled reactivity to non-gaseous components. While the lesions of severe equine asthma manifest predominantly with inflammation, smooth muscle hyperplasia and fibrosis of the peripheral airways and surrounding tissues, the larger airways are exposed to the same inhaled substances and also have morphological, functional and molecular changes.(3)

Our research initially focused on the role of club cell secretory protein (CCSP), a member of the secretoglobin family produced by non-ciliated epithelial cells concentrated within the epithelium at the transition from bronchi to bronchioles. Club cells are recognized as epithelial progenitor cells that can differentiate into ciliated and other specialized cells of the airway epithelium, participate in reduction of reactive oxygen toxicants through cytochrome enzymes, and their hydrophobic secreted protein inactivates a range inflammatory mediators. Horses with severe asthma had fewer club cells and lower concentration of CCSP in airway fluids, which may be a function of chronic inflammation resulting in reduced regenerative capacity of the airway epithelium.(4) Unique relative to other mammals, equids have two expressed CCSP genes that differ in 12 of 70 amino acids, and also in their interaction with hydrophobic molecules. (5,6) Recombinant eCCSP increased neutrophil oxidative burst, phagocytosis and extracellular trap
formation, lending support to the notion that loss of club cells has deleterious effects on lung health.(7)

Subsequently, we investigated whole transcriptomic changes in endobronchial epithelial biopsies from sites from 5th to 12th generation bronchi with next-generation sequencing. Each horse served as its own control to identify changes in gene expression associated with an inhaled challenge since inter-individual variability exceeded changes attributable to the challenge. A bioinformatics pipeline including quality control measures to account for duplicates, variable sequencing depth and dispersion was implemented, results were mapped to the equine genome, and predicted proteins were procured with a combination of software and manual approaches to assign appropriate Ensemble IDs for analyzing interactions. An overall conservative analytic approach yielded 111 genes differentially expressed in horses with severe asthma as a result of a challenge, with the majority up-regulated.(8) Not surprisingly, many up-regulated genes pertained to inflammatory mediators and effectors, and were well known members of protein interacting networks. However somewhat more surprisingly, genes with altered expression also concerned more broadly epithelial cell formation and maintenance, and the circadian rhythm, suggesting that multiple cell properties are affected in exacerbated asthma at the transcriptomic level. Subsequent analysis of enriched gene sets in asthmatic horses further highlighted the importance of cell cycle regulation and repair pathways.(9)

Transcriptomic studies of this nature yield a great deal of information, which requires subsequent confirmation regarding cell specificity, correlation with protein expression and function, and extension to a more robust number of affected and unaffected individuals. Albeit, there is strong evidence to indicate that the bronchial epithelium is profoundly altered during exacerbation of severe asthma in horses, and this insight offers new venues for investigating the role of specific proteins and for potential therapeutic targets.(10;11)

References


Genetic risk factors of equine asthma

Gerber V, DVM, PhD, DACVIM, DECEIM
Institut Suisse de médecine équine (ISME), Vetsuisse Faculty, University of Bern, and Agroscope, Switzerland

Interactions of environment and genetics likely influence the entire spectrum of equine asthma (EA), but almost all research in this field has focused on the severe clinical phenotype. While no specific genetic risk factors have been reported for mild to moderate forms of EA, genetic susceptibility to certain bacterial lower airway infections (1) could potentially be relevant. Furthermore, mild but persistent respiratory signs such as occasional coughing and nasal discharge may represent early phenotypic indicators for an increased risk to later development of severe EA (2). This suggests that the genetics of milder forms of EA may be worth investigating in longitudinal studies.

Severe EA has been shown to be partly heritable in several breeds and has been the focus of genetic research involving family and epidemiological studies, whole-genome scans and investigation of candidate genes. Reports of marked familial aggregation of severe EA date back 70 years (3). Parent, age, and stable environment have significant additive effects (4, 5) that increase the risk for developing severe EA as defined by a history of persistent frequent coughing and/or increased breathing effort. Offspring of affected sires have a more than four-fold increased risk for developing severe EA (6).

Whole genome scans in high-prevalence families indicate two chromosome regions with a genome-wide significant association with severe EA (7). Importantly, the associations differ between the families: on ECA13 in one family and ECA15 in another family. Further association and gene expression studies indicate IL4R as a candidate gene in a subset of RAO-affected horses. Molecular pathway analyses (Ingenuity Pathway Analysis®) of genomic and proteomic data showed interactions between IL4R and SOCS5 upstream of an important molecular cascade involving NFκB (reviewed in 8).

So far, no causal genetic variant has been identified in IL4R. An allelic case-control genome-wide association study in the general Warmblood population revealed another region on chromosome 13. The best associated marker was located in the protein-coding gene TXNDC11, which may be involved in regulating H2O2 production in the respiratory tract epithelium as well as MUC5AC mucin expression (9). No genomic copy number variations were found to be associated with severe EA (10), integrative analyses combining GWAS, differential expression (DE), and expression quantitative trait loci (eQTLs) were not able to discover causative genetic variants that contribute to severe EA through gene expression regulation. However, results showed interesting similarities to human asthma with disease associated genetic variants in CLEC16A that also regulate gene expression of DEXI (11). Furthermore, global gene expression studies of mRNA and miRNA levels in these high-prevalence families have shown impaired cell cycle regulation and CD4⁺ T cell differentiation into Th2/Th17 cells, respectively, in severe EA (12, 13).

At present, none of these associations can be applied as useful genetic markers in the general population. Most of the findings pertain to Warmbloods only, or even only to certain lines and families. The fact that the chromosomal regions and the mode of inheritance do not agree between families indicates genetic heterogeneity for severe EA: depending on the genetic make-up of affected horses different genes confer the susceptibility for the disease. It appears that the genetic basis of severe EA is robust, but remarkably complex. Polygenic complexity, potentially with a larger number of genes that each may only contribute less than 10 % to the total genetic
effects, may make it difficult to discover causative variants. Nevertheless, the genetics of severe EA has revealed interesting links of severe EA with allergic skin diseases and susceptibility to intestinal parasites (14, 15, 16).

ACKNOWLEDGEMENTS

The author wishes to acknowledge the contributions of all co-investigators, graduate students and horses-owners. Major contributions to this work have come from the Institute of Genetics, the Institute of Parasitology of the Vetsuisse-Faculty, University of Bern, the Animal Health Trust, the University of Giessen und Purdue University. Parts of this abstracts are based on a published review [8].

FUNDING

Studies have been funded by The Swiss National Science Foundation, the Berne Equine Lung Group and The Horse Trust.

REFERENCES


Equine Respiratory Tissue Biobank for Collaborative Studies on Equine Asthma

Jean-Pierre Lavoie, DMV, DACVIM

Faculty of Veterinary Medicine, University of Montreal, Quebec, Canada

The Equine Respiratory Tissue Bank (ERTB) has the main objective to foster collaboration between researchers interested in the study of equine asthma by facilitating access to tissues of interest from different sites for large studies. Signalment, housing, and appropriate physiological measurements for each sample are archived into the tissue bank web application. Such systems have become essential for optimizing the sharing capabilities of the tissue samples between distant laboratories for the study of many human diseases.

The outline of the ERTB was drafted in 2006 in the Cell and Molecular Biology Respiratory Laboratory of the Université de Montréal in collaboration with the Laboratoire de Télématique Biomédicale of the Respiratory Health Network (RHN) of Quebec and the Tissue bank of RHN. The first version of the bank was born after an Havemeyer workshop held in Montreal in 2009 where 17 Canadian, American and European researchers interested in equine respiratory diseases contributed to defining the needs for the bank. At present, thousands of samples from well characterized mild to severe asthmatic horses have been entered into the bank from 2 university sites (Montreal and Purdue). The second version of the software (http://www.ertb.ca/en_index.html) is now implemented and facilitates the inclusion of other centers into the bank.

Participating centers remain owners of their samples and manage the tissue materials according to their specific needs. The bank allows the archiving of physiological measurements and other pertaining information to aliquot, extract, slide, pellet, or anatomic piece specimens (fixation, embedding, etc...) as it functions in nature. Importantly, each sample is assigned a unique code and a single physical location.

The web application that supports the bank allows archiving historical, medical and physiological elements related to the specimens. This information is grouped into five sub-categories: Subject, Housing, Medication, Prophylaxis and Biological tests and information (including clinical examination, lung function, endoscopy, bronchoalveolar and tracheal lavages, blood tests, performance evaluations, thoracic radiographs, etc.). One of the multiple advantages of the ERTB is that it permits easy access to samples collected over extended periods of time from the same horse, whether it is a client-owned animal, or it belongs to a research herd. While to date, the bank has allowed collaborative studies by sharing lung and blood tissues from asthmatic horses (Canada, USA, Europe and South America), more sites from different countries would be needed for the discoveries of new phenotypes and endotypes in equine asthma. Funding for the bank would be required to support the development of the bank website and the training of the personal in the new centers.
Friday, May 24th: Etiology & pathophysiology of equine asthma

SESSION III

(Chairperson: E. Richard)

RESEARCH ABSTRACTS
The Lung Transcriptome of Horses with Pasture-Associated Severe Equine Asthma Identifies A TH17-High TH2-Low Phenotype.

Frodella CM, Thomas KA, Bowser JE, Mochal CA, Eddy AL, Claude A, Swiderski CE.

Department of Clinical Sciences, College of Veterinary Medicine, Mississippi State University. Starkville, MS 39762.

Severe equine asthma (SEA) is characterized by reversible airway obstruction, non-specific airway hyper-responsiveness and chronic neutrophilic airway inflammation. Two forms of SEA are described: one elicited by barn dust in association with indoor housing in continental climates, the second associated with grazing pastures during conditions of high heat and humidity. Airway inflammation is predominantly neutrophilic in SEA, presenting a conundrum because TH2 cytokine responses identified in both conditions are predicted to precipitate eosinophilic inflammation. TH17 responses leading to neutrophilic inflammation have been identified by increases in IL17 in barn-dust SEA, but have not been documented in horses with pasture-associated SEA. In certain human severe asthmatics, a predominance of TH17 cytokines relative to TH2 cytokines (TH17high/TH2low) has been observed, but this phenomenon has not been documented in SEA. Accordingly, we hypothesized that TH17 and TH2 phenotypes co-exist in pasture-associated equine asthma in a TH17high/TH2low relationship. To test this hypothesis and identify relevant upstream regulators, we contrasted the lung transcriptomes of horses with pasture-associated SEA (N=6) in serial lung biopsies collected during disease exacerbation and remission. Reads were aligned to EquCab3.0 with differential expression analysis and modeling using CLC Genomics Workbench and Ingenuity Pathways Analysis, respectively (Qiagen). IL-17 signaling (p=7.6x10E-11) was the top canonical pathway, supporting a predominance of TH17 responses in pasture-associated SEA. Expression of molecules supporting TH2 signaling was less robust (p=3.2x10E-4). We conclude that horses with pasture-associated SEA mirror a subset of severe human asthmatics, exhibiting a TH17 high/TH2 low phenotype in association with seasonally induced asthma exacerbations.

This project was supported by the Agriculture and Food Research Initiative (AFRI) Animal Health Program competitive grant no. 2015-67016-23172 from the USDA - National Institute of Food and Agriculture.
An Integrated Analysis of Lung Tissue microRNA and mRNA Expression Profiles from Horses with Severe Equine Asthma

Matthias F. Kraft1,2, Alicja Pacholewska1,2, Amandine Vargas3, Jean-Pierre Lavoie3, Vincent Gerber1, Vidhya Jagannathan2

1Swiss Institute of Equine Medicine, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, Switzerland
2Institute of Genetics, Vetsuisse Faculty, University of Bern, Switzerland
3Faculty of Veterinary Medicine, Université de Montréal, Canada
e-mail: vinzenz.gerber@vetsuisse.unibe.ch

MicroRNAs (miRNAs) negatively regulate post-transcriptional gene expression by binding their target mRNAs. As miRNAs are key regulators in most biological processes they have been widely implicated in the pathophysiology of several diseases. Our study aimed at investigating miRNA and mRNA expression in lung biopsies from horses affected by severe equine asthma. To achieve this, we extracted high quality mRNA and smallRNA from lung tissue samples of symptomatic horses in exacerbation (n=7), affected asymptomatic horses in remission (n=5) and healthy controls (n=8) and used them for mRNA and smallRNA Illumina sequencing. Differential expression analyses of the miRNA-seq and mRNA-seq identified 8 miRNAs and 274 mRNAs between symptomatic and control horses, 14 miRNAs and 53 mRNAs between asymptomatic and healthy horses, and 2 miRNAs and 257 mRNAs between symptomatic horses and asymptomatic horses. Interestingly, miR-142-3p and miR-223 were differentially expressed in two of the three comparisons, and have been implicated in the pathophysiology of severe neutrophilic asthma in humans. Additionally, we examined miRNA-mRNA pairs that showed significant negative Pearson correlation coefficients. Applying this approach, we identified 14 miRNA-mRNA pairs showing negatively correlated expression levels between symptomatic horses and controls and 2 significant miRNA-mRNA pairs between asymptomatic and control horses. This integrative analysis of miRNA-mRNA profiles between symptomatic, asymptomatic, and control horses provides novel insights into the etiology and development of severe equine asthma.

Ethics statement
This research project was performed on archived samples that have been used for previous research projects. In these studies no explicit owner informed consent for inclusion of animals was included.

Conflicts of interest
The authors declare no conflict of interest.

Funding statement
The presented study was funded by the Swiss National Science Foundation (Grant Number 31003A-162548/1) and by the Internal Research Fund Number 33-890 of the Swiss Institute of Equine Medicine in Bern (Switzerland). Additional support was provided by the Québec Respiratory Health Network and the Equine Respiratory Tissue Biobank of the Université de Montréal.
Metabolomics of bronchoalveolar lavage fluid samples in horses with naturally-occurring asthma and experimentally-induced airway inflammation

Uberti B\textsuperscript{a}, Albornoz A\textsuperscript{b}, Burgos R\textsuperscript{b}, Alarcon P\textsuperscript{b}, Morales N\textsuperscript{b}, Henriquez C\textsuperscript{b}, Moran G\textsuperscript{b}.

\textsuperscript{a} Department of Clinical Veterinary Sciences, Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile
\textsuperscript{b} Department of Pharmacology, Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile. gmoran@uach.cl

Purpose of the study: Metabolomics has gained increasing attention in biomedical research. The detection and quantification of cellular metabolites in equine asthma may provide unique insights into metabolic changes occurring in tandem with alterations in gene and protein activity, and could provide evidence of the validity of experimental models of this disease.

Objectives: The aim of this work was to characterize metabolomic profiles of bronchoalveolar lavage fluid (BALF) samples in healthy horses and horses with naturally-occurring asthma, or induced airway inflammation.

Methods: Four study groups were used: healthy horses (n=6), horses with naturally-occurring asthma and active airway obstruction (n=3), horses with airway inflammation induced by Lipopolysaccharide nebulization (LPS, 200 µg/10 mL, n=3), and horses with airway inflammation induced by \textit{Aspergillus fumigatus} inhalation (n=3). BALF samples were collected from each animal and investigated by gas chromatography–mass spectrometry (GC–MS) analysis for detection and quantification of metabolites.

Results: Metabolomic processing allowed quantification of 52 molecules. There were multiple significant differences (p <0.05) between healthy horses and groups of animals with airway inflammation. Globally, high concentrations of oxidant metabolites (e.g. leucine) and low concentrations of antioxidant metabolites (e.g. ascorbic acid) were found in the groups of horses with induced and natural asthma. The healthy group expressed higher levels of nonadecanoic acid. The naturally-occurring asthma group expressed higher levels of 4-hydroxybutyric and 2-hydroxybutanoic acids than any of the disease groups. Experimentally, \textit{A. fumigatus} inhalation produced more profound changes than LPS nebulization, with observed differences in citric acid, leucine, glycine and lyxitol.

Conclusions: The increase in oxidant agents and decrease in antioxidant metabolites confirm that oxidative stress is strongly involved in asthma pathogenesis. Metabolomic analysis of BALF samples by GC-MS could help to identify novel biomarkers for diagnosis or treatment of equine asthma.

Declarations:
- All procedures were approved by the Universidad Austral de Chile Bioethics Committee for the Use of Animals in Biomedical Research (approval resolution n° 251/2016).
- Funding: FONDECYT N° 1160352 and Fondecup EQM130257 (Conicyt- Chilean Government).
- None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this work.
β-glucan receptor dectin-1 in equine asthma

Leclere M.1, Manguin E.1, Boivin R.1, Vesper S. J. 2

1 University of Montreal, 3200 Sicotte, St-Hyacinthe, Canada. 2 The Environmental Protection Agency, Cincinnati, USA.

mathilde.leclere@umontreal.ca

Purpose of the study: Equine asthma exacerbations are induced by exposure to moldy hay, but a clear allergic reaction to specific molds is not consistently present. Dectin-1, a signaling pattern-recognition receptor is the major β-glucan receptor in mammalian cells.

Objectives: To determine if dectin-1 is present and upregulated in equine asthma.

Methods used: Endobronchial biopsies (EB) and bronchoalveolar lavage fluid (BALF) were collected in 6 asthmatic horses and 6 controls housed on pasture, and then stabled and fed hay. BALF concentrations of 36 fungi were measured by qPCR and intracellular spores were counted on cytospins. Dectin-1 mRNA expression was quantified by qPCR in BALF and EB. Two rabbits were immunized to produce polyclonal anti-dectin-1 antibodies.

Results: In BALF, the functional isoform and, to a lesser degree, the non-functional dectin-1 isoform increased with hay exposure in horses with asthma. Intracellular particles increased with stabling in controls, but not in horses with asthma. In both groups, there was a correlation between the functional isoform and fungal DNA in BALF.

Conclusions: The β-glucan receptor dectin-1 is expressed by BALF cells and in endobronchial biopsies of horses. The functional isoform of the β-glucan receptor appears to be upregulated in equine asthma exacerbation. The lower number of intracellular particles observed in the BALF of asthmatic horses in exacerbation could be due to particles being trapped more proximally in the airway tree, or to a more efficient removal by alveolar macrophages.

Declarations:

- All experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee of the Université de Montréal.

- Supported by the Veterinary Comparative Respiratory Society Research Fund and Fonds en Santé Équine of the University of Montreal.

- No conflict of interest.
Evaluating Myristoylated Alanine Rich C Kinase Substrate (MARCKS) family proteins as potential therapeutic targets in equine asthma syndrome

Davis K.U.¹, Schirmer J.¹, Sheats M.K.¹

¹Department of Clinical Sciences, North Carolina State University College of Veterinary Medicine, 1060 William Moore Dr., Raleigh, NC, 27606, United States of America
mkpeed@ncsu.edu

Neutrophils are potential therapeutic targets for equine asthma syndrome (EAS). Previous work from our lab determined that MARCKS (Myristoylated Alanine Rich C Kinase Substrate) protein is an essential regulator of neutrophil functions. MARCKS protein is upregulated in neutrophils responding to inflammatory signals. We hypothesize that MARCKS, and a related protein knowns as MARCKSL1, will be increased in BAL cells from horses with EAS, and that inhibition of MARCKS in LPS-stimulated equine BAL cells ex vivo will diminish production of inflammatory mediators.

Our objective is to obtain proof of principle data to support MARCKS family proteins as potential therapeutic targets for treatment of EAS.

BAL cell lysates were prepared from horses with no (n=4), mild/moderate (n=10) and severe (n=5) EAS. Relative MARCKS and MARCKS-like 1 (MARCKSL1) protein expression were determined using equine specific MARCKS and MARCKSL1 ELISA. Cultured BAL cells were pretreated with control peptide, MARCKS inhibitor peptide or VC and stimulated with LPS for 18 hours, or left unstimulated. An equine specific TNFα ELISA (Genorise) was used to quantify TNFα in supernatants and cell lysate. Data were analyzed by One-way ANOVA (p<0.05).

Relative expression of MARCKS and MARCKSL1 proteins are significantly increased in BAL cell lysates from horses with mild/moderate or severe EAS. Preliminary findings also suggest that peptide inhibition of MARCKS in LPS-stimulated equine BAL cells ex vivo attenuates levels of TNFα.

These findings point to a possible role for MARCKS family protein in the pathophysiology of EAS and support MARCKS inhibition as a potential therapeutic strategy for EAS.

Declarations:

- Animal use was approved by NC State IACUC (protocol #16-074-O).
- Funding for this study was provided by Morris Animal Foundation Grant ID D17EQ-029.
- The authors have no conflicts of interest.
Investigating the Salivary Scavenger and Agglutinin protein in Equine Asthma

Lee G.K.C. (glee09@uoguelph.ca), Tessier L, Bienzle D
Ontario Veterinary College, University of Guelph, Guelph, ON, Canada

**Purpose:** Salivary Scavenger and Agglutinin (SALSA, also known as Deleted in Malignant Brain Tumors 1; DMBT1) is a protein with putative functions in innate immunity and tissue repair. In humans, the protein has been localized mainly to mucosal epithelia and secretions, including those of the airways. Knowledge regarding SALSA in horses is limited, but transcriptomic analysis of bronchial biopsies indicated low expression during remission and exacerbation in asthmatic relative to non-asthmatic horses. This study aims to analyze the structure and function of SALSA in horses.

**Objectives:** Our objectives were to determine the sequence of the SALSA gene and to characterize tissue expression in horses.

**Methods:** The SALSA gene from bronchial cDNA samples of multiple horses was amplified and sequenced. Tissue microarrays from 4 horses containing 21 tissues each were constructed. Immunohistochemical assays for SALSA were validated and applied to equine tissue microarrays.

**Results:** The gene in horses includes five scavenger receptor cysteine-rich (SRCR) domains, two CUB (C1r/C1s, uegf, bmp-1) domains and one zona pellucida domain. These domains mediate microbial agglutination and the binding of ligands such as those involved in innate immunity. The nucleotide and amino acid sequences varied between horses (95-99% identity), suggesting the presence of isoforms. SALSA was highly expressed at mucosal sites, including tracheal, bronchial and bronchiolar epithelium.

**Conclusions:** SALSA is a multifunctional protein with multiple isoforms in different individuals and a predilection for mucosal cells. Future studies will delineate the role of SALSA in enhancing or attenuating neutrophilic inflammation in the airway epithelium of horses.

**Declaration:**

- Ethical Animal Research: Institutional Animal Care Committee of the University of Guelph, Protocol R10-031
- Sources of funding: Equine Guelph, the Natural Sciences and Engineering Research Council, the Ontario Ministry of Agriculture, Food and Rural Affairs, and the University of Guelph.
- Conflict of interest: There is no conflict of interest.
Friday, May 24th: Etiology & pathophysiology of equine asthma

SESSION IV

(Chairperson: R. Leguillette)

ROTATING ROUND TABLE DISCUSSIONS
Saturday, May 25th: Reaching a consensus on Equine Asthma

SESSION V

(Chairperson: M. Mazan)
Are pertinent biomarkers of equine asthma already available to practitioners/researchers?

Niedźwiedź A, DVM, PhD

Department of Internal Diseases with Clinic for Horses, Dogs and Cats; Wroclaw University of Environmental and Life Sciences, pl. Grunwaldzki 47, 50-366 Wroclaw, Poland

Email: artur.niedzwiedz@upwr.edu.pl

There are several definitions of biomarkers in literature, and they all sound very similar. According to the National Institutes of Health, a biomarker has been defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention. In practice, biomarkers include tools and technologies that can help in understanding the prediction, cause, diagnosis, progression, and outcome of treatment of a disease. Although bronchoalveolar lavage (BAL) has been recognized as the gold standard for diagnosing respiratory diseases such as equine asthma, currently, sensitive and specific biomarker tests useful in routine laboratory diagnostics are being sought in such horses. Finding a new test would be particularly important in horses with dyspnea in the acute stage of the disease, where BAL is contraindicated. A simple biomarker would help in distinguishing between animals with lower airway infections and those with allergic problems. Although the diagnosis of severe cases of equine asthma in horses is relatively easy, it is difficult to diagnose remission cases or horses with a mild form of the disease, as it can imitate mild equine asthma. Ideally, molecular biomarkers should reflect a feature of relevant pathological processes or mechanisms. In addition, biomarker assessment should be easy, low-cost, technically accurate, repeatable and have an acceptable risk. Therefore, a measurement from easily obtainable body fluids or tissues is preferred, like for example from blood, urine, exhaled breath condensates vs. BAL, transbronchial biopsy or lung biopsy (Ley et al. 2014).

A number of biomarkers have been shown to be present/altered in the airways or circulation of horses with asthma.

Among others, inflammatory markers such as several acute phase proteins and cytokines, have been studied as markers of systemic inflammation. However, the available literature on markers of systemic inflammation in horses with heaves is not well characterized and contains inconsistent results (Riihimäki et al. 2008, Leclere et al. 2011, Lavoie-Lamoureux et al. 2012, Niedźwiedź et al. 2014). Apart from papers on the expression of different cytokines in the course of severe equine asthma, only a few papers contain results of levels of acute phase proteins which are easy to determinate ex tempore. Haptoglobin was found to be a suitable marker for both acute and chronic systemic inflammations, whereas high concentrations of serum amyloid A (SAA) indicated acute inflammation. In reference to mild equine asthma, the literature on a systemic inflammatory process is rare. One study found no difference in the acute phase proteins levels (SAA, c-reactive protein, haptoglobin) measured between horses with mild equine asthma and other horses with exercise intolerance (Leclere et al. 2015). Another study on mild equine asthma horses found haptoglobin elevated, supporting the hypothesis of disease progression to severe equine asthma (Bullone et al. 2015).

Surfactant protein D (SP-D) is a large multimeric collagenous glycoprotein produced mainly by type II epithelial cells in the lungs and is also detectable in the serum. Serum SP-D has been
identified as a potential systemic biomarker for some pulmonary diseases in humans, such as idiopathic interstitial fibrosis and acute respiratory distress syndrome. Elevated levels of SP-D have been detected in sera of horses with mild equine asthma (Richard et al., 2012, Bullone et al., 2015). Authors found SP-D a potentially valuable and readily accessible blood biomarker of equine lower airway inflammation.

Circulating immune complexes (CICs) are protein structures that are formed as a consequence of an immune response of an organism to antigens of various origin. In humans, CICs are detectable in a variety of systemic disorders such as autoimmune diseases, allergies and infectious diseases (Wener 2014). The results of one study in horses with severe equine asthma indicate that this condition in horses is associated with the formation and high level of CICs (Niedźwiedź et al. 2014). Moreover, another study found CICs useful in differentiation of healthy vs. heaves horses, and in corticosteroids therapy monitoring (Slowikowska et al. 2018).

The main group of enzymes responsible for collagen and other protein degradation in the extracellular matrix (ECM) are matrix metalloproteinases (MMPs); while tissue inhibitors of metalloproteinases (TIMPs) lead to fibrosis formation. Collagen is the main structural component of connective tissue and its degradation is a very important process in development, morphogenesis, tissue remodeling, and repair. Several studies have been performed in horses with severe equine asthma, in which MMPs and TIMPs were evaluated in respiratory tract secretion samples. Findings of one study support the usefulness of MMPs, TIMPs, and their ratios in the evaluation of the severity of respiratory disease and in identifying the subclinical cases (Barton et al. 2015). Furthermore, another study found the concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 to be significantly decreased after therapy with inhaled glucocorticoid therapy (Barton et al. 2016).

Exhaled breath condensate (EBC) has been found in humans as a promising source of biomarkers of lung disease. What is important, EBC itself is not a biomarker but rather a matrix in which biomarkers may be identified, in that way equivalent to other body fluids. EBC hydrogen peroxide ($H_2O_2$) concentrations and pH have been found to be higher in horses with mild equine asthma, vs. control ones (du Preez et al, 2019). Additionally, both $H_2O_2$ and pH had a positive association with BALF neutrophil percentage, while LTB 4 demonstrated a positive association with BALF eosinophil percentage. Another study characterized the metabolomic profile of tracheal wash (TW) and EBC in healthy horses and those with severe equine asthma (Bazzano et al. 2018). Higher concentrations of histamine and oxidant agents, such as glutamate, valine, leucine, and isoleucine, as well as lower levels of ascorbate, methylamine, dimethylamine and O-phosphocholine, were found in the group of heaves-affected, compared to healthy ones.

Many biomarkers for the diagnosis and monitoring of equine asthma have been studied — some are already being used in clinical settings, while others require further studies. Taken together, the studies cited above clearly indicate possible usefulness of some biomarkers. However history, clinical evaluation, and BAL still lie as the base of correct diagnosis of equine asthma.

Reference list:

How do we standardize immunologic laboratory testing?

Richard E.A., Legrand, L.

LABÉO (Frank Duncombe), 1 route de Rosel, 14053 Caen cedex 4
Normandie Université, UniCaen, EA 7450 BIOTARGEN, 14000 Caen

Immune response has largely been investigated in the airways of horses with severe equine asthma (sEA), and more recently moderate equine asthma (mEA), while still represents one of the futures directions stated in the 2016 Revised Consensus Statement. Such characterization has mostly been performed through relative mRNA expression of a various number of cytokines in bronchoalveolar lavage fluid (BALF), while several publications also reported protein concentration in BALF for a more limited panel of cytokines. As per BALF sampling and cytological investigation, various methodologies have been published among research groups in terms of cytokine mRNA expressions (e.g. SYBR Green or Taqman technology, design of primers and probes, relative quantitation …).

These methodological variations may ultimately prevent from objective comparisons between published results, as well as the implementation of prospective multicentric studies. Such diversity should however not be considered as a scientific weakness, and methodological homogenization among the various research groups neither represents a prerequisite nor a final goal to be reached. However, evaluation of the methodological performances of different research teams might represent a relevant perspective. In this manner, implementation of interlaboratory comparisons (ILC), i.e. based on (or even according to) ISO/IEC 17043 and ISO 13528 warrants further consideration. Let’s consider for example BALF samples, for mRNA expression of two different cytokines by PCR.

As a first (and informal) procedure, a simple “blind test” could be performed among up to e.g. four different teams. In this procedure, the ‘reference lab’ will provide the three other ones with aliquots of the same sample(s). Each team will evaluate mRNA expression for these two cytokines based on their own procedures, and comparisons of the results obtained and agreement among the teams can be evaluated. This “blind test” might then be repeated on a regular basis, systematically alternating the ‘reference lab’ within the group. In the end, this procedure will provide an objective evaluation of the results diversity among the teams, but clearly will not determine whether several teams are more efficient than others for these specific analyses.

A second (more structured) procedure would require the specific synthesis of standards (mRNA for two different cytokines in this case), and the development/validation of relevant conditioning and conservation procedures. A similar group of four different teams will firstly evaluate their ability in detecting and quantifying predetermined amounts of analytical standards (evaluation of the detection, not of the sample extraction etc.). This step is a necessary preliminary, in the absence of reference method. A panel of ≥ 10 samples (previously calibrated with standards) will then be implemented, including several identical ones (for repeatability) and submitted to the group (including a “self-shipment”) for testing and further statistical analyses (agreement etc.). Once the methodological performance of the group is considered acceptable for this panel, the
procedure might then be repeated with another two cytokines and so on … In the end, the whole panel of standardized samples might allow the establishment of a labeling, accessible to any voluntary laboratory involved in equine asthma.

Mandatory considerations about such comparisons are that there is no trap, and this does not represent overall examination of laboratories, but simple evaluations of procedures. All labs/teams are expected to use their own methodologies, whether or not the technologies, design et al. are similar within the group. Among others, samples conditioning, conservation, shipment and their associated costs will represent major issues to be considered, and should be this should be more broadly associated with virtuous initiatives such as i.e. the Equine Respiratory Tissue Biobank (ERTB).
Saturday, May 25th: Reaching a consensus on Equine Asthma

SESSION V

(Chairperson: M. Mazan)

SUMMARY OF TABLE DISCUSSION TOPICS

ROUND TABLE DISCUSSION – REACHING A CONSENSUS REGARDING FUTURE DIRECTION FOR EQUINE ASTHMA RESEARCH