RESEARCH REPORT
SWINE HEALTH

Title: Pilot study to develop ante-mortem synovial fluid and synovium sample collection techniques in growing swine and to refine a *Mycoplasma hyosynoviae* (MHS) arthritis case definition (453-23-79) – **NPB #15-143**

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Industry Summary:

Infectious lameness in growing pigs is an emerging problem in the United States. Lameness is an animal welfare concern and increases production costs. *Mycoplasma hyosynoviae* is considered to be the most common cause of infectious lameness. It is a dynamic organism in that its colonization of the joint can be transient and only a fraction of colonized pigs develop disease. Conventional polymerase chain reaction (PCR) testing for the pathogen is done post mortem and can be difficult to interpret without additional information about the pig, joint and joint fluid. Antemortem tests that are applicable to a broad range of lameness agents would significantly improve diagnostic testing for infectious lameness. Antemortem tests would also allow producers to monitor treatment success in affected pigs. In human and canine literature, clinical pathology of joint fluid is a core ante mortem diagnostic test for multiple type of arthritis. Clinical pathology is an analysis of the protein, pH and cell types within the joint fluid. Combined with PCR and culture, clinical pathology could be a key diagnostic tool for swine. However, a technique for the ante mortem collection of joint fluid in the field and reference ranges for normal animals for clinical pathology must be developed before these tools can be applied to commercial swine operations.

The objectives of this study were:

1. To create standard case definition of Mycoplasma hyosynoviae (MHS) arthritis in growing swine
2. To do a retrospective case review of lameness cases at the Iowa State University Diagnostic Lab to understand the common causes of lameness at the diagnostic lab
3. To create reference range values for clinical pathology parameters for swine synovial fluid from clinically normal pigs
4. To apply and evaluate antemortem joint fluid collection techniques and clinical pathology to non-lame and lame pigs under field conditions

Materials and Methods:

For objective one, a literature review using PubMed, Agricola and the AASV swine information library was conducted to develop a clinical case definition for MHS arthritis in swine. To perform the retrospective lameness case review, all cases related to lameness in swine were pulled from 2010 to 2015. Cases that did not meet the age, weight, project type, billing and diagnostic testing criteria were removed. Cases were then

reviewed manually to verify relevance to lameness in growing hogs. The cases were then assigned a main and other diagnosis using specific diagnostic criteria from Disease of Swine. Frequency counts of the cases in each diagnostic category were tabulated.

In objective 2 four biopsy instruments were assessed in their ability to successfully collect a joint tissue sample in cadaver pig legs. These tools were tested repeatedly using 12 rear and four front legs from euthanized finisher pigs. Usability of each instrument was recorded on a scoring rubric. Elbow, stifle, and hock joints were biopsied then opened for gross examination to assess the impact of the technique and instrument on the native tissue. Samples from each biopsy instrument were pooled in formalin and submitted to a state veterinary diagnostic laboratory to evaluate tissue type and specimen quality.

In objective 3, the main objective was to collect joint fluid samples from clinical normal finisher pigs to create clinical pathology reference intervals for collected volume, total nucleated cell count (TNCC), total protein, pH, red blood cell count (RBCC), percentage of neutrophils, percentage of lymphocytes, and percentage of large mononuclear cells for each joint. To accomplish this, 54 healthy finisher pigs were anesthetized and antemortem joint fluid was performed. On pigs in which an antemortem sample could not be attained, a post-mortem sample was collected. The joint fluid was submitted for clinical pathology analysis (for reference interval creation) and for bacterial culture, *Mycoplasma hyosynoviae* (MHS) PCR, *Mycoplasma hyorhinis* (MHR) PCR. Twelve joints on each pig were also opened and assessed for cartilage and joint tissue abnormalities.

This objective also investigated the utility of several anesthetic protocols for this procedure, including combinations of telazol, ketamine, acepromazine and lidocaine.

In objective 4, seven clinically lame commercial finishing pigs in a production flow with a history of infectious lameness received an intramuscular (IM) injection of Telazol (4.4mg/kg), Ketamine (2.2mg/kg) and Xylazine (4.4mg/kg) (TKX). Once under anesthetic, two joint samples were collected per pig, one carpus and one hock. Joint samples were submitted for bacterial culture, *Mycoplasma hyosynoviae* (MHS) PCR, *Mycoplasma hyorhinis* (MHR) PCR and clinical pathology. The clinical pathology parameters on the lame pigs were compared to the reference ranges generated in objective three.

Results

Literature review has been completed and used to formulate a case definition for the diagnosis of *M. hyosynoviae* (MHS) in growing swine. A case was deemed positive for MHS if specimens submitted from an affected group of animals demonstrating clinical lameness, had at least one positive MHS PCR on joint fluid or joint tissue and histopathological lesions consistent with MHS. From the diagnostic lab lameness case review, *Mycoplasma hyosynoviae* and infectious arthritis caused by other bacteria cases represented about 40% of the 464 lameness cases examined.

For the creation of the reference ranges, there were 37 hock samples and 46 carpus samples that were eligible for inclusion into the reference range dataset as some samples were removed for insufficient volume, blood contamination, culture contamination or synovial tissue abnormalities.

Telazol, ketamine and xylazine was the only protocol that was suitable for collection of joint fluid from market sized animals. The depth of anesthesia produced by the other protocols was insufficient. The recovery time for these procedures is several hours.

Lastly, in the clinical lameness field case, antemortem joint samples were successfully collected in 7/7 pigs. Sampled pigs recovered well and did not have adverse effects from the procedure. Diagnostic testing and clinical pathology results indicate that there did not appear to be an infectious cause of lameness in these pigs.

Industry Impact

The creation of a reference range dataset for synovial fluid from non-lame healthy finisher pigs provides a novel diagnostic tool to practitioners and production companies. In human, equine and canine medicine, clinical pathology is a core diagnostic test for lameness and arthritis. Coupled with culture, molecular testing for mycoplasma species and histology on the joint tissue, clinical pathology completes the diagnostic picture for a joint. Due to the transient nature of many infectious arthritis agents, multiple pieces of evidence indicative of infectious agents are critical for accurate diagnosis. This complete diagnostic picture allows the veterinarians to

more confidently and accurately determine a diagnostic and treatment plan to the diagnostic case. Improved diagnosis and treatment plans are a direct benefit to pigs and caretakers alike.
The implications of improving our ability to collect necessary diagnostic samples and to develop useful diagnostic assays for infectious lameness are directly and immediately relevant to swine producers. Economic losses due to lameness are far reaching, including reduced average daily gain, culls, euthanasia, cost of labor and treatment and loss of high value genetic stock. For the pig, infectious lameness is painful and reduces the pig's ability to access vital resources including feed and water. As there is increased public attention to swine production, particularly with respect to welfare and the judicious use of antibiotics, it is essential that the swine industry drives the development of tools to better identify and diagnose lameness. The resources and deliverables generated by this project are critical to informing treatment options and ensuring the judicious use of antibiotics.

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Scientific Abstract:

Arthritis reduces welfare and causes severe pain for growing swine. *Mycoplasma hyosynoviae* is believed to be a prominent cause of infectious arthritis. This proposal serves to build the basic foundation necessary to do further, solution-oriented, applied research on *Mycoplasma hyosynoviae* by building these four resources:

- Generate a standard case definition of *Mycoplasma hyosynoviae* (MHS) arthritis in growing swine based on available information from a systematic literature review and a summary of MHS cases submitted to the Iowa State University (ISU) Veterinary Diagnostic Lab (VDL) from 2010 through 2015.
- Develop, refine and field test joint fluid and joint tissue collection techniques in live pigs
- Create reference ranges for clinical pathology parameters for swine synovial fluid from clinically normal pigs and evaluate the suitability of various anesthetic protocols for ante mortem joint fluid collection.
- Compare and analyze clinical pathology parameter data for clinically lame finisher pigs from commercial operations to reference ranges from normal animals

Materials and Methods:

A literature review using PubMed, Agricola and the AASV swine information library was conducted to develop a clinical case definition for MHS arthritis in swine. Cadaver legs were used to refine joint fluid collection and joint tissue collection techniques. Various techniques were videotaped, timed and assessed in terms of the utility in procuring a joint fluid and tissue sample.

To create clinical pathology reference intervals for collected volume, total nucleated cell count (TNCC), total protein, pH, red blood cell count (RBCC), percentage of neutrophils, percentage of lymphocytes, and percentage of large mononuclear cells for each joint, 54 healthy finisher pigs were anesthetized and antemortem joint fluid was performed. On pigs in which an antemortem sample could not be attained, a post-mortem sample was collected. This objective also investigated the utility of several anesthetic protocols for these procedures, including combinations of telazol, ketamine, acepromazine and lidocaine. The joint fluid was submitted for clinical pathology analysis (for reference interval creation) and for bacterial culture, *Mycoplasma hyosynoviae* (MHS) PCR, *Mycoplasma hyorhinis* (MHR) PCR.

Lastly, seven clinically lame commercial finishing pigs in a production flow with a history of infectious lameness received an intramuscular (IM) injection of Telazol (4.4mg/kg), Ketamine (2.2mg/kg) and Xylazine (4.4mg/kg) (TKX) combined in the same syringe. Once under anesthetic, two joint samples were collected per pig, one carpus and one hock. Joint samples were submitted for bacterial culture, MHS PCR, MHR PCR and clinical pathology.

Results

Literature review has been completed and used to formulate a case definition for the diagnosis of *M. hyosynoviae* (MHS) in growing swine. A case was deemed positive for MHS if specimens submitted from an affected group of animals demonstrated clinical lameness, at least one positive MHS PCR on joint fluid or joint tissue and histopathological lesions consistent with MHS.

For the creation of the reference ranges, there were 37 hock samples and 46 carpus samples that were eligible for inclusion into the reference range dataset. Samples were removed for insufficient volume, blood contamination, culture contamination or synovial tissue abnormalities.

Telazol, ketamine and xylazine was the only protocol that was suitable for collection of joint fluid from market sized animals. The depth of anesthesia produced by the other protocols was simply insufficient to inhibit the foot withdrawal reflex, facilitate epidural placement or in some cases, achieve unconsciousness.

Discussion

The creation of a reference range dataset for synovial fluid from non-lame healthy finisher pigs provides a novel diagnostic tool to practitioners and production companies. In human, equine and canine medication, clinical pathology is a core diagnostic test for lameness and arthritis. Coupled with culture, molecular testing for mycoplasma species and histology on the joint tissue, clinical pathology completes the diagnostic picture for a joint. Due to the transient nature of many infectious arthritis agents, multiple pieces of evidence indicative of

infectious agents are critical for accurate diagnosis. This complete diagnostic picture allows the veterinarians to more confidently and accurately determine a diagnostic and treatment plan to the diagnostic case. Improved diagnosis and treatment plans are a direct benefit to pigs and caretakers alike.

Introduction:

Infectious lameness in growing pigs is an emerging problem in the United States. Lameness is an animal welfare concern and increases production costs. *Mycoplasma hyosynoviae* is considered to be the most common cause of infectious lameness. It is a dynamic organism in that its colonization of the joint can be transient and only a fraction of colonized pigs develop disease. Conventional polymerase chain reaction (PCR) testing for the pathogen is done post mortem and can be difficult to interpret without additional information about the pig, joint and joint fluid. Antemortem tests that are applicable to a broad range of lameness agents would significantly improve diagnostic testing for infectious lameness. Antemortem tests would also allow producers to monitor treatment success in affected pigs. In human and canine literature, clinical pathology of joint fluid is a core ante mortem diagnostic test for multiple type of arthritis. Clinical pathology is an analysis of the protein, pH and cell types within the joint fluid. Combined with PCR and culture, clinical pathology could be a key diagnostic tool for swine. However, a technique for the ante mortem collection of joint fluid in the field and reference ranges for normal animals for clinical pathology must be developed before these tools can be applied to commercial swine operations.

This grant will generate the framework needed to advance the swine industry's understanding of infectious lameness in growing pigs and create, refine and field test novel sampling techniques and diagnostic assays to better diagnosis lameness. The welfare and economic benefits of this project will be received directly by swine producers, veterinarians and the US commercial swine population.

Objectives:

1. Generate a standard case definition of *Mycoplasma hyosynoviae (MHS)* arthritis in growing swine based on available information from a systematic literature review and summary of MHS cases submitted to the Iowa State University (ISU) Veterinary Diagnostic Lab (VDL) from 2010 through 2015.
2. Develop, refine and test joint fluid and joint tissue collection techniques in live pigs
    - **Phase 1***
3. Create reference ranges for clinical pathology parameters for swine synovial fluid from clinically normal pigs. Evaluate several anesthetic protocols for suitability for antemortem joint fluid collection.
    - **Phase 2 and 3***
4. Compare and analyze clinical pathology parameter data for clinically lame finisher pigs from commercial operations to reference ranges from normal animals collected in objective 3.
    - **Phase 4***

*Phase refers to alive animal study

Objective 1: Generate a standard case definition of *Mycoplasma hyosynoviae (MHS)* arthritis in growing swine based on available information from a systematic literature review and summary of MHS cases submitted to the Iowa State University (ISU) Veterinary Diagnostic Lab (VDL) from 2010 through 2015.

Literature review has been completed and used to formulate a case definition for the diagnosis of *M. hyosynoviae* (MHS) in growing swine. A case was deemed positive for MHS if specimens submitted from an affected group of animals demonstrated clinical lameness, at least one positive MHS PCR on joint fluid or joint tissue and histopathological lesions consistent with MHS. There are no lesions that are pathognomonic for MHS, but the synovial tissue (joint tissue) in MHS-associated arthritis tends to be infiltrated with lymphoplasmacytic inflammation. Other types of bacterial arthritis tend to produce a neutrophilic or mixed

lymphocytic inflammation. This case definition was then applied to the retrospective case study described below.

Retrospective review of diagnostic lab cases of lameness in growing pigs greater than 7 weeks of age from 2010 to 2015.

A systematic review of cases submitted to the ISU Veterinary Diagnostic Lab between 2010 and 2015 for lameness or locomotor dysfunction was conducted for pigs greater than 7 weeks of age. To return case numbers of all cases involving lameness or locomotion dysfunction, a search using 23 diagnostic codes and 3 diagnostic tests relevant to lameness was applied to the ISU VDL case database. This VDL case database contains all diagnostic cases (accessions) generated during the specific time frame. The search strategy was developed through consultation with VDL diagnosticians and information technology specialists. From the returned cases (n=1847), information was extracted from the submission sheet and final report into a Microsoft Excel spreadsheet. These cases (n=1847) they were then further analyzed to meet inclusion criteria related to age/weight (7 to 40 weeks or >35 lbs), inclusion of appropriate samples (case must have included tissue) and case type (must be a field case). The submission sheet and final report of all remaining cases (n=1019) were then reviewed to ensure lameness or locomotor dysfunction was truly a component of the case. After non-relevant cases were removed, 464 cases involving lameness in growing pigs remained (Figure 1).

These lameness cases (n=464) were then assigned a main and additional diagnosis in accordance with the results of the final diagnostic report for that case (Table 1). Specific criteria and cases definition for each diagnosis was created and applied uniformly to the 464 cases. There were 93 cases that were considered to be primarily a case of MHS, as defined above. There were four diagnoses that made up about 80% of the cases. These four diagnoses are a diverse group including MHS, metabolic bone disease, infectious arthritis due to bacterial infection and lameness of no specific etiology. For the no specific etiology category, these are cases where there are abnormal findings in the joint, but no pathogen can be identified from specimens submitted for that case.

The 93 MHS cases were then reviewed to identify case characteristics, submission trends and co-factors common between these cases. To complete this, all the information from the submission form and final report was entered into an excel spreadsheet for these cases.

A review of case characteristics determined that the mean age of pigs diagnosed with MHS is 18.3 weeks (range 10-32 weeks). Cycle threshold values for MHS PCR were typically around 30 to 35 (range 20.3 – 42.92) and cases requesting 3 or more MHS PCR tests returned on average about 50% positive results.

An analysis of submission trends returned that 99% of cases included a history. As well, 71% of cases listed differential diagnoses with their submission form and 80% of these cases listed multiple possible differentials. The majority of submitting veterinarians selected diagnostics at the discretion of the diagnostician (81%) and submitted tissue samples representing at least 3 to 4 animals. In summary, although these cases all fit the MHS case definition, the submitting veterinarians applied a broad approach to diagnostics by listing multiple differential diagnoses, submitting samples from several pigs, and testing for several pathogens.

A review of co-factors in conjunction with a diagnosis of MHS revealed that 30 of 93 cases returned multiple diagnoses, with the most common additional diagnoses being OCD, bacterial arthritis and metabolic bone disease. The most commonly requested secondary test was culture which returned no significant growth on 78% of these cases. Erysipelas was listed as a differential and tested for in 29% of these cases, however, only returned a positive result in 1 case, and therefore is likely not an important co-factor. Other respiratory (PRRSv, IAVS, and *M. hyopneumoniae*), enteric (*Lawsonia intracellularis* and *Brachyspira spp*) and lameness (*M. hyorhinis* and *H. parasuis*) co-factors that may be identified on PCR were rarely tested for in these cases. Of

that cases that did test for co-factors, few cases returned a positive PCR for any of the cofactors. For these 93 MHS cases, there is little information that can be gleaned about the importance of co-factors. A comparison of the role of co-factors between MHS and non-MHS cases would be more informative.

The important take home messages from this review of MHS cases is as follows:

- MHS is considered to be the most common cause of grow finisher lameness
- It can be challenging to diagnosis MHS associated lameness due to the transient nature of the pathogen. There are three other causes of lameness that are as common as MHS based on the findings from the diagnostic lab case review.
- For the 93 cases, which demonstrated testing results consistent with the MHS case definition, the submitting veterinarians cast a broad net in terms of sample submission and diagnostic testing. They did not simply test for MHS.
- Therefore to increase the likelihood of diagnosis, submit high quality specimens and apply a broad diagnostic approach.

Objective 2: Develop, refine and field test joint fluid and joint tissue collection techniques in live pigs

Phase 1:

Material and Methods:

Four biopsy instruments made by Argon Medical Devices, Inc: the SuperCore™ (biopsy gun, semi-automatic, half core), Biopince™ (biopsy gun, automatic, full core), Jawz™ (forcep, manual), and TruCore™ II (biopsy gun, automatic, half core) were assessed. The assessment consisted of multiple parameters: anatomical approach to the joint, leg position, usability (time required to collect sample, durability, personnel required), utility of a sheath or guide needle, successful attainment of target tissue (synovial or joint capsule) and biopsy quality. These tools were tested repeatedly using 12 rear and four front legs from euthanized finisher pigs. Usability of each instrument was recorded on a scoring rubric. Elbow, stifle, and hock joints were biopsied then opened for gross examination to assess the impact of the technique and instrument on the tissue. Samples from each biopsy instrument were pooled in formalin and submitted to a state veterinary diagnostic laboratory to evaluate tissue type and specimen quality.

Results:

Three of the four instruments returned samples containing synovium/joint capsule (Table 2). Stifle and hock joints were the easiest joints from which to obtain a biopsy sample based on the scoring rubric criteria.
The biopsy instruments are disposable but were used multiple times in this project. The SuperCore™ was easy to position for synovial tissue collection but could bend if maneuvered aggressively in the joint without ensuring the protective sheath was fully extended beyond the sample needle. The Biopince™ was extremely sturdy during multiple uses, but hitting bone resulted in destruction of the biopsy mechanism of this gun. The Jawz™ was easily controlled but required two people to operate and was the largest (7 French). The sample collection portion bent easily on the TruCore™ II and that impeded the collection of synovial tissue significantly for this intrument. The SuperCore™ was the best biopsy instrument for collecting samples for histopathology at a 62% success rate (Table 2).

Discussion:

Applied practically to antemortem sampling, the tissue collected via biopsy can be used for PCR or histopathology. Joint fluid could also be collected at the time of biopsy to be used for PCR and culture. Together, these results can aid in the diagnosis of infectious causes of lameness via clinical pathology, bacterial culture, PCR and histopathology while avoiding euthanasia and allowing the observation of treatment efficacy in the affected animal. Due to the cost associated with the instruments and the inability to reuse them between farms due to biosecurity, it was deemed that the utilization of biopsy instruments in the field is not practical at this time. For subsequent objectives in this grant, collection of joint tissue using biopsy tools was not pursued further.

Objective 3: Create reference ranges for clinical pathology parameters for swine synovial fluid from clinically normal pigs

Phase 2 and 3

Introduction

Clinical pathology as detailed above could be a useful tool for the diagnosis of infectious arthritis. There are current no reference ranges for clinical pathology for swine joint fluid. Until a reference range for normal, non-lame animals is established, practitioners are unable to apply this tool to lameness cases in the field. Collection of joint fluid for clinical pathology can be an antemortem procedure if the pig is placed under short term anesthesia during the fluid collection. Due to reflex arcs that are associated with the extremities, pigs must be in a deep plane of anesthesia to prevent the abrupt foot withdrawal reflex during sample collection. There are many protocols published for swine anesthesia however the deep of anesthesia and suitability of the protocols for specific procedures is rarely available. Thus, the objective of phase 2 and 3 was to compare four anesthetic protocols for suitable for antemortem joint fluid collection and collect joint fluid samples from normal pigs to use for the creation of clinical pathology reference ranges.

Materials and Methods Phase 2 and 3:

This study makes use of pigs from phase 2 and 3. These two phases are separated temporally by approximately 1.5 months. Thirty healthy, non-lame pigs (phase 2 pigs) and 24 healthy, non-lame pigs (phase 3 pigs) were weighed and moved into individual pens during the studies. Both groups were finisher pigs and were an equal mix of barrows and gilts. All pigs were fed ad libitum commercial finisher feed without antibiotics for the duration of the trial.

A summary of the procedures for phase 2 and 3 is available in Figure 2 in the appendix.

Phase 2 pigs received an intramuscular (IM) injection of telazol (4.4mg/kg), ketamine (2.2mg/kg) and xylazine (4.4mg/kg) (TKX) combined in the same syringe. Phase 3 pigs were randomly allocated to one of four treatment groups:

- Group one received an IM injection of telazol, ketamine and xylazine (TKX) combined in the same syringe. The doses are 2.2 mg/kg of ketamine (100mg/ml) and 4.4 mg/kg of xylazine and telazol.
- Group two received an intramuscular injection of acepromazine, ketamine, and telazol all from the same syringe. This is composed of 0.03 mg/kg of acepromazine, 2.2 mg/kg of ketamine, and 4.4 mg/kg of telazol.
- Group three received an IM injection of 0.3 mg/kg of acepromazine and 4.4 mg.kg of Telazol all from the same syringe and an epidural. The lumbosacral epidural consisted of 2% lidocaine doses at 2.2 mg/kg up to 10mL.
- Group four received an IM injection of 0.5 mg/kg of acepromazine and 5 mg/kg of ketamine all from the same syringe and an epidural. The lumbosacral epidural consisted of 2% lidocaine doses at 2.2 mg/kg up to 10mL

Five to ten minutes from the initial IM injection, pigs were assessed for depth of sedation based on their behavior and reflex responses. To be considered eligible for the joint fluid collection procedure, the pig must have been recumbent, with a negative palpebral response, weak or negative toe withdrawal response. If these criteria were not met or the pig reacted to the insertion of the needle in the joint, then additional action was taken:

- Ensure noise levels and lighting were kept to a minimum
- Wait another 5 minutes and re-assess. If no change, administer an additional dose of 25% to 100% of the dose originally given. Additional dose volume up to discretion of attending veterinarians. Wait 10 minutes and reassess until appropriate plane of anesthesia is attained or maximum drug dose given.

Pigs had their left carpus (except group 3 and 4 in phase 3 in which carpi were not sampled) and left hock sampled and were positioned in dorsal recumbency. A sterile preparation was performed on the joints prior to sampling. The prep consisted of shaving followed by three steps: a chlorhexidine soap scrub, alcohol scrub and final prep with tincture of chlorhexidine. Steps one and two were repeated three times. Once the joints were prepared for sampling, the joint was dried and a sterile adhesive drape will be applied (Phase 2 only). Pigs in phase 3 were not draped. Sterile needles on syringes were used for the joint fluid aspirations. Clean gloves and garments were worn. Half of the volume of joint fluid collected was placed in an EDTA tube and sent for clinical pathology for each joint. The remaining aliquot for each joint was pooled by pig and sent for bacterial culture, *Mycoplasma hyosynoviae* (MHS) PCR, and *Mycoplasma hyorhinis* (MHR) PCR at the ISU VDL (figure 2). From the anesthetic injection onwards, pigs were monitored closely. Heartrate, respiratory rate, temperature and depth of sedation will be monitored at least every 10 minutes. While under sedation, the pigs were in a temperature controlled indoor environment.

Phase 2 pigs only:
Once the ante mortem sampling is completed, the pigs were euthanized in the pens. A systematic post mortem was completed for each pig including the examination of joints and the collection of tonsil for *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* PCR. Please see below for a list of samples collected at euthanasia.

Phase 3 pigs only:
After antemortem sampling, pigs in phase 3 were recovered from anesthesia, monitored for seven days and then euthanized. During the anesthesia process, the following data points were recorded:

- Number of animals for which sufficient surgical plane was achieved to allow for joint aspiration
- Mean cost of anesthesia protocol, based on total dose given to each pig
- Mean time to joint sampling from first injection, in minutes
- Mean time to sternal for pigs that reached sufficient surgical plane for sampling, in minutes
- Mean time to ambulatory for pigs that reached sufficient surgical plane for sampling, in minutes

After antemortem sampling was completed and pigs were recovered they were scored for lameness, joint swelling, and recovery as described below in the procedure for post-procedure observations. After the scoring was completed, pigs will return to their original pens.

Procedure for post-procedure observations:
During the next seven days, pigs were assessed for locomotion. Video was recorded of each animal walking towards and away from the videographer and pictures were taken of each joint that was sampled. Lameness and joint swelling were evaluated on the day of the procedure, days two and four post-procedure, and immediately prior to euthanasia using scoring system described in Nielsen et al 2006.

Euthanasia:
On day seven of post procedural observation animals were euthanized by captive bolt and exsanguination in accordance with AVMA and AASV guidelines. Insensibility was determined by absent corneal reflex and death confirmed by absence of respiration and heartbeat. Please see below for a list of samples collected at euthanasia.

Summary of sample collection for all pigs in Phase 2 and 3:
Ante mortem:

- Joint aspiration from carpus and hock
    - 0.5mL to 1mL of joint fluid into EDTA tube
    - Remainder of aliquot for MHR and MHS PCR and culture
    - Gross path description of the synovial fluid
- Assessment and scoring of joint swelling

Post-mortem:

- Tonsils collected for MHS and MHR PCR (phase 2 only)

- Joint aspiration from carpus and hock if antemortem samples was not suitable due to blood collection or small volume
    - 0.5mL to 1mL of joint fluid into EDTA tube
    - Remainder of aliquot for MHR and MHS PCR and culture
    - Gross path description of the synovial fluid
- Assessment and scoring of joint swelling
- Internal organ evaluation
    - Collect samples for diagnostic work up if abnormalities identified
- Open and evaluate the follow joints:
    - Carpus, hock, stifle, hip, elbow and shoulder
    - Assess joints for synovium, cartilage and joint fluid gross abnormalities
- Collect histopathology of synovial tissue for sampled hock and carpus
    - Submitted to Iowa State Veterinary Diagnostic Laboratory for histopathology scoring of synovial tissue abnormalities

Creation of reference intervals:
Reference intervals were established in accordance with the published guidelines for reference interval determination by the American Society of Veterinary Clinical Pathology (Vet Clin Path. 2012. 41:441-453). Reference intervals were established for collected volume, total nucleated cell count (TNCC), total protein, pH, red blood cell count (RBCC), percentage of neutrophils, percentage of lymphocytes, and percentage of large mononuclear cells for each joint. Data was evaluated with the D'Agostino and Pearson omnibus test to determine normality; data was assessed with the Tukey test for identification and removal of statistical outliers. For parametric variables, Robust methodology on native data was used to calculate the upper limit reference point, lower limit reference point, 90% confidence interval of the upper limit reference point, and 90% confidence interval of the lower limit reference point. For nonparametric variables, a Box-Cox transformation was first performed and then Robust methodology on transformed data was used to calculate the upper limit reference point, lower limit reference point, 90% confidence interval of the upper limit reference point, and 90% confidence interval of the lower limit reference point. Additionally, for each joint the assessed variables were evaluated to determine if differences existed between antemortem and postmortem collected samples. For parametric variables, a simple T-test was used to compare the means of the antemortem and postmortem samples; a Wilcoxon rank sum test was used for nonparametric variables to compare medians. Statistical significance was set at $p < 0.05$. Reference interval determination and statistical comparisons were performed with the software packages Graphpad Prism 6 and Reference Value Advisor.

Results Phase 2 and 3:
There were a total of 50 hock and 53 carpus joint fluid samples from 54 finisher hogs collected ante mortem or immediately after euthanasia. Mean weight of pigs (standard deviation) was 130.3kg (8.2). A separate reference range database was created for carpus and hock samples. Six samples, representing carpus and hock samples from three animals, were removed due to tonsil positive PCR results for *Mycoplasma hyorhinis*. Ten samples were removed due to abnormal synovium histology. Of these ten samples, seven were hock samples and three were carpus samples. Four samples, three hock samples and one carpus sample, were removed due to positive culture results. All culture results were considered skin contaminants. For the creation of the reference ranges, there were 37 hock samples and 46 carpus samples that were eligible for inclusion into the reference range dataset. Oral fluid testing for MHS and MHR from pens holding pigs used in phase 2 and 3 was negative one week prior to the start of both phases and throughout the duration of both projects. All joint fluid and tonsils were negative for MHS.

Fluid analysis was unable to calculate TNCC, RBCC, protein and pH data due to clotting in the EDTA tube for seven hock and three carpus samples. There was one carpus sample where protein and pH could not be

determined due insufficient quantity of fluid. For the hock samples, insufficient sample volume prevented the determination of protein in two samples and pH in one.
The final sample size for each of the clinical pathology parameters is available in tables 3 and 4. For the antemortem vs. postmortem analyses, there are no differences in ante and post mortem samples for the carpal samples and these two datasets were combined. For the hock samples, the antemortem samples had higher median volume and RBCC relative to the postmortem samples. The main determinant of RBCC and volume is the sample collection process itself. RBCC generally reflects blood contamination as opposed to pathological changes related to joint inflammation. For this reason, antemortem and postmortem samples were combined for the hock and used to create the reference ranges. References ranges for the carpus and the hock are listed in tables 3 and 4.

For the evaluation of each anesthetic protocol in phase 3 for suitability for joint fluid collection, it was found that TKX was the best protocol. Table 5 provides summary information about each anesthetic protocol. All of the pigs that received TKX were able to be sampled and the cost for TKX was the lowest. TKX also had the fastest recovery time. One of pigs that received TKX died before it recovered fully.
None of the pigs in any of the groups were lame or has joint swelling during the seven-day monitoring period.

Discussion
Phase 2:
The creation of a reference range dataset for synovial fluid from non-lame healthy finisher pigs provides a novel diagnostic tool to practitioners and production companies. In human, equine and canine medication, clinical pathology is a core diagnostic test for lameness and arthritis. Changes to the cell counts and specific cell types in the joint fluid can be representative of acute, chronic, infectious and non-infectious processes occurring the joint. Clinical pathology changes associated with bacterial arthritis, viral arthritis and osteochondrosis are well elucidated for humans, canine and equines. Coupled with culture, molecular testing for mycoplasma species and histology on the joint tissue, clinical pathology completes the diagnostic picture for a joint. Due to the transient nature of many infectious arthritis agents, multiple pieces of evidence indicative of infectious agents are critical for accurate diagnosis. This complete diagnostic picture allows the veterinarians to more confidently and accurately determine a diagnostic and treatment plan to the diagnostic case. Improved diagnosis and treatment plans are a direct benefit to pigs and caretakers alike.

Phase 3:
Telazol, ketamine and xylazine was the only protocol that was suitable for collection of joint fluid from market sized animals. The depth of anesthesia produced by the other protocols was simply insufficient to inhibit the foot withdrawal reflex, facilitate epidural placement or in some cases, achieve unconsciousness. The recovery time for these procedures is several hours. During this time pigs are susceptible to chilling or over-heating due to their inability to move around the pen. Therefore, if applying the TKX protocol in the field, it is important to check the rectal temperature of the pig every hour and rotate the pig into a different position. Active cooling or warming of the pig may be necessary if the pig temperature changes beyond normal limits. Prolonging resting on the same side could cause muscle damage and pigs should be rotated every hour until they are able to sit up independently. Recovery time is thought to decrease in smaller/young pigs due to different body composition and metabolism rate.

Objective 4: Compare and analyze clinical pathology parameter data for clinically lame finisher pigs from commercial operations to reference ranges from normal animals collected in objective 3.

Phase 4: Field application of anesthetic protocol and ante mortem sampling technique in clinically lame pigs in a commercial production site

Materials & Methods:

In Phase 4, seven clinically lame commercial finishing pigs (approximately 125 to 150 lbs) in a production flow with a history of infectious lameness were ear tagged with a unique numerical identifier and housed in a empty pen at two wean to finish sites (site 1=4 pigs, site 2=3 pigs). The pigs had full access to feed and water for the duration of the trial. The pigs were given received an intramuscular (IM) injection of Telazol (4.4mg/kg), Ketamine (2.2mg/kg) and Xylazine (4.4mg/kg) (TKX) combined in the same syringe. Once under anesthetic, two joint samples were collected per pig, one carpus and one hock. The clinical pathology parameters on the lame pigs were compared to the reference ranges generated in Phase 2 and 3 to identify parameters (pH, cell count, protein, specific gravity and cytology) that may differ significantly between clinically affected and non-clinically affected pigs. Additional testing such as joint fluid culture, *Erysipelas* spp PCR and MHS and MHR PCR was also conducted for each pig.

Results:

All oral fluid and joint fluid samples collected were negative on culture, *M. hyosynoviae* PCR, *M. hyorhinis* PCR and Erysipelas PCR. All pigs recovered within two hours of the procedure and their lameness score remained the sample pre and post procedure. The pigs were monitoring for seven days after the joint fluid collection. Lameness scores remained the same or improved for all but one pig who became lame on the hock joint that was not sampled. This pig was treated as per farm protocols. Five of seven carpus and six of seven hock samples were submitted for clinical pathology due to gross blood contamination of three samples. For the clinical pathology results, these are available in table 6. Results that are above stated reference ranges are shaded. Estimated time to complete the joint fluid collection was <5 minutes per pig, once the pig was under anesthesia.

Discussion:

This project demonstrates that antemortem joint taps using TKX are practical to perform in field conditions and did not result in the worsening of clinical lameness in sampled pigs. For this particular case, it does not appear that infectious agents were the primary cause of lameness. As such, the clinical pathology reflected this and there were minimal abnormalities outside of the reference ranges described previously. It is interesting to note that the joint fluid appearance was considered mild to moderately abnormal (increased turbidity and red/straw discoloration) and yet no infectious agents or clinical pathology abnormalities were identified. This indicates that mild or moderate changes to the gross appearance of joint fluid are not a sensitive indicator of etiologies or clinical pathological abnormalities, despite clinical lameness. Severe gross changes to joint fluid (extremely dark colored or high volume) are likely better associated with infectious agents and changes to clinical pathology. The recovery time in these animals was much shorter than documented in Phase 3, likely due to the smaller size of these pigs. This study indicates that antemortem joint taps are a practical tool that can be applied successfully in the field under commercial conditions. With the clinical pathology reference ranges now available and current culture and PCR tests, antemortem joint fluid collection is a useful and economical tool for veterinarians.

Appendix A: Figures and Tables

Figure 1. Summary of case selection process for retrospective lameness case review

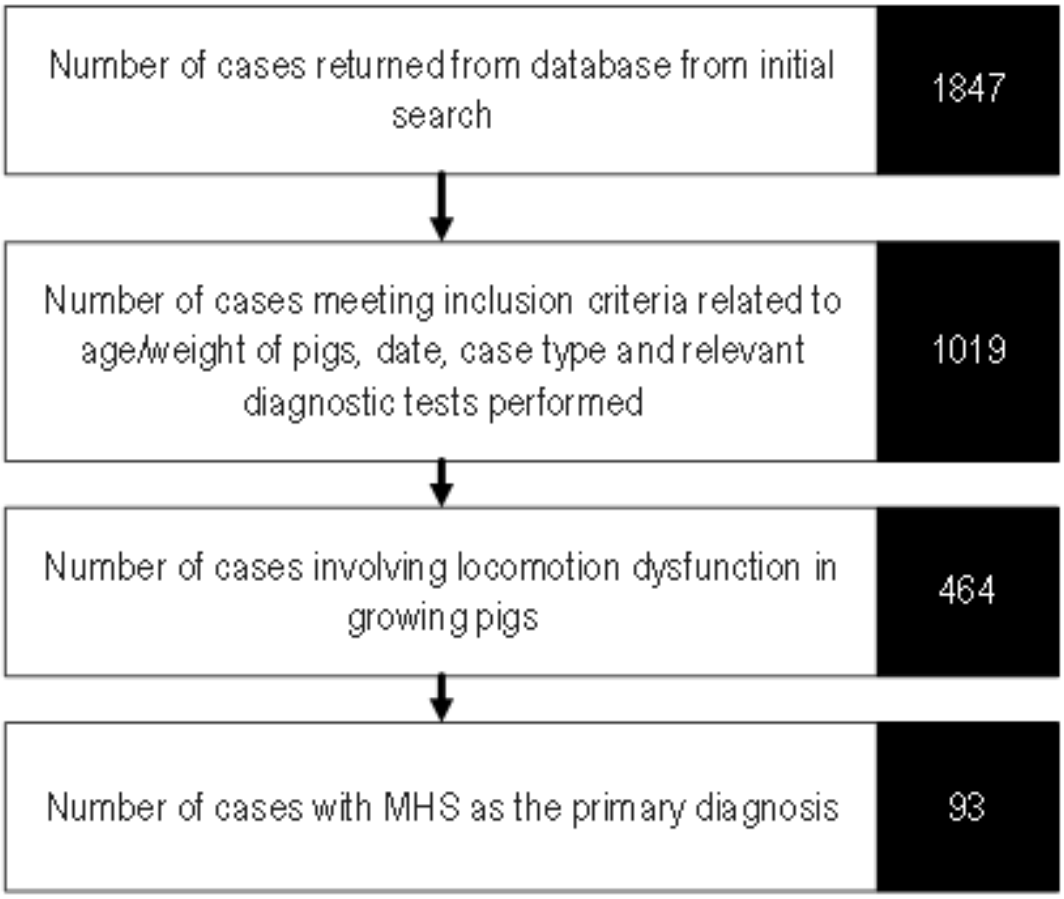

Table 1. Main diagnosis and additional diagnosis for 464 lameness cases in growing pigs

Diagnosis	Main Diagnosis		Other Diagnosis	
Lameness: significant findings of no specific etiology	101	22%	27	25%
Mycoplasma hyosynoviae	93	20%	1	1%
Metabolic (Vit D, cal/phos, rickets)	86	19%	31	29%
Infectious (bacterial)	81	18%	18	17%
Lameness documented clinically; no significant findings	43	9%	0	0%
Osteochondrosis (OCD)	29	6%	18	17%
Mycoplasma hyorhinis	19	4%	4	4%
Trauma	10	2%	8	7%
Osteomyelitis	2	0.4%	1	1%
Total	**464**	**100%**	**108**	**100%**

Table 2. Results from assessment of biopsy instruments to collect joint tissue on cadaver legs

Biopsy Instrument	Instrument Description	Picture	Usability	Samples Examined	Synovium/ Joint Capsule Samples	Synovium/ Joint Capsule Samples	Sample Quality
SuperCore™	Biopsy gun; Semi-automatic; Half core		Easy to position; Bent if maneuvered aggressively if protective sheath not fully extended	21	13	62%	Minimal crush factor
Biopince™	Biopsy gun; Automatic; Full core		Extremely sturdy during multiple uses; Hitting bone resulted in destruction	9	4	44%	Good quality; Minimal crush artifact
Jawz™	Forceps; Manual		Easily controlled; Required 2 people to operate; Largest gauge	7	3	42%	Fair quality; Moderate crush artifact
TruCore™ II	Biopsy gun; Automatic; Half core		Sample collection portion bent easily	1	0	0%	N/A

Figure 2. Phase 2 and 3 flow chart detailing methods performed and sampled collected on pigs

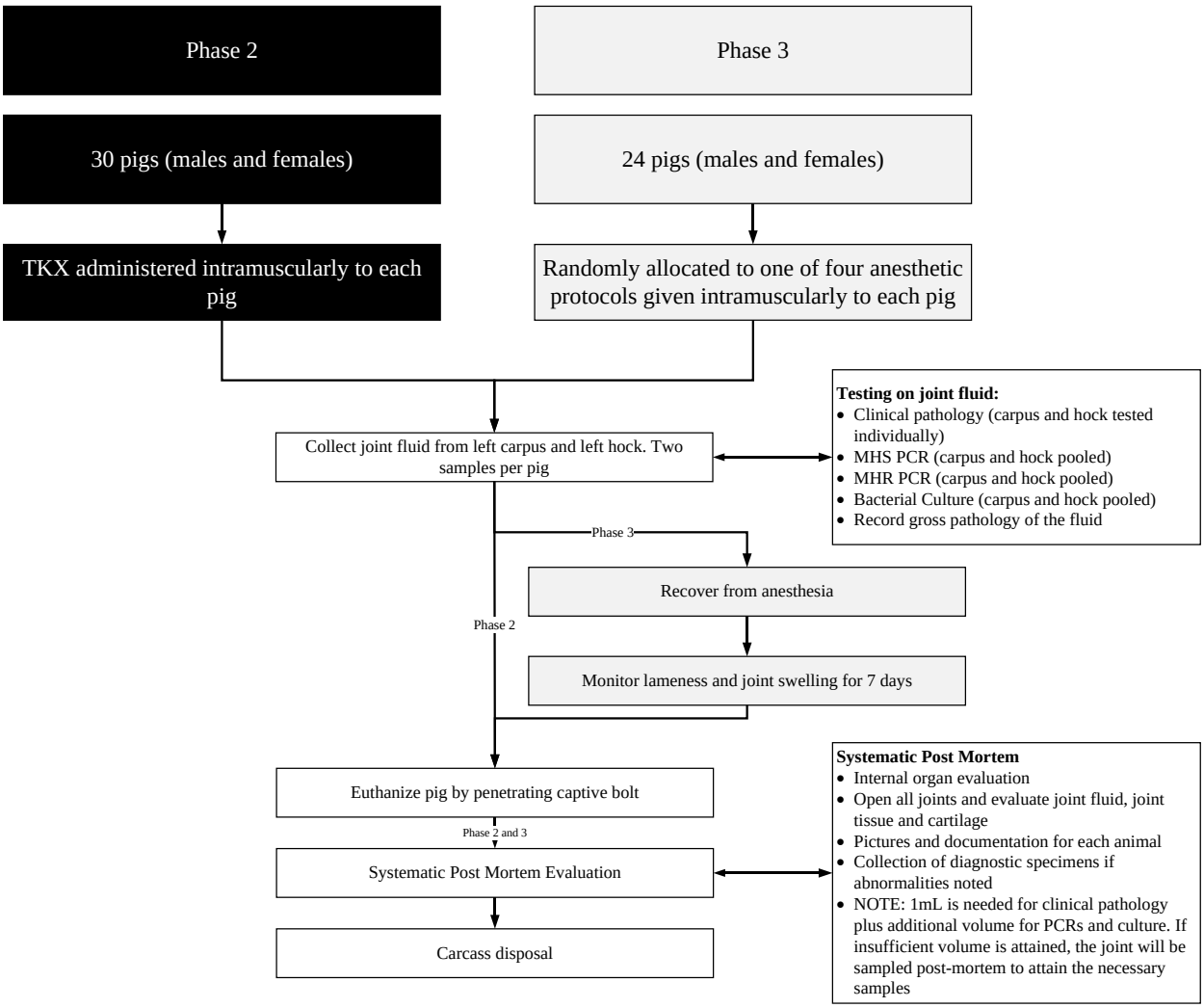

Table 3. Reference ranges for clinical pathology parameters from carpal joint fluid samples from normal, non-lame commercial finishing hogs.

Parameter	Volume	TNCC (cells/uL)	Protein	pH	RBCC	Neutrophil %	Lymphocyte %	LMC %
Lower limit of reference interval	0.36	199.4	2.01	6.08	0.002	0.27	2.61	27.46
Upper limit of reference interval	2.7	3280.7	3.64	7.18	0.27	46.49	40.58	92.01
90% CI for lower limit	0.337	186	1.85	6.005	0.0015	0.13	0	0.47
	0.383	228.4	2.23	6.155	0.0025	0.58	5.89	42.99
90% CI for upper limit	2.1305	1977.3	3.44	7.125	0.0255	31.99	35.86	88.47
	3.2695	5164.8	3.83	7.235	0.5145	65.98	44.81	95.02
Gaussian distribution of data (Y/N)	No	No	Yes	No	No	No	Yes	No
Number of samples	46	43	42	42	43	46	46	46
Number of sample for reference intervals	46	43	42	42	43	46	46	46
Outliers Removed	0	0	0	0	0	0	0	0
Type of Interval	Box-Cox Robust	Box-Cox Robust	Robust	Box-Cox Robust	Box-Cox Robust	Box-Cox Robust	Robust	Box-Cox Robust

Table 4. Reference ranges for clinical pathology parameters from hock joint fluid samples from normal, non-lame commercial finishing hogs.

Parameter	Volume	TNCC (cells/µL)	Protein	pH	RBCC (1x10^6 RBC/µL)	Neutrophil %	Lymphocyte %	LMC %
Lower limit of reference interval	0	189.2	1.27	6.15	0	0.71	0.45	24.37
Upper limit of reference interval	2.12	2367.6	3.57	6.95	0.09	33.7	56.27	95.31
90% CI for lower limit	0	154.8	1.06	6.13	0	0.15	0	15.7
	0.246	235.8	1.49	6.19	0.001	1.92	7.36	33.74
90% CI for upper limit	1.821	1488.7	3.14	6.85	0.054	26.23	48.45	87.52
	2.462	4094	3.96	7.04	0.173	41.78	63.85	104.84
Gaussian distribution of data (Y/N)	Yes	No	No	No	No	No	Yes	Yes
Number of samples	36	30	28	29	30	37	37	37
Number of sample for reference intervals	36	28	28	28	27	36	37	37
Outliers Removed	0	2	0	1	3	1	0	0
Type of Interval	Robust	Box-Cox Robust	Box-Cox Robust	Box-Cox Robust	Box-Cox Robust	Box-Cox Robust	Robust	Robust

Table 5. Comparison of various anesthetic protocols with respect to cost, success of procedure and mean recovery times

	TKX	KA + lidocaine epidural	TA + lidocaine epidural	TKA
Number of pigs	6	3	6	6
Number of animals for which sufficient surgical plane was achieved to allow for joint aspiration	6	1	6[1]	2[1]
Mean cost of anesthesia protocol (SD)	$24.98 (4.16)	$22.37 (0.82)	$38.96 (4.65)	$50.99 (3.65)
Mean time to joint sampling from first injection, in minutes (SD)	13 (9)	74 (-[2])	40 (7)	82
Mean time to sternal for pigs that reached sufficient surgical plane for sampling in minutes (SD)	125 (26)	151 (-[2])	198 (28)	363 (258)
Mean time to ambulatory for pigs that reached sufficient surgical plane for sampling in minutes (SD)	266 (73)	317 (-[2])	378 (79)	267 (-[4])
Mean lameness score during 7 day recovery[3]	0	0	0	0
Mean lameness joint swelling during 7 day recovery[3]	0	0	0	0

[1]in one pig, the joint was sampled but fluid was not collected
[2]n=1
[3] for pigs in which sufficient surgical plane was achieved to allow for joint aspiration
[4]one pig died after achieving sternal recumbency
TKA: telazol, ketamine and xylazine; KA: ketamine and acepromazine; TA: telazol and acepromazine; TKA: telazol, ketamine and acepromazine.

Table 6. Results for clinical pathology for joint fluid samples collected from seven pigs.

Joint	Color	Turbidity	TNCC (cells/µL)	Protein	pH	RBCC (1x10^6 RBC/µL)	Neutrophil %	Lymphocyte %	LMC %
Carpus	Red	Cloudy	4610	3.4	6.4	0.3	26.00	41.33	32.67
Carpus	Straw	Cloudy	45050	3	6.4	0.04	69.33	18.00	12.67
Carpus	Orange	Hazy	570	2.5	6.4	0.02	3.67	32.33	64.00
Carpus	Straw	Hazy	1360	2.5	6.4	0.01	7.67	40.33	55.33
Carpus	Red	Cloudy	950	2.6	6.8	0.14	10.67	35.33	54.00
Hock	Red	Cloudy	3790	2.9	6.4	0.3	1.00	44.67	54.33
Hock	Pink	Hazy	2660	2.2	6.4	0.02	1.67	62.00	36.33
Hock	Straw	Hazy	3930	1.8	6.8	0.02	1.00	54.67	44.33
Hock	Red	Cloudy	1090	1.3	6.8	0.23	26.67	42.67	30.67
Hock	Red	Cloudy	2190	2.3	6.8	0.45	41.00	24.33	34.67
Hock	Straw	Hazy	840	1.7	6.4	0	0.67	38.67	60.67

Five of seven carpus and six of seven hock samples were submitted for clinical pathology due to gross blood contamination of three samples.
Grey shading indicates values that are above upper limit reference ranges in table 3 and 5.