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Laura Michelle Arnold, Student

Dr. Wayne T. Sanderson, Committee Chair

Dr. Sarah Wackerbarth, Director of Graduate Studies

**An Assessment of Agreement in Detection Methods for *Histophilus somni*
in Bovine Lungs**

Capstone Project Paper

A paper submitted in partial fulfillment of the requirement for the degree of

Master of Public Health in

The University of Kentucky College of Public Health

By

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Abstract

Objectives: To examine the inter-laboratory agreement between multiplex real-time quantitative polymerase chain reaction (qPCR) assay results and aerobic culture results on bovine lung samples for detection of *Histophilus somni* (HS), and to assess associations of laboratory-derived factors on test agreement.

Methods: A survey of records from the University of Kentucky Veterinary Diagnostic Laboratory (UKVDL) was conducted to evaluate test results from grossly pneumonic bovine lung samples submitted during the period April 1, 2015 through August 31, 2018. Cohen's kappa coefficient with 95% confidence interval (CI) was calculated to describe the extent of agreement. Animal, environmental and laboratory factors were examined for associations to culture results using logistic regression analysis.

Results: Of the 417 cases analyzed, 56 were qPCR positive and culture positive for HS, 90 were qPCR positive and culture negative, 3 were qPCR negative and culture positive, and 268 were negative on both tests. Calculations yielded a Cohen's kappa coefficient of 0.43 [CI: (0.35, 0.51)], considered moderate agreement, for the qPCR assay versus aerobic culture. Results from the final model revealed male gender and cycle threshold (Ct) value measured by qPCR were significantly associated with the probability of a positive HS culture result.

Conclusion: The specificity of qPCR for detection of HS, when evaluated against the gold standard of aerobic culture, is falsely low due to the challenges of growing this organism. Bacterial qPCR assays should be routinely performed

on all cases of bovine respiratory disease, in addition to aerobic culture, to enhance organism detection.

Introduction

Bovine Respiratory Disease (BRD) is the most common cause of morbidity and mortality in cattle worldwide.¹ The traditional disease model consists of a primary viral respiratory infection in a stressed or immune-compromised calf followed by invasion of a secondary bacterial opportunist from the upper respiratory tract to the lungs, resulting in a fatal bronchopneumonia.² This model is being challenged as pathogen identification methods improve and their specific roles in pathogenesis are investigated. Indeed, the development of BRD is a complex interaction of factors associated with the animal, the multiple bacterial and viral pathogens present, and the surrounding environment which makes control efforts difficult.³ Despite decades of research and millions of dollars to improve vaccines and antimicrobials, the disease continues to have significant economic effects on the cattle industry worldwide.⁴

Calves are at highest risk for BRD development, particularly those that are lighter weight (<200 kg body weight), weaned at time of sale, commingled with susceptible (non-vaccinated) animals from multiple farms, purchased at auction, experienced an abrupt dietary change from forage to grain, or are trucked long distances.⁵ Environmental factors including dust, cold coupled with dampness, and extreme temperature fluctuations are widely accepted as risk factors, but are not well-defined.³ These risk factors have been managed with vaccine and

treatment protocols designated for “high risk calves” to reduce morbidity and mortality.⁶ One tool commonly used, “metaphylaxis” or mass medication of all new arrivals to a feeding facility with a long-acting antimicrobial, is undergoing intense scrutiny as a potential cause of antimicrobial resistance. In USDA’s Feedlot 2011 publication, it was reported that 92.6% of large feedlots (>8000 head capacity) in the U.S. mass-treat with antibiotics when a BRD outbreak was anticipated in lightweight calves.⁷ Antimicrobial use in food producing animals in the U.S. may become severely limited and tightly controlled, as it is currently in many European countries.⁸ Elaborate vaccination protocols and mass medication provide inconsistent control, therefore an effective approach to BRD will require more precise interventions to control this disease.⁹

Veterinarians and veterinary diagnostic laboratories must correctly identify pathogens in diseased lungs in order to recognize disease trends and determine how the population was exposed to the etiologic agent or agents in question. For example, the bacteria most frequently associated with BRD include *Mannheimia haemolytica* (MH), *Pasteurella multocida* (PM), *Histophilus somni* (HS) and *Mycoplasma bovis* (MB). Of those agents, HS can persist on the respiratory mucosa for long periods in the absence of clinical disease and has been considered a commensal with a relatively minor role in BRD development. However, under favorable conditions, individual HS strains can become primary pathogens. HS is known to exist within biofilms, which are highly organized aggregates of bacteria connected by an extracellular matrix that enable bacterial colonization, resistance to antimicrobials and protection from host defense

mechanisms.¹⁰ In addition, synergism between HS and bovine respiratory syncytial virus (BRSV) is proven to result in more severe respiratory disease, increased HS isolation from the lungs at postmortem, and higher IgE and IgG responses to HS antigens than in calves without concurrent BRSV infection.¹¹ The frequency of HS isolation from respiratory deaths has been increasing in Kentucky cattle with a history of rapidly fatal pneumonia and limited response to antimicrobial therapy. Detection and isolation of this bacterial strain will allow further research to determine what allows this organism to become a major pathogen and potentially lead to more effective treatment, control and vaccine improvement.

Laboratory identification of HS from diseased lungs is traditionally accomplished by aerobic bacterial culture. However, detection of HS is challenging due to its slow growth on culture plates with small pin-point colonies that can easily be overgrown by other organisms. Its growth is also inhibited by the presence of therapeutic antibiotics remaining within lung tissue.¹² Molecular diagnostics, specifically the real-time quantitative polymerase chain reaction (qPCR) assay, is an emerging technology in veterinary laboratories used to identify the nucleic acids of viral and bacterial pathogens (alive or dead) within specimens. These nucleic acid-based tests offer the ability to run many samples quickly, and can detect miniscule amounts of genetic material. Additionally, this technology allows rapid identification of pathogens that are either difficult to grow on routine aerobic culture or very time consuming and expensive to isolate (e.g. respiratory viruses).¹³

This is a retrospective study of BRD test results from a veterinary diagnostic laboratory from 2015 through 2018 to evaluate the contribution of inter-laboratory variation in detecting HS from diseased lungs. The aim is to assess the agreement between HS results from molecular assays versus traditional isolation methods, as well as examine factors such as season of the year, breed, sex, age, trace mineral status or laboratory personnel as potential confounders or effect modifiers on the test results. The secondary goal is to develop standard sampling and testing protocol recommendations for respiratory disease investigation. If BRD pathogen identification can be improved, then optimal treatment and vaccine usage can be ascertained, decreasing the need for mass medication with antimicrobials and slowing the development of resistant organisms.

Research Question: To examine the agreement between multiplex qPCR assay results and aerobic culture results on samples from bovine lung for detection of *Histophilus somni*, and to assess if agreement is associated with animal, environmental or laboratory variables.

Literature Review

The following literature review is a summary of the key concepts surrounding the bacterium *Histophilus somni*, its re-emerging role in the pathogenesis of Bovine Respiratory Disease (BRD), and the diagnostic tests used to detect its presence. A literature search was conducted using PubMed on the key words and phrases *Histophilus somni*, *Haemophilus somnus*, bovine respiratory disease, diagnostic tests, real-time quantitative PCR, culture, and Histophilosis. References cited in

key works from the initial search were examined for additional publications. The works cited were collected from published journal articles, conference proceedings, government and agricultural station reports, and book chapters primarily from the veterinary medical field.

The BRD complex affects beef cattle production worldwide.¹ It is primarily a disease of weaned calves, 7-12 months of age, and begins 7-10 days after entering a feedlot or similar feeding operation and typically reaches a peak at 14 days after arrival.¹⁴ The common BRD-associated bacteria (*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis*) are considered normal flora in the nasal passages of healthy cattle. However, with physiologic stress (such as transportation over long distances, abrupt weaning and sale through an auction, and commingling with other calves of unknown health status³) and concurrent viral infection(s), these bacteria can evade host defense mechanisms and descend into the lungs causing severe respiratory disease, specifically fibrinous bronchopneumonia.¹ Multiple risk factors contribute to disease susceptibility through complex interactions of the environment, the bacteria and viruses, and the calf's immune system. Stressed cattle are more susceptible to the viral components of BRD, including Bovine Herpesvirus-1 or BHV-1 (also known as Infectious Bovine Rhinotracheitis (IBR) virus), Bovine Viral Diarrhea (BVD) virus, Parainfluenza 3 (PI₃) virus, Bovine Respiratory Syncytial virus (BRSV) and potentially another commonly found agent, Bovine Coronavirus (BCV).

Viruses are known to damage the lining of the respiratory tract and certain viruses can suppress the immune system, allowing secondary bacterial invasion in the lungs.³ Once established in the lung, the bacterial components are responsible for triggering the immune response and the clinical signs of fever, depression, anorexia, nasal discharge, rapid breathing, and cough that often leads to death of the animal. Combinations of different bacterial and viral species can work synergistically to create even more severe disease than if operating alone.³ *Mannheimia haemolytica* (MH) is known as the most pathogenic bacterium in BRD as it causes major damage to the lungs quickly through a variety of mechanisms.¹ Most BRD therapy is directed towards killing or stopping the replication of the MH organism. However, when treatment with these antimicrobials is unsuccessful and cattle morbidity and mortality events continue to increase, it is unknown if drug resistance has developed or if a different pathogen that is not susceptible to the selected drug has become the major virulent organism. Indeed, there is no method to distinguish which bacteria or combinations of bacteria are at work in an animal with BRD just by observation of clinical signs or physical examination alone.¹⁵ Detection of the causative agent or agents through diagnostic testing is essential to assess emerging trends in order to develop effective methods to combat them.

Recently, *Histophilus somni* (HS)--formerly known as *Haemophilus somnus* or “somnus” in the cattle community vernacular, a Gram negative bacterium, has been recognized as a re-emerging pathogen in BRD in feeder cattle in the Southeastern U.S. after many years of confinement to the northern areas of the

U.S. and Canada.¹⁶ To date, it has proven difficult to treat since the organism is protected within a biofilm, a matrix that serves as a shelter from antibiotics and host immune system responses.¹⁷ Stress can trigger dispersal of large numbers of bacteria from the biofilm that can then invade and colonize the lower respiratory system.¹⁰ Unlike typical BRD outbreaks that peak at 14 days after arrival to the feedlot, HS acute pneumonia cases tend to peak much later at 25 days on feed.¹⁸ The *Histophilus somni* Disease Complex (HSDC) is the disease spectrum which occurs when HS reaches the lungs and then travels systemically to the brain, heart and joints. HSDC-affected calves may develop bronchopneumonia, severe pleuritis (infection of the membrane surrounding the lungs and heart), myocarditis (infection in the heart muscle), infectious thrombotic meningoencephalitis (infection in the brain), tenosynovitis (infection within joints), and otitis media (middle ear infection). The disease complex can occur throughout the year but most clinical cases are diagnosed between October and January.¹⁶ In the absence of consistently effective vaccine options, metaphylaxis, where treatment is applied to the entire group of cattle with an extended duration antimicrobial (either on arrival to the feed yard or administered when 10-20 % of the calves are showing clinical signs of BRD), along with 10 total days (two 5-day pulse doses) of the feed additive chlortetracycline mixed in the ration is the current recommendation for control.¹⁶

HS is a capnophilic gram-negative bacillus, considered normal flora in the urogenital and upper respiratory tract in cattle.¹⁹ Histologic lesions of vasculitis and thrombosis are hallmarks of infection.¹⁹ Studies reporting the prevalence of

the HS organism vary considerably depending on where the sample was taken within the respiratory tract, if the animal was healthy, diseased, or dead, and the method of organism detection employed. The prevalence of HS in the upper respiratory tract was found to be 42% by qPCR in healthy beef cattle prior to export from Australia.²⁰ A feedlot study published in 2017 assessing bacterial pathogens in Western Canada found HS by culture (incubated in 10% CO₂) in 22.9% of calves with BRD from trans-tracheal washes.¹⁸ In contrast, isolation of HS from the lungs in cases of fatal feedlot pneumonias was 10% in the U.S.²¹ and 14% in Canada²² by aerobic culture.

In BRD cases, HS may be found alone but often acts in concert with other pathogens.⁴ There is recognized synergism between BRSV and HS in the respiratory tract of calves that enhances disease compared to infection with either pathogen alone.¹¹ Serologic studies have concluded IgG2 antibodies are most protective against HS while IgE antibody responses (typically initiated with allergic and anti-parasitic reactions) are associated with more severe disease due to HS and of longer duration.²³ Researchers identified an IgE antibody response to HS in calves when dually infected with BRSV, at least partially accounting for the synergism observed.¹⁹ Though vaccines are available for HS and BRSV, both vaccines are known to stimulate production of IgE antibodies.²⁴ Vaccination against HS is widely practiced in the U.S. but efficacy is unproven²⁵ and is postulated to contribute to pathogenesis through IgE production.²⁴ Virtually all discussions on diagnostic testing for bacterial pathogens deem aerobic culture the “gold standard” against which all other bacterial detection

tests are judged. However, isolation of HS by aerobic culture often fails because it is difficult to grow in the laboratory; isolates prefer CO₂ for growth and, even under those conditions, the colonies are slow-growing, very small and easily overgrown by other pathogens (e.g. *Mannheimia haemolytica* and *Pasteurella multocida*) or post mortem contaminants (e.g. *Proteus* sp.).¹² Secondly, HS is particularly difficult to grow if the sample was removed from lung tissue containing residual antibiotics administered during the terminal stages of pneumonia.¹² Other factors including where within the respiratory system the sample was taken, by whom, how long after death of the animal, and transport conditions to the laboratory will affect the quality and quantity of viable bacterial cells. A ten-year retrospective study of 838 outbreaks of fatal HS infections diagnosed at veterinary diagnostic labs from Western Canada found the crude rate of isolation of HS was 34.4% (249/723) and in 205 of the 723 cases cultured, the cattle had been treated previously with antibiotics. The isolation rate from treated animals was 31.7% (65/205) compared to 35.5% (184/518) for those without treatment.²⁶

A combination of independent tests is a common method to improve validity of laboratory diagnostic tools.²⁷ To enhance the detection rate of HS, a more sensitive test that does not require growth of live organisms is needed to supplement aerobic culture. Molecular diagnostic tests such as polymerase chain reaction (PCR) assays target specific nucleic acid regions (DNA or RNA) of pathogens and amplify them for identification. Early conventional PCR assays were qualitative, indicating only presence or absence of nucleic acids. However,

current quantitative real-time PCR (qPCR) assays use a thermocycler to amplify the target regions of DNA which subsequently generates a fluorescent signal recognized by the instrument.¹³ With real-time qPCR, the cycle of threshold (Ct) or quantification cycle is the cycle at which this amplification process crosses the threshold of detection. Lower Ct values indicate more RNA or DNA in a given specimen. At the UKVDL, Ct: 20 = strong positive; Ct: 35 = weak positive; and Ct: 39 = limit of detection. No Ct value is produced if the specimen does not contain the target nucleic acid region. Therefore, a qPCR assay performed from a swab of lung tissue or bronchial content will detect the organism's nucleic acids as long as there is intact DNA or RNA present. Nucleic acid-based tests can detect growth-inhibited and dead bacteria in very low numbers, even in the presence of contaminants, resulting from common conditions such as suboptimal transport or the presence of antibiotics.²⁸ These assays offer rapid turnaround time and many samples may be run concurrently, permitting large numbers of results to be generated in hours compared to culture requiring several days.¹³ The new multiplex qPCR assays permit detection of multiple viruses and bacteria with one test. The UKVDL offers a bacterial/viral multiplex qPCR that detects four viruses (IBR, BVD, BRSV, BCoV) and four bacteria (MH, PM, HS and MB) simultaneously from one swab. A potential drawback with the qPCR assays is the ability to detect very low numbers of bacteria that may be incidental and unassociated with pneumonia.¹² However, small quantities of bacterial DNA present in a sample should be reflected in larger Ct values, close to the limits of detection for the assay.

Development and adoption of qPCR assays to rapidly detect fastidious pathogens and the comparison of the two techniques is not new in veterinary medicine. The causative bacterial agent of Johne's Disease, a chronic wasting disease of adult ruminants, is *Mycobacterium avium* subsp. *paratuberculosis* or "MAP". The traditional gold standard for MAP detection, fecal culture, can take up to 15 weeks for growth in the laboratory and has an estimated sensitivity of 30-50%.²⁹ Meanwhile, the sensitivity of fecal qPCR (using culture as the gold standard) ranges from 75-100% in cows shedding the organism in their feces in medium to high numbers, respectively.³⁰ Researchers postulated positive qPCR results are incorrectly recorded as false positives because the corresponding culture was negative, effectively reducing the calculated qPCR specificity.³⁰ Currently, researchers recognize these inherent difficulties with culture, therefore, qPCR is frequently performed first to determine the existence of MAP DNA in a sample before culture is attempted.³¹ Comparison of agreement for the two methods of detection is most often described by the kappa coefficient^{27,31,32} but a recent study utilized mixed linear modeling to identify associations between fecal qPCR and fecal culture in individual animals while adjusting for variables that could potentially alter this relationship.³³

Similarly, detection of HS by aerobic culture is known to be inherently difficult with low diagnostic sensitivity but 100% specificity. Early work published in 2000 comparing various HS detection methods including conventional PCR to bacterial culture from lung tissue concluded PCR was the most sensitive, rapid and relatively inexpensive technique available as a supplementary tool for detection

of HS.¹² An investigation³⁴ published in 2014 into the ability of qPCR assays to detect five bacterial pathogens in BRD in diseased lungs compared with culture techniques found histologic evidence of bacterial involvement in a majority of cases but only 54.6 % (82/150) yielded bacterial culture-positive results. In contrast, qPCR demonstrated positive results for 74% (111/150) of those same cases. Of the five target BRD organisms, HS was qPCR positive/culture positive in 4 cases but qPCR positive/culture negative in 31 cases. Interestingly, HS was only isolated once by culture if another bacterium was present but qPCR demonstrated frequent HS co-infection with other pathogens.³⁴ A recent (2017) study comparing sensitivity and specificity of a multiplex qPCR for bacterial pathogens in bronchoalveolar lavage fluid (BALF) versus culture found the qPCR assay was more frequently positive than the bacteriological examination for four bacterial organisms evaluated. However, the lowest kappa values for agreement between results was for HS (0.17), considered poor agreement.³⁵ All of the aforementioned studies relate a low sensitivity of the bacteriological examination for HS to its slow growth and small colonies easily overgrown by other organisms and its lack of growth when antimicrobials were utilized for treatment. Indeed, in this investigation, nearly 1/3 of HS positive cultures at the UKVDL grew too slowly to perform antimicrobial susceptibility testing (data not shown).

Methods

A retrospective approach was used for the study design. All results from the Bovine Respiratory Disease(BRD)-Bacterial Panel qPCR assays conducted on pneumonic bovine lung samples between April 1, 2015 through August 31, 2018

were utilized in this study. The final database included 417 laboratory accessions from regions across Kentucky. Cases were removed if the age was recorded as zero or the diagnosis field contained “fetal”, “abortion”, “stillbirth”, or “perinatal”. The bacterial qPCR assay results, measured in cycle threshold (Ct) values, were merged by accession number to aerobic culture results and individual animal descriptions and diagnoses. Moreover, date of submission, animal age (months), breed (dichotomized into dairy or beef), sex, season of the year submitted (winter, spring, summer, or fall), county of origin (within Kentucky), and the laboratory person responsible for collection of samples on the case (designated as 1,2,3,4, 5, other), were obtained from laboratory records. In addition, liver selenium and copper levels, measured in parts per million (ppm), were obtained and categorized as low, normal or high based on published data ³⁶ or as ‘missing’ if the test was not ordered.

Statistical Analysis

Results were examined by the four possible combinations of qPCR assay and culture results. Sensitivity, specificity, positive predictive value and negative predictive value and corresponding confidence intervals were determined with aerobic culture results as the reference method. Aerobic culture was considered the gold standard for bacteriological identification while qPCR was the comparative test. The Ct value was determined by a multiplex real-time qPCR assay and scored as negative when the generated Ct value was >39, the limit of detection. Cohen’s kappa coefficient with 95% CI was calculated to describe the extent of qPCR results agreement with the bacteriological results. Kappa values

were interpreted as follows: kappa= 0.00-.20, poor agreement; kappa= 0.21-0.40, fair agreement; kappa=0.41-0.60, moderate agreement; kappa= 0.61-0.80, good agreement; kappa= 0.81-1.00, near perfect agreement.³⁷ A Receiver-Operator Characteristics (ROC) curve was generated to visually evaluate the optimal cut-off point of the qPCR test. Calculations for the sample size, kappa statistic and ROC curve were performed using MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium).

Exploratory analyses included the use of descriptive statistics. Specifically, means, standard deviations, quartiles, and ranges were examined for continuous variables, and frequencies and percentages were examined for categorical variables of interest. Season was classified as follows: summer (June-August), fall (September-November), winter (December-February), spring (March-May). To better evaluate the relationship of qPCR Ct value to aerobic culture result, the 56 qPCR positive/culture positive cases were directly compared to the 90 qPCR positive/culture negative cases. Differences in means were assessed with a two sample independent t-test while frequencies were compared using a chi-square test for independence using the open source calculator Winpepi Version 11.65.³⁸ Due to the lack of sensitivity for aerobic culture as the gold standard, logistic regression procedures were employed to evaluate the relationship of qPCR Ct value to aerobic culture result utilizing a direct comparison of qPCR positive/culture positive against qPCR positive/culture negative results which is of primary interest in this study. The dependent variable, HS aerobic culture result, was dichotomized into growth (culture positive) or no growth (culture negative).

Regression analysis examined various factors including age, breed, sex, season of the year submitted, trace mineral (selenium and copper) status, and the laboratory employee responsible for the case. Univariate logistic regression models were first carried out to determine unadjusted associations between variables and a positive HS culture result. Variables showing a univariable association (p-value < 0.2) with the outcome were used to develop a multivariable model by a backwards stepwise selection process, retaining variables at the 5% significance level. The aim of this multivariable analysis was to determine if the association observed between the qPCR assay result and culture result was influenced by the aforementioned factors. Confounding was assessed by comparing the change in parameter estimate of the Ct value variable in the model with and without the suspected confounder. A 15% change in the Ct value estimate was considered indicative of a confounder that would subsequently be retained in the final model. All two-way interactions were evaluated in the model building process. The goodness-of-fit of the final model was assessed using the Hosmer-Lemeshow goodness-of-fit test and predictive ability was assessed by plotting the Receiver-Operator Characteristics (ROC) curve. All descriptive statistics and logistic regression analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary, NC).

Results

Table 1 presents qPCR assay and culture results. Of the 417 cases analyzed by qPCR, 56 were qPCR positive and culture positive for *Histophilus somni*, 90 were qPCR positive and culture negative, 3 were qPCR negative and culture positive,

and 268 were negative on both tests. Calculations yielded a sensitivity of 94.92% [95% CI: (86.08, 98.26)], specificity of 74.86% [95% CI: (70.12, 79.07)], positive predictive value of 38.36% [95% CI: (30.86, 46.45)], negative predictive value of 98.89% [95% CI: (96.8, 99.62)] and Cohen's kappa coefficient of 0.43 [95% CI: (0.35, 0.51)], considered moderate agreement, for the qPCR assay versus aerobic culture. The positive (sensitivity) and false-positive (1-specificity) rates of qPCR using aerobic culture as the reference method is displayed in the Receiver-Operator Characteristics (ROC) curve (Figure 1). Table 2 presents descriptive statistics for samples in each of the four possible categories. Overall, a majority of cases were male beef calves, under 1 year of age, submitted in either the fall or winter. Trace minerals, analyzed from liver samples, were within normal limits for the sample population. Utilizing a direct comparison of the 56 qPCR positive/culture positive cases to the 90 qPCR positive/culture negative cases, the mean Ct value was significantly lower (p -value < 0.0001) for culture positive cases (Mean=22.64 [95% CI: (21.82, 23.46)]) than culture negative cases (Mean=25.58 [95%CI: (24.35,26.81)]) (Figure 2). Additionally, the frequency of males having culture positive results was significantly higher ($p=0.039$) than females.

Table 3 presents results from univariate logistic regression. Variables showing a univariable association (p -value < 0.2) including Ct value, age, sex, and selenium status were used to develop a multivariable model by a backward stepwise selection process retaining variables at the 5% significance level. No variables changed the parameter estimate of the Ct value variable 15%, indicating a lack of

confounding. Likewise, all two-way interactions were evaluated and eliminated in the backward selection process (p-value > 0.05). Copper status, season, employee, and breed were omitted prior to final model evaluation.

Results from the final model, shown in Table 4, reveal both Ct value and sex have a statistically significant association with HS aerobic culture results.

Adjusting for sex, an increase in Ct value decreases the probability of a positive culture result (p-value = 0.001). Specifically, for every 1 unit increase in Ct value, holding sex constant, the estimated odds in favor of a positive culture result decreases by 13.2% [95% CI: (5.40, 20.40)]. Sex is also shown to be associated with probability of positive culture result. Specifically, the odds of a male having an HS positive lung culture result was 2.49 times that of a female, given the same qPCR Ct value. The percentage in total variation in aerobic culture results that was explained by this multiple logistic regression model, or area under the curve (AUC), was 71% (Figure 3).

Discussion

This study assessed the agreement between two diagnostic methods of detecting the organism *Histophilus somni*, from the bovine respiratory tract, using data from 417 bovine submissions to the UKVDL. The qPCR assay detected a significantly higher number of cases as HS positive than culture (n=90), similar to many other studies.^{12,34,35,39} This is not surprising in light of the qPCR assay's ability to detect the nucleic acids, in very small quantities, of growth-inhibited and dead bacteria, even in the presence of residual antimicrobials. The qPCR assay was found to be 94.92% sensitive and 74.86% specific on detecting the HS

organism when aerobic culture serves as the gold standard. Agreement between the qPCR assay and aerobic culture was considered moderate with kappa= 0.43 (0.35,0.51) although recent studies have found lower kappa coefficients for agreement.^{34,35} The higher level of observed agreement in our data was due to the high number of cases (N=268) of negative agreement (qPCR negative/culture negative), representing 64.2% of the results. Directly comparing the 56 qPCR positive/culture positive cases to the 90 qPCR positive/culture negative cases, the mean Ct value was significantly lower (p-value < 0.0001) for culture positive cases than culture negative cases. This was anticipated since the smaller the Ct value, the more infectious agent is presumed present in the sample, increasing the likelihood of viable organisms for growth. These results suggest that relying on aerobic culture alone will underestimate the presence of HS in diseased lungs.

The second aim of this investigation was to assess the potential effect of animal, environmental and laboratory factors, accessed from the laboratory information system, on the association of Ct values (continuous variable) from the bacterial qPCR assay on HS aerobic culture results (dichotomous outcome). Presenting Ct value as a continuous variable avoided the loss of information from dichotomization.³³ The results of the multivariable logistic regression revealed that Ct value is a significant predictor of HS aerobic culture results (p-value = 0.001). Specifically, a lung sample from a male calf with a Ct value above 27 or a female calf with Ct value above 24 on qPCR performed at the UKVDL has a less than 50% estimated probability of being HS culture positive. This finding

demonstrates the importance of routine bacterial qPCR testing in cases of BRD to detect HS because of the inherent difficulty of growing this organism under standard aerobic culture conditions. If HS is detected on the qPCR assay, adjustments should be made to enhance the ability to grow the organism in the laboratory, specifically incubating the inoculated blood agar plates in 10% CO₂.¹⁶ Growth of pure colonies should still be attempted to determine antimicrobial susceptibility data and, if desired, to use the organism for further study such as for vaccine development or DNA sequencing.

Interestingly, an independent variable representing male gender was a statistically significant predictor of positive HS growth. Several studies have found males at higher risk for BRD than females^{40,41} but there are conflicting results in the literature.³ The increase in the probability of HS found in this investigation is likely due to the practice of castration of bull (intact male) calves on arrival to the livestock facility after purchase. Castration is a major risk factor for BRD⁴² in addition to the stress of weaning, commingling, transport, diet change, and weather events experienced by males and females alike.⁵ A second factor may have been a shorter duration of illness for these male calves; the additional stress of castration likely contributed to quicker death and less time to implement additional antimicrobial therapy as well as less growth of opportunistic bacteria in the lungs. HS growth in the laboratory is known to be hampered by antibiotic therapy administered in the terminal stages of life. Additionally, HS cannot compete well when grown with other organisms, especially fast-growing opportunistic bacteria. A second possibility to explain the increased risk in the

male is “sex” may be a surrogate representing the purchaser of these types of calves. Typically, abruptly weaned bull calves are relatively less expensive so individuals preferentially buy these calves then castrate, vaccinate, deworm and place them on feed or grass until they reach a target weight (generally 350-400 kg body weight) before sending them to feed yards in Kansas, Iowa, Nebraska and other western states. These bull calves are less expensive because they are considered mismanaged and at “high risk” for BRD; a majority have no history of vaccination and are often trace mineral deficient due to lack of adequate supplementation at the farm of origin.³ A recommendation borne out of this study is the need for data collection on submission regarding procurement of calves, vaccination history, antimicrobials used, if castration was recently performed and by what method to better assess how these gender-related risk factors’ affect health status.

Surprisingly, other potential factors were not found to be significantly associated with HS culture positive results including age, breed, trace mineral (selenium and copper) status and laboratory employee. Age is difficult to assess since date of birth is seldom known. However, age generally correlates well with body weight unless there is an underlying disease or nutritional issue affecting growth.⁴³

Because young, lighter weight calves are known to be at higher risk for respiratory disease compared to yearlings,⁴⁴ a better parameter to assess in the laboratory setting is carcass weight measured on submission rather than age.

Breed was heavily weighted towards beef, specifically, Angus and Angus mix breeds (37.77% and 31.72%, respectively) were by far the most common breeds

submitted. The dairy industry has suffered significant decline throughout the southeastern U.S. and the dairy breed category in this study (16.22%) reflects the loss. Although overall trace mineral status was within normal limits, a qPCR positive case with low selenium status was found nearly twice as likely to have a positive HS culture result as one with normal selenium in the univariate analysis (p-value=0.09). This finding is similar to the variable “sex” in that low selenium level is most often indicative of a management problem on the farm of origin with inadequate trace mineral supplementation. Selenium is exceptionally important for proper immune function, thus low levels in the liver indicate depletion of the stored element and increased risk of infectious disease.⁴⁵ Lastly, although different persons were responsible for sample collection in the laboratory, there were no significant individual differences between results.

Season was not significantly associated with culture results although the highest incidence of BRD has been reported in the fall of the year.^{46,47} Fall is traditionally when spring-born calves are weaned and sold at auction, resulting in many calves congregating at sale barns where disease-causing organisms can easily be exchanged.³ Weather, especially the sudden and extreme changes in conditions experienced in the fall, is thought to contribute to BRD development although this link has not been confirmed.³ In a recent multivariable assessment of cohort-level factors for mortality and culling risk in US feedlots, there was a significant 3-way interaction of gender, weight and month of arrival to Midwestern feedlots in spring and summer (March-September).⁴³ Research in Australia found calves inducted into feedlots in summer and fall were at increased risk for BRD.⁴⁸

It is likely the effects of “season” reflect different factors in different geographic regions.⁴⁸ Fall and winter are generally considered the two worst seasons for calf mortality in Kentucky with 61% of the samples in this study collected during those months.

Unlike previous studies, this investigation examined associations between HS detection and risk factors gathered strictly from laboratory data, that could account for the differences in results of the qPCR assay and aerobic culture. Therefore, this retrospective analysis did have certain limitations. Because the study set was drawn only from the UKVDL, the submissions are unlikely to be representative of the national population. Distance from the farm to the lab, the fee for the postmortem examination, as well as the number and rate of mortalities on the farm likely result in selection bias.⁴⁹ Missing values were of major concern when analyzing potential risk factors. Submission forms rarely contain detailed information on the deceased animal and there was no consistent, standardized testing protocol for BRD cases during this timeframe. Although the sample size is similar to other studies of this nature, it is much smaller than feedlot studies investigating risk factors for BRD and, consequently, significant associations may have been missed. Delimitations to the study were imposed to narrow the focus to mortalities due to bronchopneumonia. The culture and qPCR data was restricted to bovine lung samples which eliminated other areas HS may be found including brain, left ventricle of the heart, multiple joints, and the larynx. Further, samples were not evaluated such as nasopharyngeal swabs or trans-tracheal washes taken from live animals as field-derived samples are subject to shipping

and storage issues that can adversely affect culture results. Perhaps more importantly, HS is present in the airways of both healthy and diseased calves so deep nasopharyngeal swabs and trans-tracheal washes may not be representative of lung infection.

It is increasingly important to meet infectious disease challenges in veterinary medicine with prevention and control measures, rather than relying on mass medication with antimicrobials. Ultimately, improvement in diagnostic techniques and analysis of the risk factors involved that cause death due to BRD can result in recommended management changes that will reduce the use of antimicrobials in food animals.

This study highlights the need for a more sensitive diagnostic test, specifically the bacterial qPCR assay, to detect *Histophilus somni*. Better methods of pathogen identification will lead to treatment and vaccine developments and allow discernment of virulence factors to differentiate commensal and pathogenic bacterial strains. Ultimately, it is the intersection of environment, host and pathogen that is crucial to understanding BRD as many factors work in concert, some manageable and others not, to create a susceptible individual or population to respiratory disease.

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Appendix

Table 1. Comparison of PCR Results to Culture Results – Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value with Aerobic Culture Results Serving as the Reference

	Culture Positive	Culture Negative	Total
PCR Positive	56	90	146
PCR Negative	3	268	271
Total	59	358	417

Sensitivity = $56/59 = 94.92\%$ [95% CI: (86.08, 98.26)]

Specificity = $268/358 = 74.86\%$ [95% CI: (70.12, 79.07)]

Positive Predictive Value = $56/146 = 38.36\%$ [95% CI: (30.86, 46.45)]

Negative Predictive Value = $268/271 = 98.9\%$ [95% CI: (96.80, 99.62)]

Kappa Coefficient = 0.43 [95% CI: (0.35,0.51)]

Figure 1: The Receiver-Operator Characteristics (ROC) Correlating PCR Positive (Sensitivity) and False-Positive (1-Specificity) Rates for a Series of Cutoff Points for the qPCR assay using aerobic culture as the reference

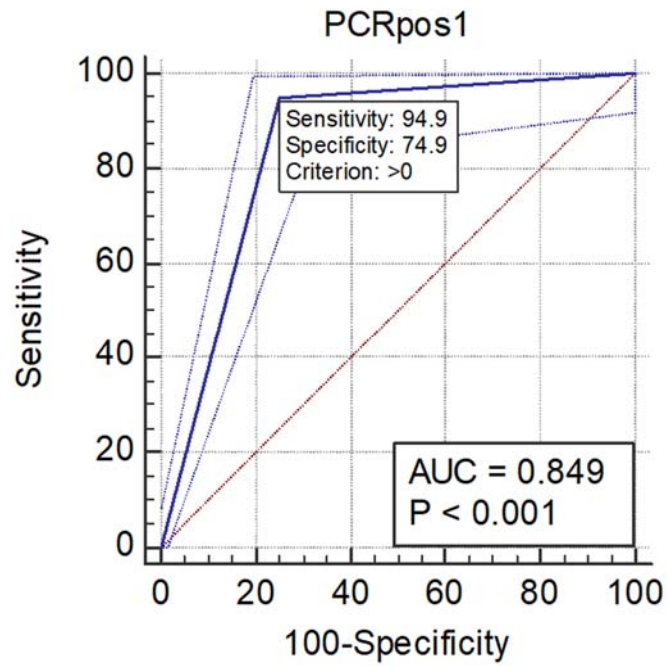
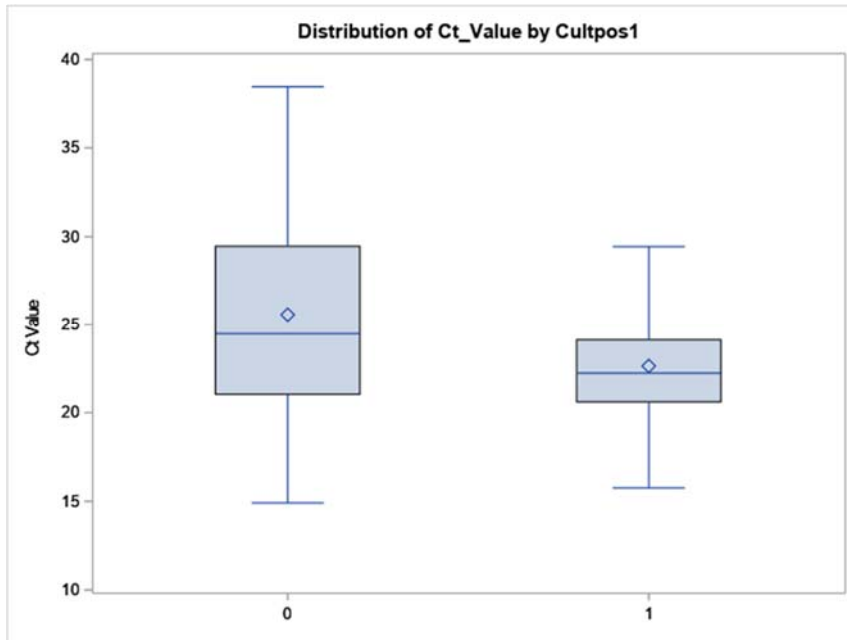


Table 2. Descriptive Statistics for Cattle in Each of the Four qPCR Assay and Culture Categories

Variable	PCR Pos/ Culture Pos	PCR Pos/ Culture Neg	PCR Neg/ Culture Pos	PCR Neg/ Culture Neg
Male	36 (73.5)	37 (44.6)	1 (100)	149 (58.0)
Female	13 (26.5)	46 (55.4)	0	108 (42.0)
Gender Missing	7	7	2	11
Less than 1 year of age	55 (98.2)	76 (89.4)	3 (100)	194 (84.4)
More than 1 year of age	1 (1.8)	9 (10.6)	0	36 (15.7)
Age Missing	0	5	0	38
Dairy Breeds	7 (14.9)	14 (16.7)	1 (50.0)	39 (16.1)
Beef Breeds	40 (85.1)	70 (83.3)	1 (50.0)	204 (84.0)
Breed Missing	9	6	1	25
Fall	14 (25.0)	23 (25.6)	2 (66.7)	91 (34.0)
Winter	15 (26.8)	24 (26.7)	0	86 (32.1)
Spring	12 (21.4)	21 (23.3)	1 (33.3)	46 (17.2)
Summer	15 (26.8)	22 (24.4)	0	45 (16.8)
Employee #1	7 (12.5)	10 (11.1)	1 (33.3)	16 (6.0)
#2	4 (7.1)	9 (10.0)	0	19 (7.1)
#3	18 (32.1)	20 (22.2)	0	83 (31.0)
#4	10 (18.0)	22 (24.4)	0	62 (23.1)
#5	12 (21.4)	23 (25.6)	1 (33.3)	69 (25.8)
Other	5 (8.9)	6 (6.7)	1 (33.3)	19 (7.1)
Liver Copper (ppm) n	41	72	3	184
Mean (SD)	55.6 (65.8)	49.0 (52.6)	77.5 (41.7)	60.1 (67.5)
Median (Q1, Q3)	32.1 (12.6, 56.7)	27.5 (14.2,62.8)	75.8 (36.7, 120.0)	33.6 (13.9, 88.9)
(Min, Max)	(1.4, 253.0)	(2.09, 237.00)	(36.70, 120.00)	(0.97, 408.0)
Missing	15	18	0	84
Liver Copper Interpretation	41	72	3	184
High	7 (17.1)	11 (15.3)	1 (33.33)	40 (21.7)
Normal	15 (36.6)	27 (37.5)	2 (66.37)	69 (37.5)
Low	19 (46.3)	34 (47.2)	0	75 (40.8)
Missing	15	18	0	84
Liver Selenium (ppm) n	41	72	3	184
Mean (SD)	0.22 (0.19)	0.31 (0.40)	0.58 (0.62)	0.34 (0.48)
Median (Q1, Q3)	0.15 (0.12, 0.22)	0.19 (0.12, 0.34)	0.33 (0.12, 1.29)	0.22 (0.14, 0.38)
(Min, Max)	(0.30, 0.89)	(0.04, 3.0)	(0.12, 1.29)	(0.04, 5.21)
Missing	15	18	0	84
Liver Selenium Interpretation n	41	72	3	184
High	4 (9.76)	14 (19.44)	1 (33.33)	25 (13.59)
Normal	4 (9.76)	11(15.28)	1 (33.33)	59 (32.07)
Low	33 (80.49)	47 (65.28)	1 (33.33)	100 (54.35)
Missing	15	18	0	84

Means and standard deviations (in parentheses) for continuous variables; frequencies and percentages (in parentheses) for categorical variables.

Figure 2: Boxplot of Mean (diamond), Median (Q1, Q3), and Minimum and Maximum (error bars) Ct (labeled “Ct”) value for Culture Positive (1) and Culture Negative (0) Results



Culture + Cases: Mean=22.64 [95% CI: (21.82, 23.46)]
Culture - Cases: Mean=25.58 [95% CI: (24.35, 26.81)]

Table 3. Univariate logistic regression results using aerobic culture positive as the outcome of interest for the 146 qPCR positive cases. Odds Ratios, 95% confidence intervals, and the p-values are presented for the assessment of unadjusted associations between Ct value, gender, age, breed, season, employee and trace mineral status (selenium and copper) on culture positive results.

Variable	HS Culture + N (%)	HS Culture - N (%)	OR Point Estimate	95% Wald Confidence Limits	Pr>ChiSq
PCR Cycle (Ct) Value	56 (38.36)	90 (61.64)	0.878	0.810-0.951	0.0014
Sex					
Male	36 (43.90)	46 (56.10)	2.227	1.034-4.800	0.039
Female*	13 (26.00)	37 (74.00)	1.000		
Age					
≤ 1 year of age	55 (40.44)	81 (59.56)	6.111	0.753-49.610	0.090
> 1 year of Age*	1 (10.00)	9 (90.00)	1.000		
Breed					
Beef Breeds	40 (36.36)	70 (63.64)	1.143	0.426-3.066	0.791
Dairy Breeds*	7 (33.33)	14 (66.67)	1.000		
Season					
Fall	14 (37.84)	23 (62.16)	0.893	0.351-2.271	0.948
Spring	12 (36.36)	21 (63.64)	0.838	0.319-2.203	0.789
Winter	15 (40.54)	22 (59.46)	0.917	0.365-2.301	0.980
Summer*	15 (38.46)	24 (61.54)	1.000		
Employee					
#1	7 (41.18)	10 (58.82)	0.840	0.182-3.880	0.778
#2	4 (30.77)	9 (69.23)	0.533	0.100-2.839	0.533
#3	18 (47.37)	20 (52.63)	1.080	0.281-4.153	0.251
#4	10 (31.25)	22 (68.75)	0.545	0.134-2.218	0.404
#5	12 (34.29)	23 (65.71)	0.626	0.158-2.481	0.630
Other*	5 (45.45)	6 (54.44)	1.000		
Copper Status					
Low	19 (35.85)	34 (64.15)	1.006	0.432-2.341	0.879
High	7 (38.89)	11 (61.11)	1.145	0.367-3.577	0.802
Normal*	15 (35.71)	27 (64.29)	1.000		
Selenium Status					
Low	33 (41.25)	47 (58.75)	1.931	0.566-6.592	0.095
High	4 (22.22)	14 (77.78)	0.786	0.159-3.873	0.378
Normal*	4 (26.67)	11 (73.33)	1.000		

*-Reference
OR-Odds Ratio

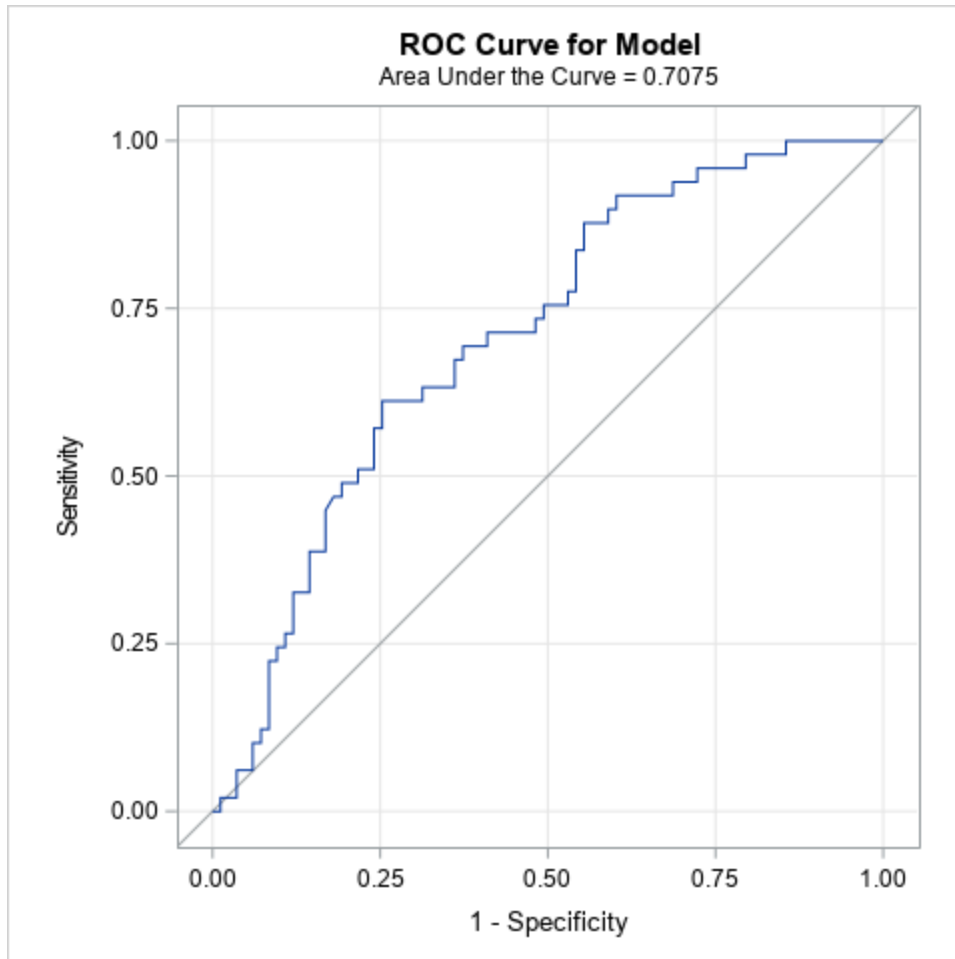
Table 4. Multivariable logistic regression model from qPCR positive cases using HS culture positive as the outcome of interest.

Variable	Beta	SE	Adjusted OR Point Estimate	95% Wald Confidence Limits	Pr> ChiSq
Ct value	-0.1420	0.0441	0.868	0.796-0.946	0.001
Gender: Male (ref: female)	0.4559	0.2064	2.489	1.108-5.588	0.027

AUC= 0.707

OR-Odds Ratio

Figure 3: Receiver Operator Characteristic (ROC) Curve generated from multivariable logistic regression analysis.



Final Model: $\text{logit}(\hat{p}) = 2.7482 + -0.1420 (\text{Ct Value}_i) + 0.4559 I_i (\text{male})$

Glossary of Terms:

Calf is any animal less than 1 year old; **Yearling** is greater than 1 year of age.

Feeders are young, weaned steers or heifers, weighing approximately 400-800 pounds. These animals may be on pasture (stocker operation) or managed in dry lot pens (backgrounding operation) and are usually fed supplementary grain. Once the target weight is met, they are marketed to feedlots and put on full feed for the slaughter market.

Feedlot or **feed yard** is a type of confinement animal feeding operation which is used for finishing livestock prior to slaughter.

Bull is an intact male bovine; **Steer** is a castrated male bovine.

Cow is a female bovine that has borne at least one calf; **Heifer** is a female bovine that has not calved.

Cow-calf operation is a farm with a permanent herd of **cows** kept by a farmer to produce **calves** for later sale.

Replacement heifer is a heifer that has been selected to be bred and placed in the beef herd.

Backgrounded cattle are feeders (steers and heifers) managed in a dry lot pen and offered supplemental feed (e.g., grain, coproducts) and forage (hay or ensiled feed). They are normally fed to approximately 800-900 pounds, then sold to a feedlot to be finished for the slaughter market.

Stocker cattle are feeders (steers and heifers) that are placed on pasture to enhance growth prior to entry into a feedlot to finish for slaughter.

Commingling beef cattle means mixing cattle from multiple source farms.

Necropsy is a surgical examination of a dead animal, in order to learn why the animal died.

Acknowledgements

First and foremost, I want to thank my two sons, Briscoe and Brody, for their unwavering support in obtaining my degree. There are not enough times I can express my gratitude for their patience and enthusiasm towards my re-entry to Graduate school. No matter what, we will always be a cord of three strands. I want to thank my chair, Dr. Wayne T. Sanderson, for suffering through my first few capstone rough drafts with nothing but support and suggestions for improvement. Perhaps more importantly, I wish to thank Dr. Sanderson for allowing me to enroll (without hesitation!) in his Epidemiology of Chronic Disease course as my first post-baccalaureate class in the spring of 2013 after 23 years away from school. Additionally, I am grateful to my other committee members, Dr. Steve Browning and Dr. Craig Carter for their time and willingness to participate in this project. A special thanks to Dr. Carter for allowing me to attend class and his ever-present optimism surrounding my degree pursuit. Finally, I would like to thank Mr. Bob Conley for his continued encouragement to finish what I started and to tackle every challenge that comes my way. You taught me the phrase, “the difficult I can do; the impossible takes a little longer.”

I have no financial or material support to disclose for this project.

Biographical Sketch

Dr. L. Michelle Arnold is a native of Louisville, Kentucky. She graduated Magna cum Laude from University of Louisville with a Bachelor's degree in Chemistry and a minor in Biology in 1986. She obtained a Doctor of Veterinary Medicine from the University of Tennessee in 1990, graduating 4th in her class. After graduation, she began her veterinary career in Sweetwater, TN at a mixed animal rural practice where she was responsible for the food animal calls, primarily dairy in nature. She launched a solo large animal exclusive practice in 1991 consisting of 95% dairy and beef and 5% equine/small ruminant. In 1997, she became part owner in a 100 cow Holstein dairy that later converted to a beef cow/calf operation in 2003. She also operated a small feeder calf weaning/preconditioning facility that handled approximately 400 calves per year on a variety of intensively grazed forages. She joined the faculty at UK in January of 2010 as the UK Ruminant Extension Veterinarian. Her areas of expertise are herd health programs, biosecurity, infectious disease and information management. She succeeded in reaching Diplomate status in Food Animal on the American Board of Veterinary Practitioners in 2012.

Michelle currently lives in Lexington, KY, and her two sons, Briscoe (21) and Brody (19) attend and reside at UK.

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