Assessment of rapid on-farm diagnostic and prognostic technologies for infectious and production-limiting diseases in dairy and beef cattle

by

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Abstract

The objective of this dissertation is to explore and assess diagnostic technologies that enable rapid, on-farm detection and prognosis of diseases in cattle. This research primarily focuses on utilizing electronic nose (eNose) technology to detect infectious and production-limiting diseases. The first chapter is dedicated to the review of volatile organic compound (VOC)-based detection technologies for disease diagnosis in cattle. The review assessed three broad categories of VOC-based detection methods, including analytical chemistry instrumentation, sensor-based devices, and biological detectors. The diagnostic performance of these VOC-based detection methods was evaluated across diseases such as bovine respiratory disease (BRD), bovine tuberculosis, Johne's disease, mastitis, metritis, ketosis, and trypanosomiasis. The review highlighted current success and existing knowledge gaps to guide future research within this field.

The second chapter evaluates the use of an eNose to diagnose BRD in Holstein calves before and after an experimental challenge with bovine herpes virus-1 and *Mannheimia haemolytica*. Twelve calves were followed over 13 days with nasal swabs and expired air collected once daily in the pre-challenge (days 1-3) and post-challenge (days 6-13) periods. The eNose was able to correctly identify pre- and post-challenge nasal swab and expired air samples with a high degree of accuracy. The eNose demonstrated potential as a field-based diagnostic tool for the detection of BRD with nasal swabs as the optimal sample type.

The third chapter expands on the work in chapter two by using the eNose for the detection of naturally occurring BRD in 363 crossbred beef cattle. The observation of clinical respiratory signs served as a comparator test to the analysis of nasal swabs by the eNose. Cattle evaluated in this study were diagnosed as either BRD or non-respiratory controls. Multiple

eNose training sets were developed and tested throughout this study for assessment of diagnostic performance. Ultimately, the eNose training set developed in chapter two provided the best agreement with clinical signs for diagnosing BRD. This research supports the development and application of universal eNose training sets that can be applied across different cattle populations.

The fourth chapter shifted the focus of chapters two and three from the diagnosis to the prognosis of BRD with an eNose. Crossbred beef cattle (n = 258) had nasal swabs collected at the time of their first treatment for BRD and analyzed by the eNose. Cattle were followed for 60 days post-sampling for determination of treatment-related outcomes. The eNose training sets were created and tested based on two classification methods: a two-outcome classification (first treatment success or first treatment failure) and a three-outcome classification (first treatment success, retreatment, or did not finish the 60-day follow-up due to BRD-related death or culling). Multiple eNose optimization methods were tested to increase the accuracy of the eNose's ability to correctly predict the observed treatment outcome. Unfortunately, the overall accuracy of the predicted treatment outcomes was poor for the eNose, regardless of which method was employed.

The fifth chapter further evaluates BRD prognosis in crossbred beef cattle; however, a different rapid detection device was utilized. Cattle (n = 84) that were evaluated for BRD a second or greater time had whole blood samples collected, which were then analyzed using a point-of-care blood analyzer to measure cardiac troponin I (cTnI) concentrations. Cattle were followed for 60 days post collection for determination of either recovery from BRD or did not finish the 60-day follow-up period due to BRD-related death or culling. Two cTnI thresholds were independently associated with an increased probability of BRD-related culling or death.

However, careful consideration regarding the test's limitations and strategic implementation is essential for effective integration into BRD management practices.

The sixth chapter compares an eNose to existing cow-side diagnostic tools for the detection of ketosis in dairy cattle. Postpartum serum, whole blood, urine, and milk samples were collected from 60 Holstein dairy cows. Laboratory serum beta-hydroxybutyrate concentrations were used to determine the true ketosis status of each cow. The eNose was used to characterize the cow's ketosis status by analyzing milk and urine samples. These eNose results were compared with the results from urine ketone test strips and a handheld blood ketone meter. Results indicated the eNose underperformed compared to conventional cow-side ketosis detection tools. Further optimization of the eNose is needed before deployment as a field diagnostic tool.

The seventh and final chapter highlights key considerations and outlines future direction for applying VOC-based detection technologies for the diagnosis and prognosis of diseases in cattle. Knowledge gleaned from chapters one, two, three, four, and six provided the framework and primary areas of focus. Emphasis is placed on the need for standardized methodologies to improve reproducibility, comparability, and eventual translation of VOC-based diagnostics into practical field applications.

In conclusion, the culmination of this work has contributed to the field of rapid, on-farm diagnostics for disease detection and prognosis in cattle. Research related to an eNose as a VOC-based detection method for both ketosis and BRD represents novel approaches for the use of this tool in live animal studies. This work supports early and accurate identification of infectious and production-limiting diseases with the goal of providing tools that help veterinarians and cattle producers improve disease management, animal welfare, and overall herd productivity.

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Chapter 1 - Volatile Organic Compound (VOC)-Based Detection Methods for Diagnosing Infectious and Production-Limiting Diseases in Cattle: Literature Review

Abstract

Volatile organic compounds (VOCs) have emerged as promising nonconventional biomarkers for disease detection in veterinary species. VOC-based detection methods including analytical chemistry instrumentation, sensor-based devices and biological detectors have enabled the characterization of these compounds. In cattle, diseases such as bovine respiratory disease (BRD), bovine tuberculosis (bTB), Johne's disease, ketosis, mastitis, metritis and others have been studied using these VOC-based detection methods. However, it is still uncertain whether characterization of VOCs can be used to differentiate healthy and diseased animals outside of controlled experimental settings. The objective of this review is to critically assess the current literature on VOC-based detection methods in cattle, evaluate reported diagnostic performance across different diseases and highlight existing knowledge gaps to guide future research within this field.

Introduction

Volatile organic compounds (VOCs) have emerged as novel biomarkers for the detection of infectious and production-limiting diseases in cattle. These compounds are found in a range of biological samples, such as breath, milk, feces, urine, and other secretions.[1] VOCs are produced as airborne metabolic byproducts of a host animal or pathogen.[2,3] Despite the diversity of sample types capable of producing VOCs, their shared property of being odor-emitting molecules makes them a robust yet underexplored approach for disease detection.

To find VOCs associated with diseases in cattle, three broad diagnostic methods have been employed including (1) analytical chemistry instrumentation, (2) sensor-based devices and (3) biological detectors. Analytical chemistry instrumentation encompasses gas chromatography–mass spectrometry (GC-MS), gas chromatography–ion mobility spectrometry (GC-IMS), proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS), proton transfer reaction-quadrupole interface time-of-flight mass spectrometer (PTR-QiTOF-MS) along with several others. These diagnostic methods provide the identification of individual VOCs and VOC profiles found within biological samples.[4] Pattern-matching algorithms can be incorporated with this technology to compare VOC profiles from healthy and diseased animals for disease classification.[5] While these instruments offer exceptional analytical precision, they are primarily limited to bench-top laboratory use although somewhat portable versions do exist.

The second category of diagnostic VOC-based detection methods are sensor-based devices, also known as electronic noses (eNoses). These tools have been demonstrated to provide rapid and potentially field-deployable diagnostics without requiring intricate laboratory analysis.[6] Differing from analytical chemistry instruments, these sensor-based devices are not designed to identify specific VOCs. Instead, they characterize an overall VOC profile ("smellprint") as different compounds bind to a variety of sensors found within the device producing unique responses.[7] Sensor responses can then be incorporated with patterns matching algorithms to differentiate disease states.

Finally, trained canines make up the primary diagnostic method within the biological detectors category. An animals' innate olfactory sensitivity to odors enables them to differentiate

VOC profiles linked to diseases.[8] This works by VOCs binding to receptors in the nasal cavity, which in turn sends chemical signals to the brain, allowing identification of distinct smells.[9] This natural olfactory system allows trained canines to recognize disease-associated VOCs. Canines have shown promise as a supplemental diagnostic method by being able to distinguish between healthy and infected animals across a variety of diseases and sample types.

Therefore, the objective of this literature review is to summarize the published literature with regard to the diagnostic performance, application and potential for VOC-based diagnostic methods to detect a variety of infectious and production-limiting diseases in cattle.

Materials and Methods

The databases utilized for the literature review include PubMed (1950 – present) and Agricola (1970 – present). An initial search strategy was created in PubMed by combining keywords and Medical Subject Heading (MeSH) terms using Boolean operators (AND, OR) to create a targeted query. Key terms, and variants of these terms, included "volatile organic compounds", "microbial volatile organic compounds", "bovine", "cattle", "cow", "diagnosis", "diagnostic tests", and "point-of-care testing". The initial query yielded only 10 results from the PubMed database. A secondary query was conducted by removing terms related to "diagnosis", "diagnostic tests", and "point-of-care testing". The results from this query yielded 570 results in PubMed and 198 results in Agricola (768 total articles). Articles captured in the second query were evaluated by the lead author (CS) for initial inclusion by reviewing the title and abstract. Articles not related to the use of volatile organic compounds for the diagnosis of diseases in cattle were removed as well as non-original and non-peer reviewed research articles (i.e., literature reviews, abstracts, conference proceedings) and duplicate articles. Of the 768 initial results, 27 articles were identified for full manuscript review. A supplemental search of the grey literature (Google Scholar) was conducted using the search string "volatile organic compounds cattle disease diagnosis". This search yielded an additional 8 articles identified for a full manuscript review.

Results and Discussion

In total, 35 original research articles met the criteria established for this literature review. The 35 articles were categorized into two groups: (1) exploratory VOC-based detection studies and (2) applied diagnostic VOC-based detection studies. Exploratory studies (n = 13) focused primarily on VOC discovery and profiling with the aim of identifying candidate biomarkers and to discriminate VOC profiles of diseased versus healthy animals. In contrast, applied diagnostic studies (n = 22) concentrated on developing and testing practical diagnostic methods. These studies reported diagnostic performance metrics capable of assessing their overall ability to diagnose disease. Table 1.1 summarizes the studies classified as exploratory VOC-based detection methods. A detailed review of this research area has been previously published.[10] The remaining 22 articles, focusing on diagnostic implementation for disease detection, are further organized by disease type and detection method and are evaluated in the following sections. Sections which do not contain studies analyzing either analytical chemistry instrumentation, sensor-based devices or biological detectors indicate that no published studies were retrieved for that disease and method type.

Bovine Respiratory Disease (BRD)

Sensor-based devices

Sensor-based devices, such as eNoses, have been evaluated for the diagnosis of bovine respiratory disease (BRD) (Table 1.2). Across two studies, eNoses have demonstrated high diagnostic accuracy, ranging from 72.5% to 100%, in correctly differentiating BRD-affected from healthy animals.[11,12] These devices have been applied to various sample types, including expired air and nasal secretions, with the latter tested on both experimentally infected and naturally occurring BRD cases.

Although both studies reported strong diagnostic performance, their methodologies differed in terms of sensor type, study design, and device training approach. Nonetheless, both investigations highlighted the reliability of nasal secretions as a diagnostic sample, with reported accuracy above 90%. Research to date has focused on identifying BRD status immediately after an acute BRD infection and has primarily been conducted in young Holstein calves. While results are limited, there is some indication that eNoses could supplement or improve upon traditional BRD diagnostic methods, which are often subjective and inconsistent.

Biological detectors

Two studies investigated the use of trained canines to detect BRD, with markedly different study designs producing different outcomes (Table 1.2). In one study, two Labrador Retrievers were trained to identify cell cultures infected with bovine viral diarrhea virus (BVDV) among samples containing bovine herpes virus-1 (BHV-1), bovine parainfluenza virus-3 (BPIV-3) or uninfected cultures.[13] Reported sensitivities for the two canines were 85% and 96.7%, with corresponding specificities of 98.1% and 99.3%. By contrast, a second study trained two scent hound-type canines to differentiate nasal swabs from cattle treated for BRD versus those not treated. Diagnostic performance was substantially lower, with detection rates between the two canines of 45.1% and 39% for positive samples.[14]

Using canines to differentiate isolated BRD pathogens in culture provided better diagnostic performance when compared to diagnosing samples collected from live cattle. While canine breed effect remains unclear, evidence suggests that targeted training on specific pathogens may improve diagnostic accuracy. However, it is unknown if training canines on a single pathogen will improve detection capabilities for diseases consisting of multiple pathogens, as is commonly found with BRD. Biological detectors offer a complementary approach to VOC-based diagnostics but require extensive training and expertise, which may limit scalability in production settings.

Comparative insights

Taken together, the available evidence suggests that eNoses currently offer a more consistent and scalable method for BRD detection than biological detectors. While one canine study demonstrated high accuracy, its findings were limited to *in vitro* conditions, whereas eNoses have shown strong performance across both experimental and naturally infected calves. Moreover, the extensive training and handler expertise required for canine deployment, especially when working around cattle, may restrict widespread application. By contrast, eNoses present greater potential for standardized use in production settings. Future work should prioritize validation of these detection methods across diverse populations, management systems and naturally occurring BRD cases to establish their practical utility in the cattle industry.

Bovine Tuberculosis (bTB)

Analytical chemistry instruments

Mycobacterium bovis and *Mycobacterium tuberculosis*, causative agents of tuberculosis in cattle and humans, can be detected using analytical chemistry instrumentation such as gas chromatography–mass spectrometry (GC-MS) and gas chromatography–ion mobility spectrometry (GC-IMS) (Table 1.3). Across three studies, the instruments' sensitivity and specificity were consistently high, and one even achieved perfect diagnostic performance.[15-17]

Crucially, these tools have been used on a variety of biological samples, such as breath and feces, and have demonstrated the ability to differentiate between vaccinated and unvaccinated animals in addition to identifying cattle infected with various *M. bovis* strains.

The majority of research using analytical chemistry instrumentation for bTB has been carried out with experimentally infected calves under controlled conditions, and despite the encouraging results, the number of animals included in these studies has been relatively small.[15,16] Stronger external validity is provided by more recent research on naturally infected adult dairy cattle, which raises the possibility that these tools could be modified for useful application in field settings.[17] All things considered, GC-MS and GC-IMS offer a strong basis for the creation of sensitive, non-invasive diagnostic instruments for bTB diagnosis in cattle.

Sensor-based devices

The ability of eNoses to identify *M. bovis* has also been assessed with both commercial and custom-built devices reporting high diagnostic accuracy (Table 7.3).[18,19] These instruments have effectively distinguished between infected and non-infected cattle when used on a variety of sample types, such as breath and sera. Both methods depended on training the device on the same population of cattle used for testing, which is common across multiple detection methods and diseases in this field of research. The device design in one study was informed by previous VOC profiling using GC-MS, while the other study used commercially available technology to achieve strong diagnostic performance results. These studies demonstrate how sensor-based VOC detection technologies can be tailored to various cattle populations and study designs. Current research suggests that eNoses can offer rapid and efficient detection of bTB, despite the small number of published studies. To verify their scalability and dependability, broader validation across larger populations and production systems is required.

Comparative insights

VOC-based detection methods for bTB, whether using analytical chemistry tools or sensor-based devices, show great promise for diagnosis. The benefits of these methods include relatively rapid analysis, non-invasive sampling methods, and the capacity to identify and classify VOC profiles unique to this disease. Even though the available data is promising, more studies with larger sample sizes and naturally infected cattle are necessary to confirm these technologies as useful diagnostic tools for everyday applications.

Johne's Disease

Analytical chemistry instruments

The most frequently studied method for identifying a *Mycobacterium avium subspecies paratuberculosis* (MAP) infection, commonly known as Johne's disease, through VOC-based diagnostic approaches is GC-MS. Across three studies evaluating cattle that were naturally infected with MAP, GC-MS showed high diagnostic accuracy (Table 1.4). Most of the studies reported sensitivities and specificities over 90%, and some studies were able to classify infected versus non-infected animals with near-perfect diagnostic accuracy.[20-22] Fecal samples were among the most studied, however, alveolar gas and air collected from cattle housing enclosures have also been investigated as non-invasive sample types. These results imply that GC-MS may provide versatile application across various biological and environmental samples and can accurately detect VOC profiles associated with MAP.

Aside from differences in the cattle population and sample source, the body of evidence suggests that GC-MS has a great deal of promise for diagnosing Johne's disease. Its value as a diagnostic tool is further demonstrated by its capacity to distinguish between MAP presence and even predict bacterial load. Like other VOC-based technologies, studies have so far been limited by small sample sizes. Larger-scale validation using the methodology described in these studies is warranted before these diagnostic technologies can be incorporated into surveillance programs.

Sensor-based devices

The detection of Johne's disease has been assessed using eNose technology in a single study (Table 1.4). An eNose, by sampling feces, exceeded the diagnostic accuracy of GC-MS in the same study by achieving a sensitivity and specificity of 100%.[22] These findings demonstrate the potential of sensor-based devices as rapid, field-adaptable substitutes for analytical chemistry instrumentation. Early results show that eNoses can effectively detect MAP-related VOC profiles in production-based settings, even though the evidence base for these devices in Johne's disease is still small. More research is required to determine reproducibility across herds, production systems, environmental contexts, and to assess device performance over time.

Comparative insights

Overall, VOC-based diagnostic methods have demonstrated great promise in the diagnosis of Johne's disease. While eNose applications show potential for on-farm deployment, GC-MS studies offer strong proof of diagnostic capability across a variety of biological and environmental sample types. Current research indicates that VOC-based diagnostics may be useful in enhancing early and precise detection of MAP, but more work is needed to validate these techniques in larger and more diverse cattle populations.

Ketosis

Sensor-based devices

Researchers have studied eNoses as VOC-based detection tools to identify ketosis in dairy cows, but the results have been variable (Table 1.5). In a study conducted on postpartum dairy cows, diagnostic performance was constrained, exhibiting inadequate sensitivity and specificity for the detection of subclinical ketosis utilizing milk and urine samples.[23] A similarly designed study indicated improved diagnostic accuracies on postpartum dairy cows, ranging from 66.7% to 100%, contingent upon the biological sample and the pattern matching algorithm employed.[24] However, in the latter study both subclinical and clinical ketosis cows were enrolled, potentially lending towards greater accuracies with a more reliable case definition. A single study explored breath samples, spiked with acetone, for experimental validation in a controlled setting.[25] The eNose in this study was able to differentiate samples of varying levels of acetone with 85.7% accuracy. This study suggested possible application for diagnosing ketosis from breath samples of dairy cattle.

Results from these studies demonstrate the eNose has the ability to detect ketosis, but reliability is not consistent across the literature. Sample and pattern matching algorithm types appear to play the largest identified role in diagnostic performance. However, disease stages likely should be explored in greater depth to assess their role in diagnostic accuracy. In general, eNoses could serve as a rapid, non-invasive test for ketosis. The identified research provides the foundation for further validation studies and refinement of the explored methodologies. Nonetheless, further comprehensive validation in field environments, especially among herds and production systems, is essential before eNoses can be regarded as dependable instruments for identifying clinical and subclinical ketosis in dairy cattle.

Mastitis

Analytical chemistry instruments

A variety of mastitis-causing bacterial pathogens have been successfully diagnosed in both experimentally inoculated and clinical milk samples using GC-MS (Table 1.6). Research has consistently shown that GC-MS has a moderate to high degree of accuracy in differentiating between various mastitis pathogens.[26-29] Overall diagnostic performance with GC-MS has been shown to increase with a decreasing number of unique pathogens presented to the instrumentation for correct classification.[26] Additionally, diagnostic performance peaked when only differentiating between Gram-positive and Gram-negative pathogens. Some pathogens were correctly classified more frequently than others, although not consistently across the studies identified in the review. It is currently unknown if certain pathogens presenting similar VOC profiles make differentiation and correct classification difficult for these devices. It has also been demonstrated that sample preparation, including incubation duration, affects diagnostic performance, with longer incubation typically increasing identification accuracy.[29] These results imply that GC-MS provides a viable method for pathogen-level discrimination in mastitis. However, dependable performance depends on sample handling and data analysis optimization.

Biological detectors

The use of trained dogs as biological VOC detectors for mastitis pathogens has also been studied. One study trained nine canines to recognize *Staphylococcus aureus* in milk samples taken from clinical mastitis cases, as well as inoculated milk samples and bacterial cultures.[30] Combined results from all canines used in the study showed sample type greatly affected sensitivity and specificity with the highest diagnostic performance from bacterial culture samples (Sn = 91.3%, Sp = 97.9%) and inoculated milk samples (Sn = 83.8%, Sp = 98%), whereas milk obtained directly from cows with clinical mastitis (Sn = 59%, Sp = 93.2%) showed lower performance. These findings demonstrate that dogs can identify volatile organic compounds (VOCs) associated with mastitis pathogens, but they also highlight the difficulties of *ex vivo* detection from field cases.

Comparative insights

VOC-based diagnostic methods for mastitis show encouraging potential for pathogen identification. Differentiation of a variety of bacterial species was possible by analytical tools like GC-MS in milk collected from clinical cases as well as inoculated samples. Sophisticated data analysis techniques and sample preparation were important factors in increasing the performance of these instruments. While biological detectors show potential for mastitis pathogen identification, they may be constrained for large scale adoptability by extensive training requirements and a lack of diagnostic accuracy with clinical samples. When taken as a whole, these studies show how VOC-based technologies can improve the diagnosis of mastitis while also emphasizing how crucial it is to optimize sample handling, analytical techniques, and field applicability for consistent and trustworthy results.

Metritis

Sensor-based devices

Only a single study has evaluated the diagnostic performance of VOC-based detection methods for diagnosing metritis (Table 1.7). An eNose was used to detect naturally occurring metritis cases in dairy cattle by analyzing uterine fluid from affected and healthy cows.[31] The eNose showed very good diagnostic performance, attaining 100% sensitivity and 91.6% specificity, despite the small sample size. These results show that in a field setting, metritis caused by multiple pathogens can be reliably detected using eNose technology over varying timepoints following parturition. Despite the scarcity of available data, this method appears promising as a rapid, non-invasive diagnostic technique that has the potential to be used in conjunction with laboratory testing and conventional clinical evaluation. To verify dependability and assess practical application in routine herd health management, more research involving larger cohorts and a variety of production systems is required.

Trypanosomiasis

Analytical chemistry instruments

VOC signatures associated with *Trypanosoma vivax* infections in cattle have been found using GC-MS (Table 1.8). In a single study, earwax samples from experimentally infected calves were examined to differentiate between healthy and infected animals.[32] Twenty VOC markers were identified and used to guide a pattern matching model to differentiate between healthy and infected calves. Results demonstrated 96.7% sensitivity and 100% specificity. Furthermore, a 13 VOC model demonstrated 94.4% sensitivity and 100% specificity in distinguishing between acute and chronic infection stages of the disease.

These findings provide evidence that GC-MS has the ability to detect trypanosomiasis accurately and non-invasively. Additionally, GC-MS may also be able to track the disease's progression. Even though the available data is restricted to a small experimental group, it shows that VOC-based methods have the potential to accurately identify parasitic infections in cattle. This research identified that trypanosomiasis and potentially other less common cattle diseases may be detected with VOC-based diagnostics and provided consideration for less commonly collected sample types, such as earwax. To evaluate field applicability and create dependable, scalable diagnostic tools, more research is required with larger, naturally infected populations.

Conclusions

VOC-based diagnostics have the potential to serve as reliable disease detection methods across a diverse range of cattle diseases. Their ability to analyze an array of noninvasive sample types and provide results more rapidly than standard diagnostic tests is promising. However, uncertainty remains around their practical application due to a lack of standardization across instrument types, device methodologies, and large differences in study designs. To advance their use, future research should prioritize validation in larger and more diverse cattle populations taking into consideration important factors such as disease model, VOC-based detection method training, disease status verification and methodology standardization to improve accuracy and reproducibility. With continued development and rigorous validation, VOC-based technologies could complement traditional diagnostics, enable earlier intervention, and ultimately enhance herd productivity, welfare, and the feasibility of routine field application.

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Table 1.1. Exploratory studies using VOCs-based detection methods for various cattle diseases.

Disease	VOC detection tool	Date Published	Study Location	Key Findings	Reference
Bovine Respiratory Disease	Gas chromatography-mass spectrometry	2004	USA	2 VOCs were associated with BRD-affected cattle.	33
Bovine Respiratory Disease	Gas chromatography-mass spectrometry	2018	USA	4 VOCs from nasal secretions and 5 VOCs from serum were different between BRD and healthy cattle, with phenol being the common marker.	34
Bovine Respiratory Disease	Proton Transfer Reaction-Quadrupole interface Time-Of-Flight Mass Spectrometry	2022	UK	Could not reliably differentiate simulated respiratory diseased vs. non-diseased cattle.	35
Bovine Respiratory Disease	Electronic nose	2010	Germany	Discrimination was possible between samples before and after BRD challenge. Correlations linked decreasing responses of multiple eNose sensors to increasing concentrations of acute phase proteins in the blood.	36
Bovine Tuberculosis	Gas chromatography-mass spectrometry	2019	USA	Identified various VOCs as potential biomarkers for identifying *Bacillus Calmette-Guérin* (*M. bovis* vaccine strain) and *M. kansasii.*	37
Bovine Tuberculosis	Gas chromatography-mass spectrometry	2023	Romania	Identification of 3 breath, 9 feces, and 3 skin tentative VOCs for disease detection.	38
Bovine Tuberculosis	Electronic nose	2015	Korea	Identified VOCs to discriminate TB-infected and TB-free sera of cattle.	39
Johne's Disease	Gas chromatography-mass spectrometry	2013	Germany	34 VOCs were identified as potential biomarkers to differentiate MAP cultures from blank cultures.	40
Johne's Disease	Proton transfer reaction time-of-flight mass spectrometry	2019	Germany	Identified 2 VOCs to discriminate groups with MAP-infected vs. non-infected and VOCs emission differences.	41
Johne's Disease	Gas chromatography-mass spectrometry	2019	USA	No unique biomarkers for *M. avium ssp. Paratuberculosis* (MAP).	37
Mastitis	Gas chromatography – mobility spectrometry	2005	Sweden	Identified VOCs to differentiate mastitic milk and milk from healthy cows.	42
Mastitis	Electronic nose	2005	Sweden	Mastitic milk from cows with acute clinical mastitis could be differentiated from healthy milk.	42
Mastitis	Gas chromatography – mobility spectrometry	2018	China	1 VOC was significantly correlated with growth of *Staph. aureus*.	43

Metritis	Electronic nose	2021	Russia	Differentiate between the composition of the VOCs of the cervical postpartum secretions with the normal and pathological course of the postpartum period.	44
Poisonous plant intoxication: *Stryphnodendron rotundifolium*	Gas chromatography – mobility spectrometry	2020	Brazil	Identified 2 VOCs to discriminate intoxicated vs. non-intoxicated.	45

Table 1.2. Bovine Respiratory Disease (BRD): applied diagnostic VOC-based detection methods.

VOC detection tool	Infection type	Study Population	Biological sample type	Date Published	Study Location	Key Findings	Reference
Electronic nose	Naturally occurring BRD	25 naturally occurring BRD and 25 clinical healthy Holstein calves	Nasal secretions	2021	Russia	Identified BRD-affected and healthy cattle with 100% accuracy.	12
Electronic nose	Experimental infection of BHV-1 and *M. haemolytica*	12 Holstein calves used as healthy and then infected	Expired air and nasal secretions	2025	USA	Expired air: 72.5% and nasal secretions: 97.8% correct identification of BRD positive samples.	11
Trained canines	Cell cultures of BVDV, BHV-1, BPIV-3, and uninfected.	65 trials (8 cultures in each trial) with up to 1 BVDV positive culture per trial. 44 blank trials with 0 BVDV positive cultures per trial	Viral cell culture	2016	USA	Sensitivity for identifying BVDV infected cell cultures vs BHV-1, BPIV 1, and uninfected cell cultures: Dog 1- 85% and Dog 2- 96.7%. Specificity: Dog 1- 98.1% and Dog 2- 99.3%.	13
Trained canines	Natural-occurring BRD	82 BRD-negative and 32 BRD-positive crossbred beef cattle	Nasal secretions	2022	USA	Dog 1: 45.1% and dog 2: 39% correct identification of cattle who developed BRD within 20 days of arrival to the feedyard.	14

Table 1.3. Bovine Tuberculosis (bTB): applied diagnostic VOC-based detection methods.

VOC detection tool	Infection type	Study Population	Biological sample type	Date Published	Study Location	Key Findings	Reference
Gas chromatography-mass spectrometry	Experimentally infected with *M. bovis*	7 non-infected and 16 infected male Holstein calves	Breath	2014	USA	83.8-97.4% sensitivity and 96.4-99.2% specificity for different *M. bovis* strains.	15
Gas chromatography-mass spectrometry	Experimentally infected with *M. bovis*	20 Holstein calves used as healthy and then infected, half vaccinated with BCG	Feces	2017	USA	100% sensitivity and 100% specificity.	16
Gas chromatography – ion mobility spectrometry	Natural infection of *M. tuberculosis*	12 bTB-negative dairy and 19 bTB-positive dairy cows	Feces	2023	Spain	91.66% sensitivity and 99.99% specificity.	17
Electronic nose	Experimental infection of *M. bovis*	8 calves used as healthy and then bTB-infected and 19 other uninfected cattle	Blood	2005	Ireland	100% correct identification of infected and noninfected cattle.	18
Electronic nose	Natural infection of *M. bovis*	8 bTB-infected and 14 bTB-free cattle	Breath	2012	USA	100% sensitivity and 79% specificity.	19

Table 1.4. Johne's Disease: applied diagnostic VOC-based detection methods.

VOC detection tool	**Infection type**	**Study Population**	**Biological sample type**	**Date Published**	**Study Location**	**Key Findings**	**Reference**
Gas chromatography-mass spectrometry	Natural infection of *M. avium ssp. paratuberculosis* (MAP)	10 MAP-negative and 10 MAP-positive fecal and tissue samples	Feces and antemortem tissue	2021	Germany	Accuracy of MAP presence for feces = 98.4% and tissue = 100% and MAP growth score for feces 88.6% and tissue = 83.9%.	20
Gas chromatography-mass spectrometry	Natural infection of *M. avium ssp. paratuberculosis* (MAP)	47 MAP-negative and 30 MAP-positive dairy cattle	Alveolar gas, enviromental air and feces	2021	Germany	Fecal- AUC-ROC: 0.94- 0.96, alveolar gas- AUC-ROC: 0.82-0.95 and stable air-AUC-ROC: 0.87-0.91 over multiple study days.	21
Gas chromatography-mass spectrometry	Natural infection of *M. avium ssp. paratuberculosis* (MAP)	48 MAP-negative and 28 MAP-positive cattle	Feces	2024	Australia	98.1% Sensitivity and 92.3% Specificity.	22
Electronic nose	Natural infection of *M. avium ssp. paratuberculosis* (MAP)	48 MAP-negative and 28 MAP-positive cattle	Feces	2024	Australia	100% Sensitivity and 100% Specificity.	22

Table 1.5. Ketosis: applied diagnostic VOC-based detection methods.

VOC detection tool	Infection type	Study Population	Biological sample type	Date Published	Study Location	Key Findings	Reference
Electronic nose	Field cases of subclinical ketosis	60 healthy and subclinical ketosis cows	Milk and urine	2023	USA	Variable and overall poor sensitivity and specificity for detecting subclinical ketosis.	23
Electronic nose	Field cases of clinical and subclinical ketosis	10 ketosis cows, 71 subclinical ketosis cows and 75 healthy cows	Milk, feces, and blood	2024	China	Accuracy of the detection of ketosis in milk = 87.5%, feces = 100% and blood = 66.7%.	24
Electronic nose	Acetone spiked samples imitating ketosis breath concentrations	Acetone spiked (180 samples distributed from 2 to 10ppm) and 179 normal air samples	Air	2025	Italy	Highest accuracy across different acetone concentrations was 85.7%.	25

Table 1.6. Mastitis: applied diagnostic VOC-based detection methods.

VOC detection tool	Infection type	Study Population	Biological sample type	Date Published	Study Location	Key Findings	Reference
Gas chromatography – mobility spectrometry	*Staph. aureus,* Coagulase-negative *Staph.* (CNS), *Strep. dysgalactiae, Strep. uberis,* and *E. coli*	50 field mastitis milk samples (5 from each pathogen) and 10 milk samples from cows without clinical mastitis	Milk	2008	Netherlands	Correct classification between 5 pathogens: 66%, 4 pathogens: 93%, gram-positive vs gram-negative: 94%.	26
Gas chromatography – mobility spectrometry	*Staph. aureus*, Coagulase-negative *Staph.* (CNS), *Strep. dysgalactiae, Strep. uberis*, and *E. coli*	25 field mastitis milk samples (5 from each pathogen) and 15 inoculated milk samples (3 from each pathogen)	Milk	2009	Netherlands	93% correct prediction for inoculated milk samples across 5 mastitis pathogens.	27
Gas chromatography – mobility spectrometry	*Staph. aureus*, Coagulase-negative *Staph.* (CNS), *Strep. dysgalactiae, Strep. uberis*, and *E. coli*	25 field mastitis milk samples (5 from each pathogen)	Milk	2009	Netherlands	Correct identification of mastitis pathogens were 88%. 8 hr milk incubation time was optimum.	28
Gas chromatography – mobility spectrometry	Experimentally inoculated milk samples of *Staph. aureus, Strep. dysgalactiae*, and *Strep. uberis*	33 samples (11 per pathogen) used for training, 15 samples (5 per pathogen) used for testing	Milk	2015	Netherlands	Correct identification of mastitis pathogens samples based on total ion count traces of the GC-MS: 67% for 6h and 60% for 8h of sample incubation. Correct identification of mastitis pathogens samples based on analysis of	29

						mass traces of the GC-MS: 80% for 6h and 93% for 8h of sample incubation.	
Trained canines	Field strain of *Staph. aures* grown and isolated on agar plates, inoculated in milk and collected from cows with clinical mastitis	Mastitis milk samples from clinical cases of Staph. aureus, E. coli, Strep uberis, Strep. dysgalactiae, P. aeruginosa, and C. albicans	Milk	2018	Germany	Identification of Staph. aureus against other mastitis pathogens on agar plates (Sn= 91.3%, Sp= 97.9%), inoculated milk (Sn=83.8%, Sp=98%), and milk from mastitic cows (Sn=59%, Sp= 93.2%) combined from 9 dogs enrolled in the study.	30

Table 1.7. Metritis: applied diagnostic VOC-based detection methods.

VOC detection tool	Infection type	Study Population	Biological sample type	Date Published	Study Location	Key Findings	Reference
Electronic nose	Naturally occurring metritis	42 metritis and 28 healthy dairy cows	Uterine fluid	2014	Germany	Sensitivity of 100% and specificity of 91.6%.	31

Table 1.8. Trypanosomiasis: applied diagnostic VOC-based detection methods.

VOC detection tool	Infection type	Study Population	Biological sample type	Date Published	Study Location	Key Findings	Reference
Gas chromatography-mass spectrometry	Experimentally infected with *T. vivax*	6 Brazilian Curraleiro Pé-Duro calves used as healthy and then infected	Earwax	2022	Brazil	Healthy vs. infected: 96.7% sensitivity and 100% specificity and acute vs. chronic infection: 94.4% sensitivity and 100% specificity.	32

Chapter 2 - Cyranose® 320 eNose Effectively Differentiates Pre- and Post-Challenge Respiratory Samples in an Induced Bovine Respiratory Disease Model

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Abstract

Field-based diagnostic technologies which aid in the early detection of bovine respiratory disease (BRD) are of great need, given the rising attention related to animal welfare and antimicrobial stewardship. This induced BRD study followed 12 Holstein calves through pre-challenge (day 1–3) and post-challenge (day 6–13) periods with daily sampling of nasal secretions with nasal swabs and expired air with air collection bags for determination of BRD status by use of an electronic nose (eNose). Animals were challenged with bovine herpes virus-1

(BHV-1) on day 3 following sample collection and *Mannheimia haemolytica* on day 5. Results demonstrated a high degree of accuracy for the eNose in correctly classifying pre-challenge samples for nasal swabs (93.5%) and expired air (96.8%). Post-challenge correct classification by the eNose was 97.8% for nasal swabs and 72.5% for expired air samples. Logistical regression was used to determine the probability of agreement between eNose classification and actual animal BRD status by study day. The largest discrepancy between nasal swab and expired air samples fell on days 6 and 7, immediately following the bacterial challenge. The eNose demonstrated potential as a field-based diagnostic tool for the detection of BRD with nasal swabs as the optimal sample type.

Introduction

Bovine respiratory disease (BRD) is the most significant health challenge facing United States commercial cattle feeding operations and consequently results in substantial economic losses for the industry each year [1]. Costs commonly associated with BRD include prevention, treatment of sick cattle, decreased productivity in the form of lower average daily gains, death loss, and labor. The development of field-based diagnostics for the early detection of BRD is gaining increasing attention, driven by growing concerns over animal welfare and the rising threat of antibiotic resistance in the livestock industry [2]. The ability to provide rapid, accurate, and easy-to-interpret results, which can be implemented shortly after diagnosis to guide timely treatment, has shown to be a definitive need for the cattle industry. However, validation of field-based diagnostic tools is complicated by the lack of an affordable and practical gold-standard diagnostic test [3].

Currently, antemortem diagnosis of BRD in commercial cattle feeding operations is primarily accomplished by animal caretakers based on visual observation of clinical signs.

Clinical illness scoring systems, such as the Wisconsin [4] and California scoring systems [5] for pre-weaned dairy calves and the less structured DART (depression, appetite loss, respiratory character, and temperature) system [6] for feedlot cattle, have been used to provide more objective criteria for BRD diagnosis and treatment. Unfortunately, these systems rely on proper training of the animal caretaker to determine a final diagnosis with test accuracy largely based on the individual scorer's ability to detect clinical signs [7,8]. Due to a consistent lack of accuracy in diagnosing BRD from clinical signs alone, ancillary tests, such as body temperature measurement [9], thoracic auscultation and lung ultrasound with associated scoring systems [10-13], are occasionally implemented to help in the diagnosis. Regrettably, these tools and associated scoring systems also require training of the animal caretaker before implementation and still vary widely based on the animal caretaker's skill in operating such modalities [14].

Field-based diagnostic tests which provide objective results pertaining to a BRD specific diagnosis and depend only minimally on the skill of an individual in operating and accurately interpreting the test are limited. Computer-aided lung auscultation (CALA) is one of the only validated field-based technologies for feedlot cattle in the diagnosis of BRD [15]. CALA allows diagnostic results to be consistent from test operator to operator [16]. Although not validated or widely used in a field setting, an on-farm colorimetric detection assay utilizing loop-mediated isothermal amplification (LAMP) has been tested for the detection of common BRD bacterial pathogens, including *Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni* [17]. It detects the presence of these specific BRD bacteria in respiratory samples based on a color change detected by computer software. A limitation inherent to diagnosing BRD with LAMP pertains to its sensitivity in detecting BRD pathogens which are commensals of the bovine respiratory tract.

Another field-based diagnostic technology which is not validated or widely used in a field setting for the diagnosis of BRD is the electronic nose (eNose). This device detects volatile organic compounds (VOCs) emitted from a biological sample via an internal bank of chemical sensors [18]. VOCs found within the respiratory tract are products of viral, bacterial, and inflammatory origin [19-21]. eNoses analyze sample VOC profiles and output categorical results based on pre-training of the device for specific disease processes or pathogens, thus allowing the animal caretaker to implement the device on-farm with ease. An eNose has been previously used to test nasal secretions obtained via nasal swabs of calves naturally infected with BRD (Mycoplasma bovis and bovine adenovirus-3). The eNose was able to correctly classify 10 calves as either "sick" or "healthy" with 100% accuracy [22]. Other studies have utilized an eNose for the detection of bovine tuberculosis in cattle. Cho et al. [23] demonstrated differentiation of 11 bovine tuberculosis-infected serum samples from 10 bovine tuberculosis-free serum samples using principal component analysis. Additionally, Fend et al. [24] were able to correctly classify 16 unknown sera samples from calves as either infected or uninfected with *Mycobacterium bovis* using a discriminant function analysis model. A single study assessed the ability of an eNose to characterize blood serum samples from cattle experimentally infected with *Mannheimia haemolytica* and non-infected cattle [25]. Results of this study compared eNose sensor responses to acute-phase protein concentrations with differentiation between infected and uninfected animals. The existing literature is deficient in studies demonstrating the use of a commercially available eNose for detecting BRD due to pathogens commonly found in United States cattle feeding operations.

This study utilizes the commercially available Cyranose® 320 eNose in calves experimentally induced with BRD pathogens (bovine herpes virus-1 [BHV-1] and *Mannheimia* *haemolytica*) to determine its capability for accurate BRD status classification. The objectives of this study are to determine the ability of the eNose to correctly differentiate calves pre-BRD challenge from the same calves post-BRD challenge and to optimize the sample type for use on the eNose to diagnose BRD.

Materials and Methods

Experimental Design and Enrollment Criteria

This study was conducted over a two-week period in July and August of 2022 at the Kansas State University College of Veterinary Medicine. The study population consisted of twelve 150 kg (range: 143–172 kg) intact male Holstein calves, approximately 5 months of age, sourced from a commercial dairy. Calves were transported approximately 129 km directly from the commercial dairy to the study location where they were acclimated overnight on the day of arrival (acclimation—study day 0) before the study commenced. Calves included in the study were not previously vaccinated or treated for respiratory disease at the source location and were determined to be clinically healthy by a large-animal veterinarian on arrival to the study location. Lung ultrasound was performed on each calf prior to enrollment. No lung lesions were observed on ultrasound for any of the animals enrolled in the study. Prior to study initiation, calves (n = 12) were individually identified with a single ear tag and then were randomly assigned to two equal groups (n = 6). Two groups of six calves each were housed in separate pens with an open-front shed and 9.2 × 18.4 m concrete pad. Study calves were fed a ration containing corn, oats, and soybean meal with monensin and ad libitum access to prairie hay and water.

During the study period, 264 respiratory samples, consisting of 132 expired air and 132 nasal swab samples, were collected from the 12 study animals. Throughout the study each calf was observed at least twice daily for signs of clinical illness. Prior to study initiation a treatment protocol was established for animals meeting clinical illness criteria in accordance with the on-site attending veterinarian. Sample collection and clinical illness observation were conducted by CSS, LFW, BVL, and MDA. On study days 1 and 2, each calf had respiratory samples collected once per day. Half of the study population (n = 6) had expired air and nasal swabs collected in the morning while the other half had respiratory samples collected in the evening. After respiratory sample collection, samples were transported to a laboratory at the Kansas State University College of Veterinary Medicine for analysis by the Cyranose® 320 eNose. Calves alternated between morning and evening sample collection on each consecutive day throughout the study period except for day 3. On study day 3, samples were collected from all calves in the morning and then all animals were challenged with BHV-1 immediately after. On study day 4, calves were only observed for clinical illness with no respiratory samples collected. On the morning of study day 5, calves were challenged with *Mannheimia haemolytica* and then followed with clinical observation every six hours for a 24 h period. Twice-daily respiratory sample collection along with clinical observation resumed for all calves on study day 6 and continued through study day 13, following the same format as detailed on study day 1 and 2. The study timeline (Table 2.1) illustrates a simplified sampling scheme with associated viral and bacterial challenge timepoints for the live animal portion of the study. On study day 14, all calves were euthanized and necropsied for assessment of lung pathology. Calves were sedated with xylazine (IM, 0.15 mg/kg) and euthanized by captive bolt and intravenous infusion of supersaturated magnesium sulfate. Gross necropsy was conducted on all study animals, and a total percent lung consolidation was estimated by a single investigator (BVL) using the equation described by Fajt et al. [26].

Viral Challenge Preparation and Inoculation

BHV-1 (Colorado strain) was propagated at 37 °C in a 5% CO2 incubator on a bovine nasal turbinate (BT) cell line. After 70–80% cytopathic effect (CPE) development in the 75 cm^2 cell culture flask, the material was exposed to two freeze/thaw cycles. The cell debris was centrifuged at low speed (1500 × g) for 10 min. The supernatant was titrated in the vials and placed on the BT cells for 48 h to determine 10^5 Tissue Culture Infectious Dose 50 (TCID50)/mL of the challenge inoculum. Individual challenge doses (4 mL) were aliquoted and stored at −80 °C. Viral challenge aliquots were thawed in a household refrigerator (~4 °C) 24 h prior to inoculation. On study day 3, all animals were restrained in a squeeze chute with a rope halter and inoculated with 4×10^5 TCID50 BHV-1 by fully inserting a 5 cm plastic nasal cannula into the left nostril.

Bacterial Challenge Preparation and Inoculation

A field strain of *Mannheimia haemolytica* serotype A1 (genome information is available at https://www.ncbi.nlm.nih.gov/nuccore/CP017519 (accessed 11 June 2025)) was grown from a characterized clinical isolate on sheep blood agar in 5–7% CO_2 for 18–24 h. A single isolated bacterial colony was inoculated into brain–heart infusion (BHI) broth and incubated for 16–18 h at 37 °C. The bacterial inoculum was centrifuged at 3000 × g for 15 min at 4 °C and washed twice with phosphate-buffered saline (PBS). After the second wash, the bacterial pellet was suspended in PBS to reach an optical density equivalent to $1.0 – 1.2 \times 10^9$ CFU/10 mL. The bacterial challenge preparation was completed the morning of inoculation (study day 5). After preparation, the inoculum was placed on ice in a light-protected cooler and transported to the study site. At the study site, all calves were restrained in a squeeze chute with a rope halter and inoculated with 10 mL of the *M. haemolytica* suspension via endoscopy into the tracheal bronchus. Following instillation of the inoculum, the endoscope was flushed with 60 mL of sterile PBS solution to achieve a total volume of 70 mL.

Expired Air and Nasal Swab Collection

Expired air and nasal swab samples were collected for eNose analysis. Samples were collected from all study animals once daily on study days 1, 2, and 6–13, split between two sampling timepoints (morning and evening). Six animals were sampled in the morning and six animals were sampled in the evening, with cattle alternating between morning and evening collection on each consecutive day. Only one sampling timepoint was used on the morning of study day 3 prior to the viral challenge. No respiratory samples were collected on day 4 and day 5 (*M. haemolytica* challenge day).

Expired Air

Prior to expired air collection, calves were restrained in a squeeze chute and a rope halter was applied to the calf's head to limit movement during expired air collection. Expired air samples were collected using the apparatus depicted in Figure 2.1. A large canine anesthetic induction mask was connected on one end to a disposable non-rebreathing T-piece. On the outflow port of the T-piece, a sealed 3.79 L mylar food storage bag was attached via a 22 mm internal diameter (ID) tubing adapter. The anesthesia mask provided an air-tight seal around the calf's mouth and nostrils and was held on the calf for the entire collection cycle. As the calf exhaled, expired air was diverted through the non-rebreathing T-piece into the mylar bag. As the calf inhaled, the valve on the non-rebreathing T-piece connected to the mylar bag closed and the valve opposite opened, which allowed fresh air into the mask. Calves would breathe into the air collection apparatus until the mylar bag was full (approximately 5–10 breaths). Once collection was complete, the 22 mm ID tubing adapter connected to the mylar bag was removed from the non-rebreathing T-piece and sealed with a 22 mm ID rubber tapered plug. After expired air collection, expired air bags were placed into a plastic storage container for safe transport. The bags were held at environmental temperature (~20–38 °C) after collection until transport to the laboratory where they were held at room temperature (20 °C) to be analyzed by the eNose.

Nasal Swab

Prior to nasal swab collection, calves were restrained in a squeeze chute and a rope halter was applied to the calf's head to limit movement during nasal swab collection. Dirt and debris were removed from the external nares with a paper towel; a 15 cm sterile rayon swab with a polystyrene handle was inserted approximately 7.5 cm into the nasal cavity and rotated across the nasal mucosa for approximately 5 s. The swab was then removed from the nares and placed in a 6 mL preservative-free blood collection tube and capped. After collection, the blood tubes containing the swabs were stored in a plastic storage container. The swab was held at environmental temperature (~20–38 °C) after collection until transport to the laboratory where it was held at room temperature (20 °C) to be analyzed by the eNose. Calf nostril sampling was alternated (left/right) each consecutive day of the study.

eNose Procedure

Respiratory samples were analyzed by the Cyranose 320® eNose according to the manufacturer's general recommendation and previous work performed by Schelkopf et al. [27]. The Cyranose® 320 eNose is a portable, handheld device composed of 32 carbon-based sensors with potential to differentiate a wide array of VOC profiles. The Cyranose 320® eNose instrument settings for analysis of expired air and nasal swab samples are outlined in Tables 2.2 and 2.3. For expired air samples, a 16 G × 1.5" aluminum hub needle was penetrated through the mylar bag. The needle was attached to a 76 cm intravenous extension set connected to the eNose sample inlet. Prior to running the expired air samples, the eNose underwent a conditioning phase as recommended by the manufacturer, consisting of a six-minute purge cycle followed by three pre-sniffs (sample runs) using expired air samples from the previous collection timepoint. Morning and evening samples were saved and maintained in their original collection container until the next collection timepoint to be used as pre-sniff in the conditioning phase of the eNose. Pre-sniffs performed at the start of the study consisted of three additional samples collected from the study animals at the first collection timepoint. Expired air samples were analyzed between one to four hours post-collection.

For nasal swab samples, a 16 G × 1.5” aluminum hub needle was penetrated through the rubber cap of the preservative-free blood collection tube. The needle was attached to the eNose by the same set-up outlined for the expired air sample. An additional 18 G × 3.5” spinal needle was penetrated through the rubber cap of the blood collection tube next to the previous needle (Figure 2.2) to eliminate negative pressure generated from the eNose inlet pump. Prior to running the nasal swab samples, the eNose underwent a conditioning phase with three pre-sniffs using nasal swab samples from the previous collection timepoint as described for expired air samples. Nasal swab samples were analyzed between one to four hours post-collection.

eNose Training and Data Processing

All samples collected during the study were analyzed by the Cyranose® 320 eNose. Raw data generated from a single sample run on the eNose was streamed into the PCnose™ software, provided with the Cyranose® 320 eNose, on an external computer and stored as a comma-separated value (CSV) file. CSV files were then loaded into the Chemometric Data Analysis (CDAnalysis™) software, provided with the Cyranose® 320 eNose, on the external computer to create a training set. A separate training set was created for expired air and nasal swab samples.

“Post-Challenge” training set classes for both expired air and nasal swabs were created by selecting 5 sample runs on study day 13 from calves with the greatest amount of lung consolidation on necropsy (day 14). “Pre-Challenge” classes used in the training sets differed between expired air and nasal swabs and were created by selecting 5 sample runs randomly without replacement from the first 3 days of the study excluding calves used in the “Post-Challenge” class of the training set. Training sets were evaluated on the PCnose™ and CDAnalysis™ software for quality assurance and quality control, as recommended by the eNose manufacturer. The two parameters evaluated were cross validation and Mahalanobis distance, both of which provide objective measurements pertaining to how well the 2 classes in the training set are separated. Once the training sets were established, the remaining individual sample data (CSV files) from previous sample runs collected on the eNose were ran as “unknown samples” in the CDAnalysis™ software to obtain a binary outcome (“Pre-Challenge” or “Post-Challenge”). Data analysis configuration settings used for the training sets are included in Tables 2.2 and 2.3.

Statistical Analysis

All expired air and nasal swab samples not used in the creation of training sets were run through their associated training set on the CDAnalysis™ software as “unknown samples” and assigned either a “Pre-Challenge” or “Post-Challenge” classification as described above. Nasal swab samples were exclusively run on the single nasal swab training set and expired air samples were exclusively run on the single expired air training set. Descriptive statistics were used to determine the count and associated percentage of correctly identified pre- and post-challenge samples on the eNose for expired air and nasal swabs.

Data were also analyzed by a logistic regression model using the “glmer” function in the ‘lme4’ package of R Studio® (R Studio®, version 2024.9.0.375; R Core Team) to determine the probability of agreement between the eNose/CDAnalysis™ software and the animal’s actual status. The model’s outcome variable was the comparison of the classification of eNose/CDAnalysis™ (“Pre-Challenge” or “Post-Challenge”) to the actual animal status (pre-challenge vs. post-challenge). Comparison was binomial with agreement (1) or disagreement (0). The model included fixed effects for sample type, day, and the interaction of sample type by day with an associated significance level of $p < 0.05$. Animal identification was included as a random intercept in the model to account for repeated measures. The “emmeans” function in the ‘emmeans’ package of R Studio® was used to calculate the probability of agreement between the eNose/CDAnalysis™ software and the actual animal status for each sample type by study day.

Results

Descriptive Statistics

Calf lung consolidation scores collected at necropsy (day 14) are individually reported in Table 2.4. Mean lung consolidation among all 12 calves was 13.17% (median: 11.85%). Cross validation of expired air training set performed on the PCnose™ and CDAnalysis™ software from the Canonical Discriminant Analysis algorithm (CDA) model was 70%, with a Mahalanobis distance between the two classes (“Pre-Challenge” and “Post-Challenge”) of 14.3. Nasal swab cross validation was 70% as well, with a Mahalanobis distance between the two classes of 5.018.

In total, 132 expired air and 132 nasal swab samples were collected during the study period; 10 expired air and 10 nasal swab samples were used to create the respective training sets, leaving 122 nasal swabs and 122 expired air samples for analysis by the eNose/CDAnalysis™

software. In the pre-challenge period (days 1–3), the eNose correctly identified 30/31 expired air samples as pre-challenge, compared to 29/31 nasal swab samples (Table 2.5). In the post-challenge period (days 6–13), the eNose correctly identified 66/91 expired air samples as post-challenge, far fewer than the 89/91 correctly identified nasal swab samples (Table 2.5). Individual animal eNose classification by day of the study is displayed for both expired air and nasal swabs in Figures 2.3 and 2.4, along with identification of samples used in the creation of the training set.

Logistic Regression

Figure 2.5 displays the model-estimated mean probability of agreement between the Cyranose® 320 eNose and the actual animal status by sample type and study day. When the logistic regression model was used to predict probability of agreement as described in the methods, the model failed to converge with animal identification accounting for repeated measures. Animal identification was subsequently removed from the model. In the final model, the interaction of sample type by day was significant ($p = 0.0493$), so main effects were not reported. In the pre-challenge period, expired air had a mean probability of agreement of 1.0 (standard error [SE] < 0.01) for study day 1 and a mean probability of agreement of 1.0 (SE < 0.01) for study day 2. On study day 3, expired air had a mean probability of agreement of 0.91 (SE = 0.09). Pre-challenge results were similar for nasal swabs with a mean probability of agreement of 0.91 (SE = 0.09) on day 1, 0.90 (SE = 0.09) on day 2, and perfect agreement (probability [p] = 1.0, SE < 0.01) on day 3. Post-challenge mean probability of agreement differed the most between expired air and nasal swabs on study days 6 and 7 (days 1 and 2 post-*M. haemolytica* challenge [expired air—day 6: $p < 0.01$ +/− <0.01 SE, day 7: $p = 0.42$ +/− 0.14 SE; nasal swab—days 6 and 7: $p = 0.92$ +/− 0.08 SE]). On days 8 through 13, nasal swabs had perfect mean probability of agreement (*p* = 1.0 +/− <0.01 SE) between the eNose and the actual animal status. Expired air mean probability of agreement increased from day 6 to perfect agreement (*p* = 1.0 +/− <0.01 SE) by days 10 and 11. The mean probability for expired air samples decreased after day 11 to 0.92 (SE = 0.08) on day 12 and 0.86 (SE = 0.13) on day 13.

Discussion

No published study has used the Cyranose® 320 eNose in the detection of BRD in cattle from respiratory samples in an induced disease model. The focus of the current study was to provide proof of concept that the Cyranose® 320 eNose has potential as a BRD diagnostic tool and to assess its ease of use for potential field-based application. The methods utilized to operate the Cyranose® 320 eNose were based on prior research to detect ketosis in dairy cattle by our research group and the general recommendations for basic operation provided by the manufacturer of the device [27]. Operation of the device was carried out in a manner that could be practically applied in a commercial livestock operation for the diagnosis of BRD.

In the absence of a practical and affordable gold-standard test for diagnosing naturally occurring BRD, the authors elected to use a challenge model with two commonly isolated BRD pathogens (BHV-1 and *M. haemolytica*) to test the utility of the eNose for BRD diagnosis. The challenge model provided the advantage of knowing when animals became exposed to the pathogens and allowed for daily sampling throughout the early period of known infection. Daily sampling after the BRD challenge was included in the study design to determine how early in the disease process the eNose could reliably make a diagnosis, if feasible at all. To minimize the impact of prior respiratory disease on study results, animals used in this study were selected based on no previous history of BRD treatment and the absence of BRD signs (labored breathing, nasal and/ocular discharge, cough, and lethargy) throughout the pre-challenge period.

The inoculation of BHV-1 and *M. haemolytica* served as the standard method for classifying animals with BRD. The presence of BRD was confirmed on the final day of the study by evidence of lung consolidation.

Overall, the Cyranose® 320 eNose was able to correctly identify pre- and post-challenge samples with a high degree of accuracy (Table 2.5). Specifically, in the pre-challenge period, expired air and nasal swab sample types both provided a high probability of agreement between eNose classification and actual animal status regardless of the day (Figure 2.5). In Figure 2.5, when evaluating the post-challenge period for both sample types, in general, the agreement increased as the challenge period progressed. This is not unexpected as the eNose was trained with day 13 samples, when study animals likely would have had more developed respiratory disease. The post-challenge period provided the greatest divergence between the two sample types. While both expired air and nasal swabs had the lowest probability of agreement in the post-challenge period on days 6 and 7, the nasal swab provided results that were still comparable to the high probability found in the pre-challenge and middle to late post-challenge periods.

The results in this study closely match the only other published research which used an eNose to provide a binary diagnosis for BRD [22]. Kuchmenko et al. [22] were able to correctly classify 5/5 calves as "healthy" and 5/5 calves as "sick" with an eight-sensor eNose trained on 20 healthy and 20 sick cattle from the same cohort. While it is common among studies using an eNose in the diagnosis of respiratory disease to analyze individual sensor responses [22,25], the current study uses the combined response of all 32 sensors present in the device to provide a singular categorical output. Individual sensor data were not explored in this study due to the high degree of accuracy already acquired from the combination of all sensors. However, the Cyranose® 320 eNose can turn on and off any of its 32 sensors, so further optimization of the device is possible through this method. Additionally, the Cyranose® 320 eNose comes equipped with other algorithms for data analysis. Only one algorithm (CDA) was applied for data analysis throughout this study due to the high degree of accuracy achieved. While the model cross validation using the CDA algorithm achieved for both training sets would be considered suboptimum (70%), previous work by Schelkopf et al. [27] determined that model cross validation on the Cyranose 320® eNose did not reliably translate to diagnostic accuracy.

It is important to note that the eNose used throughout this study was trained on raw respiratory samples collected from live cattle enrolled in the study. The eNose was not specifically trained to detect certain bacteria, viruses, or specific inflammatory responses present in respiratory disease cases. Additionally, the eNose was optimized by sample selection to differentiate live animals with no clinical BRD signs from live animals which had lung consolidation on necropsy ranging from 12.30% to 24.65%. It was no surprise to the researchers that samples representing animals immediately post-BRD challenge, which likely have a minimal amount of lung consolidation, had lower agreement than those samples later in the post-challenge phase of the study. Of note, clinical signs of illness observed from the study animals did not warrant treatment of any calf at any timepoint throughout the study. Animals did not experience severe BRD signs, denoting that the eNose was trained on animals experiencing moderate to mild BRD signs only.

In terms of field application, this study differs primarily due to the collection of all samples and associated data files prior to creating a training set and running unknown sample files on the designated training set through the CDAnalysis™ software after study completion. Ideally, samples could be analyzed in real time after collection on an eNose that is already trained to detect BRD and provide results within minutes. Sample type selection in this study was also based on ease of on-farm application consisting of a hand-made expired air collection bag and a nasal swab with an associated collection system. Aside from the nasal swab providing better statistical results, it was also much easier to store, transport, and collect from cattle. The use of a blood collection tube with a pierceable rubber cap to store and analyze the nasal swab with the eNose ensures sample headspace stability. The nasal swab and blood tube system also provided better biosecurity, as collection tools were not shared between cattle, and collected samples were protected from human exposure after collection. These results and observations suggest that the nasal swab is the ideal sample in this study and warrants its use in future similar studies. While only a single nasal swab was collected from a one nostril during the sampling of each calf, no discernible difference in nasal laterality was noticed in the results of the study. In an attempt to replicate a fast-paced commercial setting, sampling one nostril rather than both provides a much more efficient collection process and limits materials needed to perform the test.

A primary limitation of this study pertains to the inability of the logistical regression model to account for repeated measures. This is likely due to the small sample size used throughout this study and the high probability of agreement within individual sampling days. Additional limitations of this study surround its applicability to naturally occurring BRD in commercial cattle feeding operations. Currently, it is undetermined how applicable the specific training set used in this study is on other cohorts of cattle or other pathogens of the BRD complex. Published studies diagnosing respiratory disease in cattle with an eNose have only trained an eNose on the same cohort it used for detection [22,25]. It is unknown if one universal training set can be created and applied to accurately detect BRD in other diverse cattle cohorts and if training sets created in challenge studies are applicable to naturally occurring disease.

Sample stability after collection is also unknown. In the current study, samples were collected and then transported to a lab for analysis on the eNose in a span of one to four hours. It is undetermined if results from samples run on the eNose immediately after collection or samples run many hours to days later could still provide reliable results. While work has been carried out to characterize specific VOCs present in respiratory secretions from cattle with BRD [28,29], this was not attempted in the current study. It is unknown what exact VOCs produced by specific biological processes or organisms are used by the Cyranose® 320 eNose to differentiate animals pre and post-BRD challenge.

Conclusions

The Cyranose® 320 eNose correctly identified pre- and post-challenge respiratory samples with a high degree of accuracy. The majority of incorrect post-challenge classifications occurred immediately following BRD challenge. Given both the accuracy and logistics of sample collection, nasal swabs were the optimum sample for detection of BRD using the eNose. This study demonstrates the potential use of the Cyranose® 320 eNose as an on-farm BRD diagnostic tool. Future research to confirm these findings in naturally occurring BRD cases is warranted.

Abbreviations

BRD	Bovine respiratory disease
CALA	Computer-aided lung auscultation
eNose	Electronic nose
VOCs	Volatile organic compounds
LAMP	Loop-mediated isothermal amplification
BHV-1	Bovine herpes virus-1
BT	Bovine nasal turbinate
CPE	Cytopathic effect
TCID50	50% Tissue culture infectious dose
BHI	Brain-heart infusion
PBS	Phosphate buffered saline
ID	Internal diameter
CSV	Comma-separated values
CDA	Canonical discriminant analysis algorithm
CDAnalysis™	Chemometric data analysis software
P	Probability
SE	Standard error

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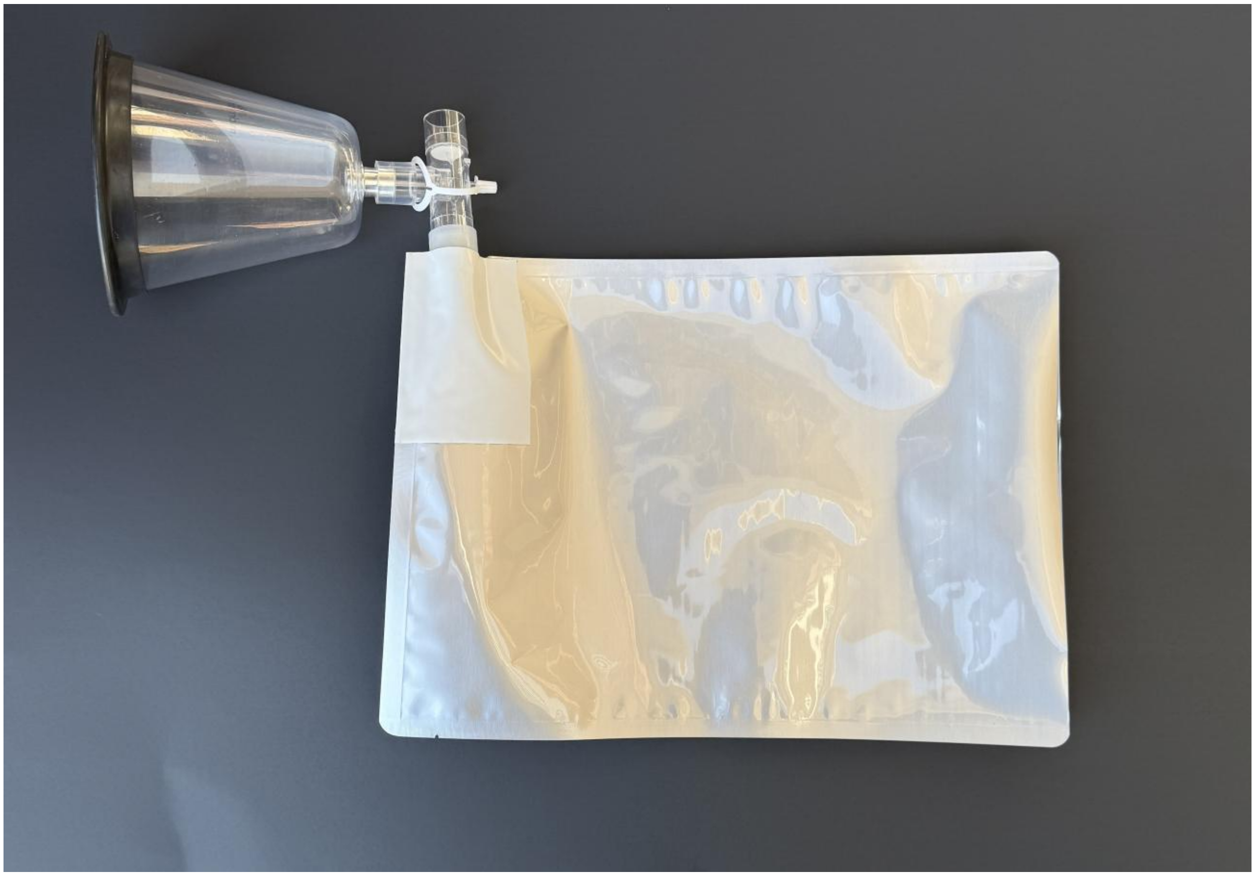

Figure 2.1. Expired air collection apparatus consisting of a non-rebreathing T-piece connected to a large canine anesthetic induction mask and a 3.79 L mylar food storage bag.

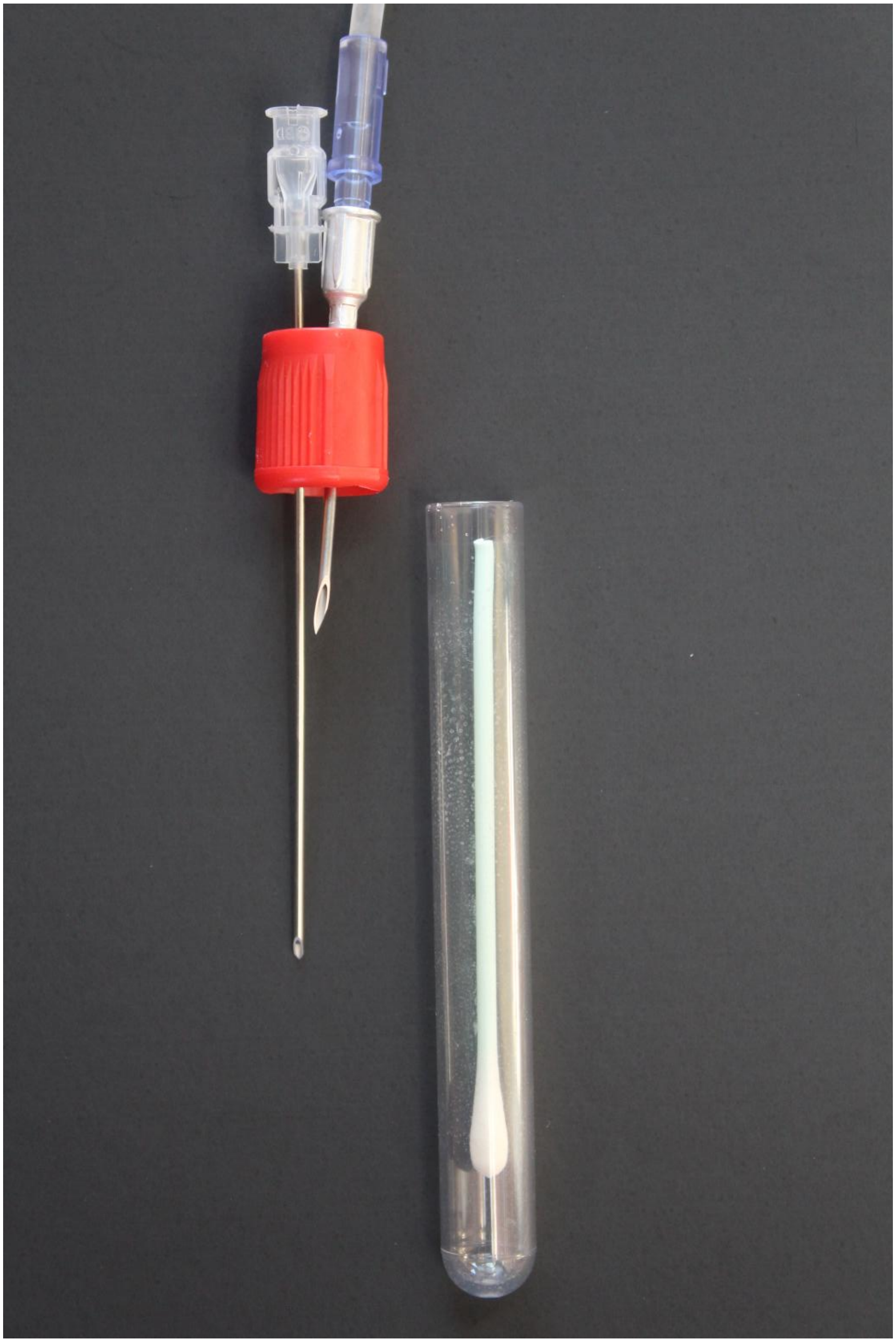

Figure 2.2. Nasal swab collection system with lid uncapped from blood collection tube storing a nasal swab. A 16 G × 1.5” aluminum hub needle attached to an IV extension set and an 18 G × 3.5” spinal needle are penetrated through the rubber cap of the tube.

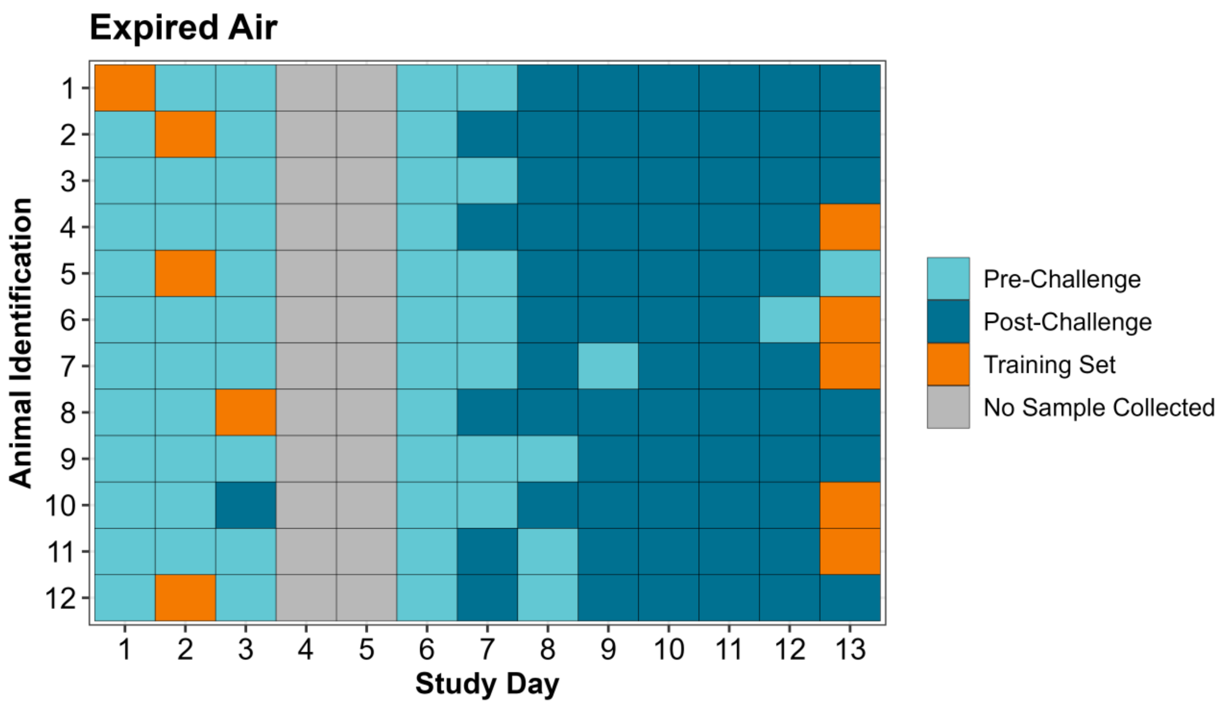

Figure 2.3. Tile map identifying individual animal expired air sample classifications by the Cyranose® 320 eNose and identification of samples used in the training set by study day.

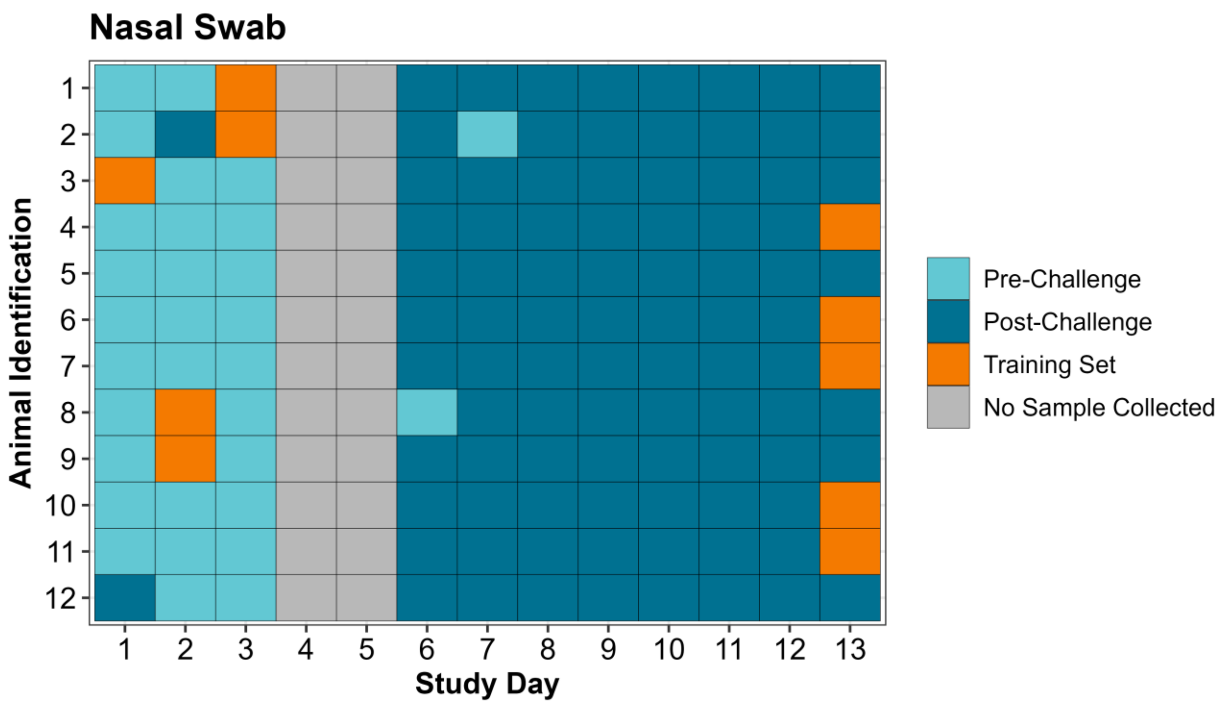

Figure 2.4. Tile map identifying individual animal nasal swab sample classifications by the Cyranose® 320 eNose and identification of samples used in the training set by study day.

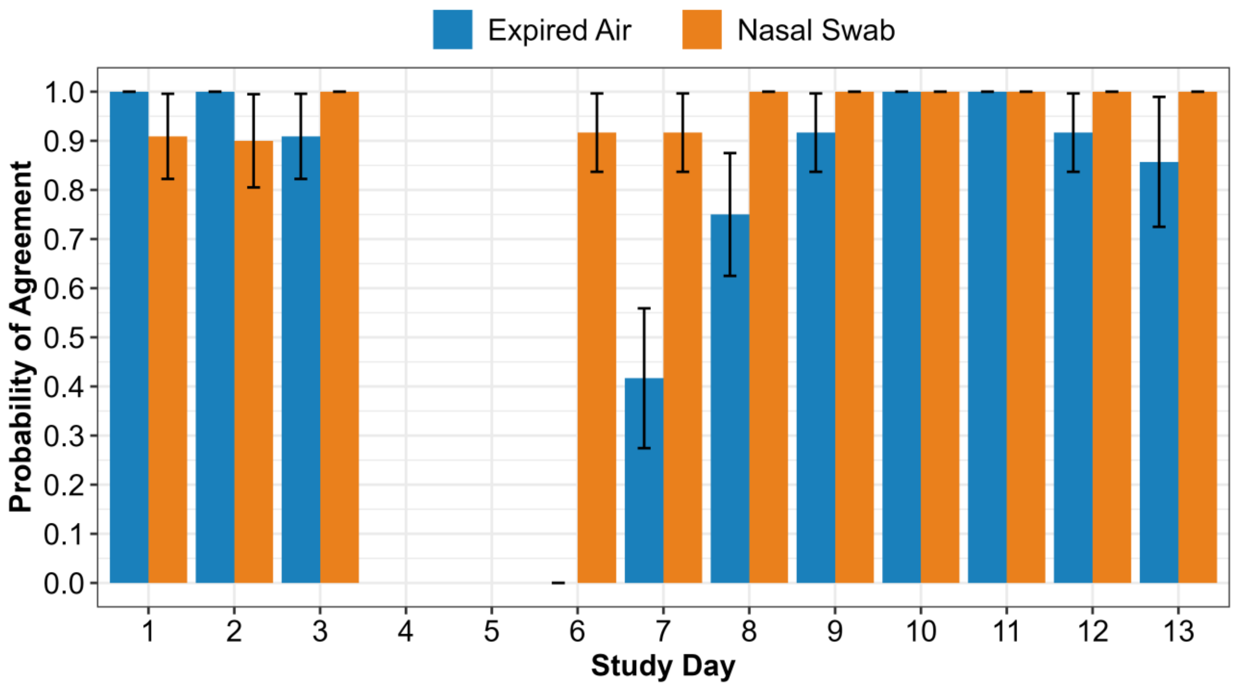

Figure 2.5. Model-estimated mean probability of agreement between Cyranose® 320 eNose and actual animal status by sample type and study day. Error bars represent standard error (SE) of probability of agreement.

Table 2.1. Study timeline of respiratory sample collection and bovine respiratory disease (BRD) challenge by study day. "X" represents all 12 animals enrolled in the study underwent the associated activity on the designated study day.

Procedure	**Study Day**												
Expired Air Collection	X	X	X			X	X	X	X	X	X	X	X
Nasal Swab Collection	X	X	X			X	X	X	X	X	X	X	X
Bovine herpes virus 1 (BHV-1) Challenge			X [1]										
Mannheimmia haemolytica Challenge					X								
	1	2	3	4	5	6	7	8	9	10	11	12	13

[1]BHV-1 challenge was administered following respiratory sample collection on study day 3.

Table 2.2. Cyranose 320® eNose instrument settings for analysis of expired air samples as displayed in the PCnose™ software provided with the device.

Class assignment	Class name	Training samples
Class 1	Pre-Challenge	5
Class 2	Post-Challenge	5
Flow operations	**Time (seconds)**	**Pump speed (cm^3/minute)**
Baseline purge	10	High (180)
Sample draw 1	60	High (180)
1st air intake purge	10	High (180)
2nd sample gas purge	180	High (180)
Data analysis configuration settings		
Algorithm:	Canonical Discriminant Analysis (CDA)	
Preprocessing:	Auto-scaling	
Normalization:	Normalization 1	
Identification quality:	Always choose	

Table 2.3. Cyranose 320® eNose instrument settings for analysis of nasal swab samples as displayed in the PCnose™ software provided with the device.

Class assignment	**Class name**	**Training samples**
Class 1	Pre-Challenge	5
Class 2	Post-Challenge	5
Flow operations	**Time (seconds)**	**Pump speed (cm^3/minute)**
Baseline purge	10	Medium (120)
Sample draw 1	30	Medium (120)
1st air intake purge	10	High (180)
2nd sample gas purge	90	High (180)
Data analysis configuration settings		
Algorithm:	Canonical Discriminant Analysis (CDA)	
Preprocessing:	Auto-scaling	
Normalization:	Normalization 1	
Identification quality:	Always choose	

Table 2.4. Individual calf lung consolidation score at necropsy.

Animal ID	Total Lung Consolidation
1	11.61%
2	2.39%
3	12.09%
4[1]	22.50%
5	9.18%
6[1]	24.65%
7[1]	17.85%
8	11.10%
9	8.85%
10[1]	12.30%
11[1]	14.79%
12	10.72%

[1]Day-13 respiratory samples collected from these animals were used to create the “Post Challenge” training set class for expired air and nasal swab on the eNose.

Table 2.5. Correctly identified expired air and nasal swab samples by the Cyranose® 320 eNose pre- and post-induced BRD challenge.

Sample Type	Pre-Challenge (Day 1-3) No. Correct/No. Tested (%)	Post-Challenge (Day 6-13) No. Correct/No. Tested (%)
Expired Air	30/31 (96.8%)	66/91 (72.5%)
Nasal Swab	29/31 (93.5%)	89/91 (97.8%)

Chapter 3 - Electronic Nose Technology Provides Good Diagnostic Agreement with Clinical Observation for Bovine Respiratory Disease Detection in Beef Cattle

Abstract

Bovine respiratory disease (BRD) is a major health concern in beef cattle. Volatile organic compound (VOC)-based point-of-care diagnostics such as electronic noses (eNose), show promise for improving BRD diagnosis. This study aimed to evaluate the ability of a commercially available eNose to distinguish cattle with naturally occurring BRD from non-BRD animals. Nasal swabs were collected from 363 crossbred beef cattle during chute-side evaluation for clinical illness and analyzed by a commercially available eNose. Nasal swabs were classified by the eNose as either BRD or non-respiratory controls (CON) based on newly developed operation specific training sets and a previously developed BRD challenge training set matched with machine learning algorithms. The eNose results were compared to clinical observation of disease performed by trained personnel by use of agreement metrics. The previously developed eNose BRD challenge training set provide good diagnostic agreement with clinical observation for detecting BRD when using the BRD challenge training set (Gwet's AC1 = 0.62, 95% CI: 0.54–0.70). All operation specific training sets used to classify nasal swabs on the eNose provided poor agreement with clinical observation. The eNose demonstrated potential as a diagnostic tool to differentiate naturally occurring BRD from non-BRD cases when compared to clinical observation. The use of the BRD challenge training set also supports the development

and application of universal eNose training sets that can be applied across different cattle populations.

Introduction

Bovine respiratory disease (BRD) imposes a significant economic burden on the cattle industry, and one aspect of its cost is related to labor.[1] A national survey of beef producers in the United States found that labor costs associated with BRD were similar to or exceeded costs of vaccines and medicines for its prevention and treatment.[2] Labor tasks performed on-farm, directly related to BRD, include gathering and sorting cattle, along with diagnosing clinical signs, and administering treatments for prevention and control. However, persistent labor shortages in the agricultural sector and high employee turnover on many cattle operations have made it increasingly difficult to consistently and effectively carry out these labor-intensive tasks.[3,4] This is exacerbated by identified knowledge gaps in animal health and behavior among agricultural laborers, potentially leading to a missed BRD diagnoses due to lack of qualified labor.[5]

BRD is primarily diagnosed by visual assessment of clinical respiratory signs including labored breathing, coughing, nasal discharge, and lethargy.[6] These signs can be evaluated in the context of an established BRD clinical scoring system with the goal of providing a more standardized treatment protocol. However, farm employees tasked with diagnosing disease must first be trained in the identification of these signs which can be time-consuming and inconsistent. With improper training, drift of the treatment protocol may occur raising concerns for treatment efficacy, increased risk of drug residues and potential development of antimicrobial resistance. Given the clinical diagnosis and qualified labor challenges described above, the development of point-of-care (POC) diagnostic technologies which provide a consistent, objective, and reliable diagnosis of BRD are of great value to cattle operations. Thoracic auscultation and thoracic ultrasound are currently among the few commercially available options that have been widely evaluated for POC BRD diagnosis. Studies using a stethoscope for thoracic auscultation have evaluated its utility for BRD diagnosis. Results have shown limited diagnostic accuracy with notably low sensitivity.[7] Additionally, inter-operator variability with a stethoscope for thoracic auscultation poses a significant challenge, as there tends to be low agreement between different individuals performing the test, potentially leading to inconsistent diagnosis.[8] To address these limitations, a computer-aided lung auscultation (CALA) system, known as Whisper® On Arrival (Merck Animal Health, USA), was developed. CALA has a reported sensitivity and specificity for BRD detection near or exceeding 90%.[9] Despite these results, this technology has yet to see universal adoption in commercial cattle operations.

Thoracic ultrasound has gained attention as a POC tool for BRD diagnosis because of its ability to visualize lung lesions and assess the severity of pulmonary disease. Multiple studies have highlighted its high sensitivity and specificity for detecting BRD-related lung pathology.[10-12] Unfortunately, thoracic ultrasound shares some of the challenges similar to traditional thoracic auscultation. Operator proficiency plays a critical role in obtaining and interpreting ultrasound images accurately. Research has shown diagnostic results remain variable between operators, and achieving competency often requires significant training and experience.[13] This learning curve can limit its practical use in fast-paced or resource-limited settings.

Recently, technologies focused on the noninvasive detection of volatile organic compounds (VOCs) have emerged as BRD diagnostics.[14] Volatile organic compound detection tools such as an electronic nose (eNose) can evaluate breath or nasal secretions from cattle within minutes and provide an objective diagnosis for BRD, making it a candidate for POC testing. While not validated in a commercial production setting, an eNose has reported accuracy as high as 100% in correctly identifying BRD-positive animals.[15] A commercially available eNose (Cyranose® 320, Sensigent, USA) was evaluated for BRD detection and demonstrated promising diagnostic accuracy of 97.8% and 72.5% in correctly diagnosing nasal secretions and breath, respectively, in an induced BRD model.[16] However, before the eNose can be effectively implemented as a POC diagnostic tool on an operation it must be trained on known positive and negative animals to develop an accurate disease profile. This initial calibration step is essential for reliable performance. Once trained, the device can deliver near-real time diagnostic results that are consistent regardless of the test operator.

Therefore, the objective of this study was to assess the ability of a commercially available eNose to differentiate cattle with naturally occurring BRD from non-BRD cases. Clinical observations of respiratory signs for the diagnosis of BRD, performed by trained personnel, served as the comparator test.

Materials and Methods

Study design and enrollment criteria

This observational study was conducted at a Kansas feedyard over a five-week period in November and December of 2024. All study procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Kansas State University (protocol #5060 approved 16 September 2024) and informed owner consent was obtained for all animals included in the study. Crossbred beef cattle housed in dry lot pens were evaluated daily for signs of clinical illness by experienced feedyard personnel. Cattle identified with any illness were removed from their pen and evaluated chute-side by feedyard personnel trained in disease detection for final diagnosis of disease following the operation’s specific protocol. Animals receiving their first diagnosis of any illness were enrolled into the study on a rolling basis.

Animal sampling

At enrollment, illness and demographic data were collected for all cattle. Illness data consisted of the clinical diagnosis for each animal and demographic data included animal identification number, lot number, animal sex, weight at enrollment and days-on-feed at enrollment. Additionally, a single nasal swab (15 cm sterile rayon swab with a polystyrene handle) was collected from each animal by inserting the tip 5 cm into the nasal passage and rotating it against the nasal mucosa for 3 seconds. Prior to sample collection, while cattle were restrained in a squeeze chute, a paper towel was used to remove dirt and debris from the external nostrils. Only a single nostril was sampled on each animal and nostril selection was determined based on convenience and safety of collection. After the nasal swab was removed from the nasal passage it was immediately sealed in a 6 mL preservative-free blood collection tube held at environmental temperature (0 to 24 °C). Nasal swabs were stored upright in a plastic storage container for transportation to an off-site laboratory for same day analysis on the eNose.

Diagnostic test 1: clinical observation of respiratory signs

Clinical diagnosis of disease was performed by trained feedyard personnel. Training of personnel and development of the operation’s diagnosis and treatment protocol was carried out by the consulting veterinarian. Animals presenting with clinical signs of any diseases were identified first in the pen. Cattle were then removed from the pen for chute-side confirmatory diagnosis of disease. Animals displaying clinical signs of respiratory disease were evaluated using a variation of the DART (depression, appetite loss, respiratory character change, elevated body temperature) system. Animals displaying severe forms of a single clinical sign, such as severe respiratory distress, or moderate forms of multiple clinical signs, such as depression, nasal discharge and elevated rectal temperature, were diagnosed with BRD. Animals presenting with non-respiratory illness were categorized as non-respiratory controls (CON), which included musculoskeletal disorders (e.g., infection, lameness, swelling), digestive disorders (e.g., diarrhea, bloat, indigestion), and other systemic or infectious conditions (e.g., ocular infection).

Diagnostic test 2: electronic nose technology

Nasal swabs collected at enrollment were evaluated by a commercially available eNose (Cyranose® 320, Sensigent, USA) from one to eight hours after collection at an off-site laboratory. This handheld eNose is equipped with 32 polymer-carbon black composite sensors used to collect and characterize VOC signatures emitted from the nasal swab samples. Samples were analyzed by the eNose using the sampling and collection set-up methods previously developed and described in detail by Schelkopf, et al. [16] Prior to performing samples on the eNose, the device was connected to a laptop computer to store the collected sensor data from each animal’s swab. Once all nasal swab samples were collected and performed on the eNose, the saved sensor data from a subset of the animals enrolled in the study were used to develop training sets to test the eNose’s diagnostic performance. Training set creation was carried out on the Chemometric Data Analysis software (CDAnalysis™, version 11.2, Sensigent, USA) on a laptop computer. Each training set included five animals per outcome class. Classes were labeled as BRD or CON, enabling the device to categorize unknown samples into one of these two groups.

High lot morbidity-early 2nd treatment (HLM-E2T) training set

After completion of the study, individual animal treatment records, from arrival at the feedyard to 60 days post-enrollment, along with lot-level health records were reviewed retrospectively to create an operation-specific training set based on health data. Animals from lots with high BRD morbidity (>25%) that received a second BRD treatment within seven days of enrollment were assigned to the BRD class of the training set. BRD lot morbidity was calculated as number of animals in the lot that received a first BRD treatment divided by total animals in the lot. The non-respiratory controls (CON) training set class consisted of animals that never received BRD treatment during their time at the feedyard and were from lots with the lowest BRD morbidity (<5%). The five animals from each class of the training set were visualized by use of a principal component analysis (PCA) score plot provided in the CDAnalysis™ software (data not shown). Several iterations of the training set were made by removing and replacing animals with similar health characteristics to maximize the visual separation between the BRD and CON classes on the PCA score plot. Characteristics of animals included in the final training set are displayed in Table 3.1. Cross-validation of all eNose algorithms was performed using CDAnalysis™ software, and the three algorithms with the highest cross validation were selected to generate HLM-E2T training set/algorithm combinations (Table 3.2), which were then used to test the remaining samples. As this was the initial evaluation of the training set, it was uncertain which algorithm would perform best; therefore, multiple algorithms were tested.

High ultrasound lung score (HULS) training set

A subset of cattle enrolled in this study and diagnosed with BRD by clinical observation were evaluated using targeted thoracic point-of-care ultrasound (TT-POCUS) at enrollment by a trained individual as part of an unrelated study. These cattle were assigned ultrasound lung scores (ULS) on an increasing severity scale from one to five, based on ultrasonographic abnormalities described by Feitoza, et al. [17] Because these data were readily available, it was utilized to create a second operation specific training set. Cattle receiving ULS of four or five were selected for inclusion in the training set. Initial training set creation consisted of the five highest ULS from cattle enrolled in the study to create the BRD class. The CON class consisted of the same five animals as the HLM-E2T training set, as no ULS were available for animals clinically diagnosed as non-respiratory controls. Several iterations of the training set were made by removal and replacement of cattle in the BRD class with similarly high ULS to maximize visual separation between BRD and CON classes of the training set on the PCA plot provided by the CDAnalysis™ program (data not shown). Table 3.1 summarizes the characteristics of animals found in the final training set. Cross validation and selection of three eNose algorithms were carried out in the same manner as described above to create HULS training set/algorithm combinations (Table 3.2).

BRD challenge training set

A final training set from a previous research study was used alongside the operation-specific training sets (HLM-E2T and HULS). This BRD challenge training set was developed and tested on five-month-old Holstein calves that were experimentally infected with bovine herpes virus-1 (BHV-1) and *Mannheimia haemolytica*.[16] In that study, eNose analysis of nasal swabs correctly identified 29 of 31 pre-challenge and 89 of 91 post-challenge samples. For the present study, the training set was modified to output categorical results of BRD or CON to ensure consistency with the other training sets. Incorporating this training set allowed evaluation of the applicability of training sets developed from different cattle populations, as well as assessment with clinical observations of BRD. The BRD challenge training set/algorithm combination and data analysis configuration settings used in the previous study are available in Table 3.2.

Nasal swab sample testing

The operation specific training set/algorithm combinations and the previously developed BRD challenge training set were used to classify all nasal swab samples that were not included in a respective training set. The BRD challenge training set was tested on all samples collected in the study while the operation specific training sets were tested on all animals in the study except the 10 used to create the respective training set. Clinical diagnosis data were removed and non-training set samples were uploaded into the CDAnalysis™ software for testing against all 7 training set/algorithm combinations (BRD challenge = 1, HLM-E2T = 3, HULS = 3) with classification as either BRD or CON based on the software assignment.

Statistical analysis

All data were analyzed in R Studio (R Studio®, version 2024.12.1.563; R Core Team).

Agreement between two imperfect tests

Diagnostic agreement between clinical observation–assigned diagnoses and eNose training set/algorithm combinations–assigned diagnoses was evaluated without assuming either method as a perfect reference standard. Agreement was summarized using the raw overall percent agreement as a crude metric of concordance between tests. Agreement was further assessed using Cohen's kappa and Gwet's agreement coefficient type 1 (AC1). Cohen's kappa measures the extent to which agreement between tests exceeds what would be expected by chance. Gwet's AC1 is another chance-adjusted index that tends to correspond more closely with the observed proportion of agreement regardless of disease prevalence.[18] Gwet's AC1 is particularly valuable in situations where the raw agreement is high, but kappa is reduced due to the so-called "kappa paradox," which can arise when the condition being measured has high or low prevalence.[19-22] Cohen's kappa and Gwet's AC1 were interpreted using previously reported guidelines: -1–0.20 = poor, 0.21–0.40 = fair, 0.41–0.60 = moderate, 0.61–0.80 = good, and 0.81–1.00 = very good agreement.[23] All agreement measures and 95% confidence intervals (CIs) were calculated using the 'irrCAC' package in R Studio.

Clinical observation as the reference standard

To further evaluate the eNose training set/algorithm combinations, clinical observation of respiratory signs was used as the reference standard for BRD diagnosis. The eNose diagnostic classification performance was assessed by calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Diagnostic performance metrics and 95% Clopper-Pearson CIs were calculated using 'epiR' package in R Studio.

Results

A total of 372 cattle were enrolled throughout the study. Nine animals were excluded from the final dataset: three were mislabeled at enrollment as first-diagnosis BRD cases (n = 3), three were clinically classified as non-respiratory controls but had a prior record of respiratory disease (n = 3), and three were diagnosed with calf diphtheria (necrotic laryngitis) (n = 3). Calf diphtheria was considered a respiratory disorder, but a distinct diagnosis apart from BRD.

The final dataset included 363 animals, of which 282 (77.7%) were clinically diagnosed with BRD and 81 (22.3%) as non-respiratory controls (CON) by feedyard personnel. Demographic characteristics and illness diagnosis of the study population are summarized in Table 3.3. Overall, 54.3% of the cattle were heifers and 45.7% were steers. The mean weight was 404.6 kg (SD = 103.0 kg), and days-on-feed ranged from 2 to 203, with a median of 41. Among the non-respiratory diagnoses, the majority were musculoskeletal disorders (78/81), with the remaining three cases including two digestive disorders and one case of infectious bovine keratoconjunctivitis (IBK).

Diagnostic agreement assuming neither clinical observation of respiratory signs nor the eNose as a perfect reference standard was evaluated for all eNose training set/algorithm combinations (n = 7) (Table 3.4). The previously developed BRD challenge training set presented the highest overall percent agreement (0.72; 95% CI, 0.68 to 0.77) with clinical observation by showing a significant difference over all other eNose training set/algorithm combinations as demonstrated by non-overlapping 95% CI. When statistical agreement metrics (Cohen's kappa and Gwet's AC1) were applied to evaluate the training set/algorithm combinations, Cohen's kappa indicated higher coefficients for the all HULS algorithms compared with the HLM-E2T algorithms and BRD challenge training set. However, when viewing 95% CIs, the only significant differences were found between the HULS/canonical discriminant analysis (CDA) vs. HLM-E2T/ k-nearest neighbors (KNN) and HULS/KNN vs. HLM-E2T/KNN. Despite these differences, all Cohen's kappa 95% CIs were interpreted as having poor agreement with clinical observation for BRD detection. In contrast, Gwet's AC1 agreement statistics provided a different perspective, with the BRD challenge training set showing the highest value (0.62; 95% CI, 0.54 to 0.70). This Gwet's AC1 statistic represented good agreement, while its 95% CI encompasses moderate to good agreement with clinical observation for BRD detection. Moreover, its CI was significantly different from those of all HLM-E2T and HULS training set/algorithm combinations, which consistently reflected poor agreement with clinical observation.

Diagnostic performance of the eNose training set/algorithm combinations were evaluated using clinical observation for diagnosing BRD as the perfect reference standard (Table 3.5). The BRD challenge training set had a significantly higher sensitivity (0.90; 95% CI, 0.86 to 0.92) compared with HLM-E2T and HULS training set/algorithm combinations, all of which were consistently low (≤ 0.50). The opposite trend was observed for specificity where all HLM-E2T and HULS training set/algorithm combinations were significantly higher than the BRD challenge training set. Among the HLM-E2T and HULS training set/algorithm combinations significant differences were observed between HULS/CDA vs. HLM-E2T/CDA, HULS/CDA vs. HLM-E2T/KNN, and HULS/KNN vs all other TS/A combinations except HULS/CDA. Despite these variations in sensitivity and specificity, no differences were observed for PPV or NPV as indicated by all overlapping CIs. Positive predictive value estimates ranged between 0.73 to 0.86 and NPV estimates remained low ranging between 0.16 to 0.25.

Discussion

While the objective of this study was to determine if an eNose could differentiate cattle with naturally occurring BRD from non-BRD cases, operational constraints provided a limitation to cattle that could be enrolled. Only animals which were evaluated chute-side for clinical illness were tested with the eNose. This included animals clinically diagnosed with BRD and non-BRD related illnesses. No clinically healthy animals were included in this population. This is a recognized limitation of this study.

In the absence of affordable and practical gold standard methods for diagnosing BRD on-farm, many operations rely solely on clinical observation of respiratory signs to complete this task. This reliance creates challenges for evaluating new diagnostic tools, as a perfect reference standard rarely exists for comparison under real-word production settings. Consequently, alternative statistical approaches beyond traditional diagnostic metrics (sensitivity, specificity, PPV, and NPV) should be considered. In this study, agreement measures such as Cohen’s kappa and Gwet’s AC1 were used to compare a non-validated diagnostic tool with the operation’s standard method for BRD clinical diagnosis.

Results of this study suggest that the BRD challenge training set, although developed and tested in a different cattle population, demonstrated good agreement with clinical observation (as measured by Gwet's AC1) when applied to this beef cattle population. The eNose was able to classify animals similarly to clinical observation despite being imperfect. These findings indicate its potential for diagnosing naturally occurring BRD and promote the investigation of universally applied training sets for disease detection. Logistically, application of the eNose could favor on-farm adoption. The procedure includes the collection of a single nasal swab which can be performed animal side on the portable eNose. Using the nasal swab collection system set-up and eNose procedures developed by Schelkopf, et al.[16] and tested in this study, results can be obtained within minutes. Given the urgent need for POC confirmation of BRD, further refinement and exploration of the eNose as a practical diagnostic tool for BRD detection is warranted.

Differences emerged between the two agreement statistics for the BRD challenge training set. Cohen's kappa indicated poor agreement, whereas Gwet's AC1 statistic suggested good agreement. Although Cohen's kappa is widely used, it is known to underperform in the presence of prevalence imbalances.[20-22] In contrast, Gwet's AC1 provides a more robust and stable measure under such conditions, making it particularly relevant for this study.[18,19] In this dataset, 77.7% of animals were diagnosed with BRD from clinical observation, while the eNose BRD challenge training set classified 90% as BRD. These substantial imbalances between BRD and CON cases resulted in misleading kappa estimates that differed greatly from Gwet's AC1 and the overall percent agreement. This supports the use of Gwet's AC1 in this study for comparing the two BRD detection methods, as its values align more closely with overall percent agreement than Cohen's kappa, which differed substantially.

The operation-specific training sets (HLM-E2T and HULS) were developed to provide a framework for other operations to build their own eNose training sets tailored to their cattle populations. The results indicated that regardless of the training set or algorithm employed, neither approach was successful in meeting this objective, as demonstrated by poor agreement with clinical observation. From these results, we can only conclude that the eNose does not agree with clinical observation. These results do not indicate whether the eNose and its operation specific training sets are better or worse at detecting true BRD status when compared to clinical observation. It is important, nonetheless, to consider the criteria used to construct each training set when evaluating their usefulness. The HLM-E2T training set selected BRD cases based on treatment records, aiming to capture animals that have failed treatment for and potentially have more severe respiratory diseases. This approach was chosen because treatment records can be readily available in operations and could provide practical guidelines for training set case selection. Despite poor agreement with clinical observation, all HLM-E2T training set/algorithm combinations classified this population with a substantially lower apparent prevalence (AP) of BRD than clinical observation, suggesting that the training sets may have been trained to detect BRD at a higher severity threshold. Similarly, the HULS training set, developed using ultrasound-based criteria to target animals with the most pronounced lung pathology or progressive BRD, also produced training set/algorithm combinations that identified BRD at a much lower prevalence. This again suggests potential bias toward detecting more severe disease. Evaluation of how well the HLM-E2T and HULS training sets differentiated animals with comparable severe respiratory characteristics to the animals included in the training set did not fall within the objective of this study.

In contrast to the operation-specific training sets, the BRD challenge training set was developed using cattle sampled before and after a BHV-1/*M. haemolytica* challenge. Although pathogen identification was not performed on the samples in this study, it is possible that this training set differentiated cattle with those pathogens from those without. Both BHV-1 and *M. haemolytica* are recognized BRD pathogens, but they can also exist as commensals in non-BRD cases, which could potentially explain the high BRD prevalence observed when applying the BRD challenge training set to this population. At present, it remains unknown which VOCs are specifically associated with BRD pathogens or inflammatory responses. Further complicating interpretation, the VOCs detected by individual sensors within the Cyranose® 320 eNose are proprietary, limiting the ability to identify or link sensor responses to pathogen-specific or disease-related VOCs with this device.

Traditional diagnostic metrics presented in Table 3.5 are included as some operations may regard their clinical observation of respiratory disease as a perfect or near-perfect reference test. However, caution is warranted when evaluating other diagnostic tests against a reference in this manner, as a new test cannot outperform the standard it is being compared to. Clinical observation of respiratory signs, with or without rectal temperature, has previously been reported to diagnose BRD in feedlot cattle with a sensitivity of 0.27 (95% Bayesian credible interval: 0.12–0.65) and a specificity of 0.92 (0.72–0.98) using the presence of lung lesions at slaughter as the reference test.[24] This highlights the inconsistency in clinical observation for BRD detection as a reference test. Despite these limitations, sensitivity and specificity remain useful for understanding how well a new test identifies positives and negatives relative to the chosen reference. For example, the 0.72 overall percent agreement observed between the BRD challenge training set and clinical observation (Table 3.4) can be further interpreted as the eNose correctly identifying 90% of clinical BRD cases (sensitivity), but only 11% of non-BRD controls (specificity). This shows that there is high agreement on positive cases, but low agreement on negative cases. Without a gold standard test, it is exceedingly difficult to conclude which test, if any, correctly diagnosed those that are truly negative for BRD. Incorporating additional diagnostic methods with stronger ability to detect BRD-negative cases would provide valuable support in this context.

When considering predictive values, an interesting pattern emerges across all eNose training set/algorithm combinations in this study (Table 3.5). The PPV and NPV are broadly similar, all with overlapping CIs. PPVs in this study are relatively reliable at finding an animal diagnosed by clinical observation as positive, ranging from 0.73 to 0.86, while NPVs are consistently low and relatively unreliable at finding an animal diagnosed as a non-BRD control by clinical observation, ranging from 0.16 to 0.25. The fact that PPV and NPV are similar across training set/algorithm combinations, despite differences in sensitivity and specificity, is explained by the varying apparent prevalences (AP) of BRD classification by the eNose training set/algorithm combinations. It would be difficult if only using sensitivity, specificity, PPV and NPV to determine the eNose training set/algorithm combination that most closely matches clinical observation. This illustrates why relying solely on traditional diagnostic metrics when comparing two imperfect tests can be misleading. Agreement statistics provide an alternative method for evaluating diagnostic performance and may offer clearer guidance for selecting the most appropriate test in practice.

Additional limitations of this study relate to the eNose's direct application as a chute-side or POC tool as tested in this study. Because the eNose required training prior to classifying the remaining samples, it was not used alongside the current operation's procedures for disease detection. Training was performed after all samples from the study were collected and treatment data was obtained to allow optimization of the training sets. The saved sensor data from the remaining samples not included in the training set were only ran on the CDAnalysis™ software after training sets were finalized. This occurred several months after the conclusion of the study. Along with this, samples were not performed on the eNose immediately after collection but instead one to eight hours later due to facility and operation constraints. It is unknown if the sample stability remains constant from collection up to eight hours. While results still support same-day testing on the eNose, chute-side testing should be further evaluated. Although data exists on the use of clinical observation of respiratory signs for diagnosing BRD, the accuracy of this method can vary widely between evaluators, resulting in large differences in reported sensitivity and specificity. In the present study, no sensitivity or specificity was calculated for clinical observation alone. While personnel at this operation were trained by a consulting veterinarian, diagnostic performance could differ compared to other operations. Therefore, operations interested in implementing this tool should first evaluate it in parallel with their current method of BRD detection to assess reliability and agreement.

Conclusions

The eNose demonstrated potential as a diagnostic tool for identifying cattle with naturally occurring BRD. Agreement with clinical observation was good when using the BRD challenge training set in this study. However, in the absence of a gold standard BRD detection method, these findings are limited to assessing consistency between the two tests rather than determining diagnostic accuracy. Implementation of the eNose should be tailored to each operation, in consultation with their veterinarian, as differences in herd management, cattle populations, and disease prevalence may influence performance. Before adoption, operations should validate the eNose alongside their existing diagnostic methods to ensure reliability and practical application. Future research should focus on validating the BRD challenge training set for universal application, refining the approach for creation of operation specific training sets and assessing performance under varied operational conditions to maximize diagnostic agreement and utility in commercial cattle operations.

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Table 3.1. Characteristics of cattle used to create the high lot morbidity-early 2nd treatment (HLM-E2T) and high ultrasound lung score (HULS) eNose training sets for the bovine respiratory disease (BRD) and non-respiratory control (CON) classes.

Animal Identification	Clinical Diagnosis	Sex	Weight (kg)	Days-on-feed	Lot BRD Morbidity (%)	Days to 2nd BRD Treatment after Enrollment	ULS
HLM-E2T BRD Class							
Animal #1	BRD	Steer	263	33	61.2	7	–
Animal #2	BRD	Heifer	259	5	46.8	2	–
Animal #3	BRD	Steer	216	10	25.3	6	–
Animal #4	BRD	Heifer	253	68	28.0	7	–
Animal #5	BRD	Steer	327	15	30.0	3	–
HLM-E2T/HULS CON Class							
Animal #1	Musculoskeletal	Heifer	558	52	0.8	–	–
Animal #2	IBK	Heifer	524	9	3.1	–	–
Animal #3	Musculoskeletal	Heifer	381	14	1.5	–	–
Animal #4	Musculoskeletal	Heifer	517	8	3.1	–	–
Animal #5	Musculoskeletal	Steer	680	104	1.8	–	–
HULS BRD Class							
Animal #1	BRD	Heifer	405	125	–	–	5
Animal #2	BRD	Steer	398	56	–	–	5
Animal #3	BRD	Heifer	365	64	–	–	4
Animal #4	BRD	Heifer	441	97	–	–	4
Animal #5	BRD	Heifer	442	81	–	–	4

BRD = bovine respiratory disease. CON = non-respiratory control. HLM/E2T = High lot morbidity/early 2nd treatment. HULS = High ultrasound lung score. IBK = infectious bovine keratoconjunctivitis. ULS = ultrasound lung score.

Table 3.2. Cyranose® 320 eNose data analysis configuration settings and associated cross validation for each training set (BRD challenge, HLM-E2T and HULS) and machine learning algorithm (CDA, KNN and SVM) combinations.

Algorithm	Preprocessing	Normalization	Identification Quality	Cross Validation	M-Distance
BRD Challenge Training Set					
CDA	Auto-scaling	Normalization 1	Always Choose	70%	5.12
HLM-E2T Training Set					
CDA	Auto-scaling	Normalization 1	Always Choose	40%	4.84
KNN	Auto-scaling	Normalization 1	Always Choose	50%	–
SVM	Auto-scaling	Normalization 1	Always Choose	80%	–
HULS Training Set					
CDA	Auto-scaling	Normalization 1	Always Choose	90%	6.17
KNN	Auto-scaling	Normalization 1	Always Choose	50%	–
SVM	Auto-scaling	Normalization 1	Always Choose	90%	–

CDA = canonical discriminant analysis. HLM-E2T = high lot morbidity-early 2nd treatment. HULS = high ultrasound lung score. KNN = k-nearest neighbors. M-Distance = Mahalanobis distance. SVM = support vector machine.

Table 3.3. Demographic characteristics and clinical diagnoses of cattle included in the final dataset for analysis.

Variable	**Value**
Total animals	363
Bovine respiratory disease (BRD)	282 (77.7)
Non-respiratory control (CON)	81 (22.3)
Sex	
Heifer	197 (54.3)
Steer	166 (45.7)
Weight (kg)	404.6 ± 103.0
Days-on-feed	41 (2–203)
Non-respiratory diagnoses	81
Musculoskeletal disorders	78 (96.3)
Digestive disorders	2 (2.5)
Infectious bovine keratoconjunctivitis (IBK)	1 (1.2)

Values reported as n (%), median (range) or mean ± SD.

Table 3.4. Diagnostic agreement metrics for detecting bovine respiratory disease (BRD) between clinical observation of respiratory signs and eNose training set (BRD challenge, HLM-E2T and HULS) and machine learning algorithm (CDA, KNN and SVM) combinations.

	Overall Percent Agreement		Cohen's Kappa		Gwet's AC1	
Algorithm	**Estimate**	**95% CI**	**Coefficient**	**95% CI**	**Statistic**	**95% CI**
BRD Challenge Training Set						
CDA	0.72	0.68–0.77	0.01	-0.09–0.11	0.62	0.54–0.70
HLM-E2T Training Set						
CDA	0.41	0.36–0.46	-0.06	-0.14–0.02	-0.14	-0.25 – -0.03
KNN	0.42	0.38–0.48	-0.10	-0.187– -0.02	-0.08	-0.19–0.04
SVM	0.37	0.32–0.42	-0.05	-0.12–0.02	-0.24	-0.35– -0.14
HULS Training Set						
CDA	0.42	0.38–0.48	0.07	0.01–0.13	-0.13	-0.24– -0.03
KNN	0.40	0.36–0.46	0.07	0.01–0.12	-0.18	-0.28– -0.08
SVM	0.50	0.45–0.56	0.02	-0.07–0.10	-0.24	-0.35– -0.14

CDA = canonical discriminant analysis. CI = confidence interval. HLM-E2T = high lot morbidity-early 2nd treatment. HULS = high ultrasound lung score. KNN = k-nearest neighbors. SVM = support vector machine.

Table 3.5. Diagnostic performance metrics for eNose training set (BRD challenge, HLM-E2T and HULS) and machine learning algorithm (CDA, KNN and SVM) combinations for detecting bovine respiratory disease (BRD) using clinical observation of respiratory signs as the reference test.

							Sensitivity		Specificity		PPV		NPV	
Algorithm	**TP**	**TN**	**FP**	**FN**	**Total**	**AP**	**Estimate**	**95% CI**	**Estimate**	**95% CI**	**Estimate**	**95% CI**	**Estimate**	**95% CI**
BRD Challenge Training Set														
CDA	253	9	72	29	363	0.90	0.90	0.86–0.92	0.11	0.05-0.20	0.78	0.73–0.82	0.24	0.11–0.40
HLM-E2T Training Set														
CDA	105	40	36	172	353	0.40	0.38	0.32–0.44	0.53	0.41–0.64	0.74	0.66–0.81	0.19	0.14–0.25
KNN	120	31	45	157	353	0.47	0.43	0.37–0.49	0.41	0.30–0.53	0.73	0.65–0.79	0.16	0.11–0.23
SVM	85	30	46	192	353	0.33	0.31	0.25–0.36	0.61	0.49–0.72	0.74	0.65–0.82	0.19	0.15–0.25
HULS Training Set														
CDA	91	60	16	186	353	0.30	0.33	0.27–0.39	0.79	0.68–0.87	0.85	0.77–0.91	0.24	0.19–0.33
KNN	79	63	13	198	353	0.27	0.29	0.24–0.35	0.83	0.73–0.91	0.86	0.77–0.92	0.25	0.20–0.30
SVM	138	40	36	139	353	0.49	0.50	0.44–0.56	0.53	0.41–0.64	0.79	0.73–0.85	0.22	0.16–0.29

AP = apparent prevalence. CDA = canonical discriminant analysis. CI = confidence interval. FP = false positive. FN = false negative. HLM-E2T = high lot morbidity-early 2nd treatment. HULS = high ultrasound lung score. KNN = k-nearest neighbors. NPV = negative predictive value. PPV = positive predictive value. SVM = support vector machine. TP = true positive. TN = true negative.

Chapter 4 - Assessment of an Electronic Nose for Predicting Treatment Outcomes at First Treatment for Bovine Respiratory Disease in Feedyard Cattle

Abstract

Bovine Respiratory disease (BRD) is the most significant health challenge in feedyard beef cattle with few available and practical methods to reliably predict treatment response. The development of predictive tools that can be implemented on-farm to provide timely and accurate results would provide substantial value in improving BRD management. The objective of this study was to evaluate the performance of a commercially available electronic nose (eNose) for predicting treatment response at the time of first bovine respiratory disease (BRD) treatment in feedyard cattle. Nasal swabs were collected from cattle (n = 258) diagnosed with BRD in the feedyard and evaluated with an eNose at the time of first treatment. Two separate training sets matched with machine learning algorithms were created from the study population to allow the eNose to predict either 2 or 3 potential treatment outcomes. The overall accuracy of the eNose in predicted treatment outcome was poor (<45%) regardless of the method employed. While the eNose did show >70% agreement between predicted and true outcome for first treatment success with all training set and algorithm combinations, this came at the cost of a high misclassification rate for the prediction of other outcomes (retreatment, did not finish and first treatment failure). This study provides considerations for future studies attempting to discriminate treatment outcomes for cattle using an eNose. Further optimization of eNose methods should be explored before application in a commercial feedyard setting.

Introduction

The application of technology to detect and characterize volatile organic compounds (VOCs) as a component of precision livestock farming in cattle remains relatively untested in commercial production settings. Technology, such as an electronic nose (eNose), gas chromatography-mass spectrometry and gas chromatography-ion mobility spectrometry, have been proposed as potential non-invasive tools for on-farm disease detection.[1-3] VOCs emitted from biological samples of cattle, such as breath, nasal secretions, feces, earwax, urine and milk, can be classified with specific diseases by these tools to guide treatment or management decisions on-farm.[4-9] However, there has been little evaluation on their overall utility and effectiveness in commercial settings where environmental conditions and logistical constraints may impact performance. This is especially true for bovine respiratory disease (BRD), the most economically important disease of feedyard cattle, which could benefit from on-farm early detection technology and predictive tools to guide clinical decision-making.[10,11]

Due to their portability and ability to provide rapid results, eNoses are among the most studied tools for VOC BRD diagnostics applied animal-side. Research to date has explored the diagnostic performance of eNoses, with studies reporting accuracy between 72.5–100% in the discrimination of BRD-affected from healthy control cattle.[12,13] Unfortunately, these studies generally included small sample sizes and were performed in controlled experimental environments, which limits study applicability to production settings. While evaluating the eNose as a diagnostic tool has been the primary focus in this field, no published studies have utilized the eNose for prediction of BRD treatment response, disease severity, or mortality. This represents a knowledge gap within this research area, highlighting the need for studies evaluating the prognostic potential of the eNose in BRD management to aid in individualized, targeted treatment regimens.

Thus, the objective of this study was to assess a commercially available eNose as a prognostic tool for the prediction of BRD treatment outcomes at the time of first treatment in feedlot cattle. The study will test if an eNose, trained on nasal secretions collected from cattle at first BRD treatment, can predict whether an animal will respond successfully to its first BRD treatment, require retreatment for BRD, or be at risk for BRD specific mortality or culling. Results will guide potential on-farm application of eNose technology as part of an integrated BRD management strategy.

Materials and Methods

Study design and enrollment criteria

This study was conducted in one commercial feedyard on crossbred beef cattle housed in dry lot pens in November and December of 2024. All study procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Kansas State University (protocol #5060 approved 16 September 2024) and informed owner consent was obtained for all animals included in the study. Feedyard cattle were evaluated daily by experienced personnel for signs of clinical illness. Cattle that showed one or more signs of depression, nasal discharge, cough, increased respiratory rate, or labored breathing were removed from their pen for chute-side evaluation. Rolling enrollment of study participants was conducted on eligible cattle during chute-side evaluation for BRD. These evaluations were performed by feedyard personnel trained in disease detection and treatment by a consulting veterinarian according to the feedyard's specific protocol. Enrollment criteria consisted of cattle diagnosed with BRD for the first time in the feedyard and at the time of diagnosis received an initial treatment for BRD. No other specific exclusion criteria were used for study enrollment.

Animal sampling

At enrollment a single nasal swab was used to collect nasal secretions from either the right or left nostril of the animal, based on safety and convenience of sampling. Prior to nasal swab collection cattle were restrained in a squeeze chute and a paper towel was used to wipe dirt and debris from the external nares. While restrained, a 15 cm sterile rayon swab with a polystyrene handle was inserted approximately 5 cm into one nostril and rotated against the nasal mucosa for approximately 3 seconds. The nasal swab was removed from the nostril and immediately stored in a 6 mL preservative-free blood collection tube held at environmental temperature (0 to 24 °C). At the end of each collection day, all swabs were transported to an off-site laboratory where they were evaluated by the eNose within one to eight hours following their collection time point. Cattle demographic information included lot number, individual identification number, sex, weight at enrollment, and days on feed at enrollment were collected from the feedyard's computerized management software at the time of study enrollment.

eNose sample collecting procedures

The eNose (Cyranose® 320, Sensigent, USA) equipped with 32 polymer-carbon black composite sensors was used to collect and characterize VOCs emitted from nasal swabs on the same day as sample collection. Individual sensor responses were provided by the eNose for each sample. For sensor data collection, the eNose was connect to a laptop computer housing software (PCnose™, version 6.8, Sensigent, USA) provided with the device. A nasal swab, remaining in the blood collection tube, was sampled using the same eNose methods and collection set-up described by Schelkopf, et al.[12] A sample's sensor data collection, using the eNose, was completed in 30 seconds. All sensor data were streamed from the eNose to the PCnose™ software and exported as a comma separated values (CSV) file. CSV files were labeled with animal identification and stored for later analysis.

eNose training for treatment outcome prediction

Individual animal 60-day treatment outcomes were collected at the conclusion of the study from the feedyard's computerized management software. Additionally, BRD treatments occurring after enrollment and BRD lot morbidity information were recorded. The ability of the eNose to determine 60-day treatment outcomes was based on 3 or 2-class outcome scenarios. The different class outcome structures were created to determine if the eNose had improved accuracy in predicting between 3 outcomes compared to 2 outcomes. The 3-class outcomes included:

- First treatment success (FTS): animals who were treated for BRD at enrollment and finished the 60-day follow-up period without receiving another BRD treatment.
- Retreated (RET): animals that received additional treatment(s) for BRD after their initial BRD treatment and finished the 60-day follow up period.
- Did not finish (DNF): animals that were culled or died of a BRD specific cause before the end of the 60-day follow up period.

A single animal could only be classified as one of the three outcomes. Animals with 60-day outcome records who did not classify into either FTS, RET, or DNF were removed from the study. The 2-class outcomes included:

- FTS: the same animals in FTS for the 3-class outcomes.
- First treatment failure (FTF): the combined RET and DNF classifications from the 3-class outcomes.

Prior to using this eNose to predict treatment outcomes, the device was trained on a subset of animals from the study. Two training sets were created, one for the 3-class outcome and one for 2-class outcome. The 3-class outcome training set consisted of 3 classes (FTS, RET, DNF) with 5 animals per class. The 2-class outcome training set contained 2 classes (FTS, FTF) with 5 animals per class. Each training set went through an extensive selection process for optimization using individual animal and lot level health data along with matching machine learning algorithms with the highest cross validations. Final training sets for the 3-class and 2-class were each matched with the same 3 machine learning algorithms (k-nearest neighbors [KNN], canonical discriminant analysis [CDA], support vector machine [SVM]) to create at total 6 training set/algorithm combinations used to predict the outcome of the remaining samples.

eNose training set selection: expanded

The eNose used in this study requires training prior to sample classification. Training was completed after all 60-day treatment outcomes were determined, using nasal swab sample sensor data collected from study animals at enrollment. The eNose was first trained to classify animals into the 3-class outcomes (FTS, RET, DNF). Individual animal records were used to select five animals from each outcome to be added to the eNose training set for initial training of the device. Criteria for initial animal selection was as follows: among the FTS animals those with the lowest lot BRD morbidity, of the RET animals those who were retreated for BRD within 7 days of enrollment, and of the DNF animals those who were retreated for BRD within 7 days of enrollment and were culled or died within the fewest number of days after enrollment. The initial training set was uploaded into the chemometric data analysis software (CDAnalysis™, version 11.2, Sensigent, USA) which was provided with the eNose, contained on a laptop computer. The CDAnalysis™ software provided a principal component analysis (PCA) score plot of the training set identifying all animals and their associated outcomes. Iterations of the training set were made by removing animals initially included in the training set and adding new ones with similar health data to create the greatest visual separation between outcomes on the PCA plot. Once a final training set was established, cross validation for all machine learning algorithms provided on the eNose were performed using the CDAnalysis™ software to determine the highest percent successful discrimination between the three outcomes.

For the creation of the 2-class outcomes training set, the same five animals were retained for FTS from the final training of the 3-class training set to create FTS for the 2-class training set. Additionally, a total of five animals were selected from the RET and DNF of the three-class training set to create FTF for the 2-class training set. This training set was uploaded to the CDAnalysis™ software, and iterations of the training were performed with only animals used in the 3-class training set to achieve the greatest visual separation between outcomes on the PCA plot. Cross validation of all machine learning algorithms was performed on the 2-class training set in the same manner as the 3-class.

The three highest percent cross validation machine learning algorithms were selected from both the 3 and 2-class training sets to be tested for agreement with an animal's true 60-day treatment outcome. This was achieved by uploading the individual animal CSV file collected from the eNose into the CDAnalysis™ software with the uploaded training set and allowing the software machine learning algorithm to predict which outcome the unknown animal most closely represented based on the combined 32 sensor response. The program assigned each animal a categorical outcome of FTS, RET, or DNF from all three of the algorithms used for the 3-class training set and either FTS or FTF for all three algorithms used for the 2-class training set.

Statistical analysis

Sixty-day outcome records were matched with the eNose predicted outcome for each training set/algorithm combination tested (n=6). Animals included in the final training sets were excluded from the analysis. Data were analyzed with descriptive statistics and by logistic regression models using “glm” function in the ‘stats’ package of R Studio® (R Studio®, version 2024.12.1.563; R Core Team) to determine probability of agreement. In total, 6 logistic regression models were used, three for the 3-class outcomes and three for the 2-class outcomes based on the number of algorithms tested. For each model, a binomial outcome variable consisting of agreement (1) or disagreement (0) based on the algorithm predicted outcome and true outcome at the conclusion of the 60-day follow-up period. Each model included a single fixed effect variable for the predicted outcome given by the eNose based on the algorithm used. Model significance level was set to $p < 0.05$.

Results

In total, 287 cattle were sampled throughout the study period. Twenty-nine animals were excluded from the final data set: 3 were mislabeled at enrollment as first-diagnosis BRD cases, 9 were culled or died due to a non-respiratory cause, 14 were shipped to harvest before the end of the 60-day follow-up period, and 3 were diagnosed with calf diphtheria (necrotic laryngitis). The final data set contained eNose sensor data from 258 nasal swabs collected throughout the study and was used for training set creation and testing. Demographic information collected at enrollment from study animals in the final data set and stratified by 3-class and 2-class treatment outcomes are displayed in Table 4.1. The distribution of treatment outcomes for the study population consisted of 74% FTS, 17.1% RET and 8.9% DNF. Of the DNF group 10 animals died and 13 were culled due to BRD.

Health demographic information used for selection of animals in the final training set for both the 3-class (n = 15) and 2-class (n = 10) outcomes are presented in Table 4.2. Figures 4.1 and 4.2 display the PCA score plots of the final training set for 3 and 2-class. Mahalanobis distances (M-distances), provided by the eNose canonical algorithm used to assess distance between classes, were below manufacturer's recommendation of ≥5 for optimum discrimination for all 3 and 2-class outcomes (data not shown). Table 4.3 shows the cross-validation of those training sets when different machine learning algorithms and data analysis configurations are applied. Cross validation, expressed as a percentage, was suboptimal when viewing the CDA and KNN algorithms for the 3 and 2-class training sets, while SVM algorithm was optimal for both the training sets (manufacturer recommends ≥ 90% cross validation for optimal discrimination between classes).

Table 4.4 and Table 4.5 show contingency tables for each training set algorithm for the 3-class and 2-class outcomes, respectively. All 3-class training set algorithms performed poorly for overall accuracy of the eNose to correctly predict 60-day outcomes. Overall accuracy increased when the eNose was used to predict 2 classes instead of 3-class. Unfortunately, accuracy was still poor regardless of the training set and algorithm type. Training set performance was further evaluated by the eNose's ability to correctly predict individual treatment outcomes. Three-class true outcome prevalences were not evenly distributed amongst the clinical outcomes evaluated (Figure 4.3). FTS exceeded over 75% of the observed true outcomes, while RET accounted for 16% and DNF for 7.4%. Three-class prevalence distribution for the eNose predicted outcomes, regardless of the algorithm type, was drastically different for the true outcomes. All algorithms predicted RET with the highest prevalence among the 3 classes while CDA and KNN both predicted FTS with the lowest prevalence among the 3 classes.

The probability of agreement between true treatment outcome and eNose predicted outcome by algorithm type was modeled. Across all 3-class training set algorithms tested on the eNose the probability of agreement between the eNose predicted outcomes and true outcomes was significantly higher for FTS compared to RET and DNF (Figure 4.4). FTS mean probability of agreement was highest for CDA (77.1%; 95% confidence interval [CI]: 64.9 – 85.9%) followed by SVM (76.8%; 95% CI: 66.5 – 84.7%) and KNN (71.6%; 95% CI: 59.8 – 81.1%). No significant difference was observed between RET and DNF within algorithm. Between algorithm types, mean differences were observed between RET and DNF, unfortunately all RET were below 20% and all DNF were below 10%.

Similar prevalence trends were observed when the RET and DNF outcomes were combined to create the FTF outcome in the 2-class training set (Figure 4.5). Two-class true outcome prevalence was highest for FTS (75%) with FTF accounting for 25%. The inverse of this relationship in prevalence was observed for all eNose training set algorithms with FTF comprising a higher number of the predicted outcomes. Agreement between eNose predicted and true outcome were modeled for 2-class algorithms (Figure 4.6). FTS had a significantly higher probability of agreement compared to FTF for all algorithm types. FTS mean probability for all 2-class algorithms was similar to mean probabilities in 3-class algorithms. CDA provided the highest mean probability of agreement for FTS with 77.8% (95% CI: 67.5 – 85.5%) followed by SVM (73%; 95% CI: 61.8 – 81.9%) and KNN (71.8%; (95% CI: 62.4 – 797.7%). Minor differences in mean probability of agreement for FTF were observed between the different eNose algorithms. While FTF probability of agreement for each algorithm was higher than either RET or DNF in all 3-class algorithms, means were still below 30%.

Discussion

This study represents the first published attempt at using an eNose to predict BRD treatment outcomes in feedyard beef cattle. This eNose was selected due to its commercial availability and rapid results. The eNose was used in a manner that could be integrated as part of an existing chute-side protocol for diagnoses and treatment of BRD without impeding the flow of operations. The use of a nasal swab with its associated collection method developed by Schelkopf, et al.[12] provided a convenient sample for collection, transportation and sensor analysis of nasal secretions by the eNose. The sample could be transported to other locations on or off the operation, as is the case in this study, for analysis without known compromise of the sample headspace used for VOC analysis.

The results of this study, however, are not promising for immediate application of this technology for chute-side prognostication of BRD treatment outcomes in a commercial feedyard. Overall accuracy of the eNose predicting treatment outcomes were low (Table 4.4 & 4.5). This low accuracy can be explained by viewing the true outcome prevalences and eNose predicted outcome prevalences (Figure 4.3 & 4.5). As shown in Figure 4.3, the eNose predicted RET as the most frequent outcome for all algorithms (CDA: 41.2%, KNN: 37.4%; SVM: 37%) when the true prevalence of RET in the study population was only 16%. The same trend was true for DNF where the eNose over-predicted the number of animals with that outcome compared to the true outcome. This over prediction led to low agreement between eNose predicted outcomes and true outcomes emphasizing the lack of confidence a test operator may have if an animal was predicted as RET or DNF by the eNose. The opposite of this can be seen with a much higher probability of agreement for FTS in Figure 4.4. A test operator would have higher confidence that an animal predicted as FTS was truly an FTS. However, when viewing these results for FTS probability of agreement in light of the true prevalence (76.5%) and eNose predicted prevalence across the algorithms tested (range: 25.1 – 33.7%), utility of the device becomes somewhat diminished (Figure 4.3). In this situation the eNose under predicted the number of FTS in a population. Due to the true prevalence of FTS comprising over 75% this inherently leads to a higher chance of the eNose predicted FTS correctly, especially when it predicts that outcome infrequently.

Predicting greater than 2 outcomes can cause challenges for the eNose's discrimination ability especially when those classes have substantial overlap with regard to sensor response. This was anticipated prior to the start of the study and an analysis of the eNose was included for predicting 2 outcomes. Because RET and DNF comprised the two smallest outcomes in the 3-class structure, these were combined to create a single class labeled FTF as both of these outcomes failed their first treatment for BRD. Better discrimination was achieved by combining these outcomes and the overall accuracy of the 2-outcome training set was higher compared to the 3-outcome but still considered suboptimal for application in a commercial production setting (Table 4.5). In Figure 4.6, the probability of agreement between eNose predicted outcome and true outcome for FTF was higher than the combination of RET and DNF in the 3-class training set. FTS probability of agreement remained largely unchanged from the 3-class training set to 2-class training set regardless of algorithm. The probability of agreement for FTS may warrant further evaluation for prognostication purposes. Although the eNose might not be suitable as a standalone test, its value as a component of a prognostic framework should be explored. Integrating the eNose with tests that provide high confidence for predicting treatment outcomes such as RET, DNF, or FTF, but low confidence for FTS could enhance overall predictive accuracy.

While the use of an eNose to predict treatment outcomes in veterinary medicine remains largely untested, previous research in human medicine has demonstrated poor prognostication for treatment response. In a study involving lung cancer patients, breath samples analyzed by an eNose had an accuracy of 58% in predicting response to treatment therapy.[14] Similarly, another evaluating rectal cancer treatment outcome by analyzing breath samples with an eNose reported a sensitivity of 64% and a specificity of 47% with an overall accuracy of 58%.[15] Results from the current study align with these findings, highlighting the challenges in using an eNose for reliable disease prognostication.

Modifications and considerations to the eNose methodology used in this study should be contemplated for potentially improving overall accuracy in future research pursuing this device for prognostic purposes. Selection of animals for inclusion in the eNose training set remains an area of contention. A major limitation of this device is the limited number of animals used to train the machine learning algorithms. In this study each training class consisted of 5 animals. This eNose has a minimum number of 5 and a maximum number of 10 animals that can be included per training class. The decision to include only 5 animals per training class was based on prior research demonstrating the effectiveness of this approach.[12] This strategy also allowed retention of the maximum number of animals in the RET and DNF groups for algorithm testing. Regarding individual animal selection for the training classes, initial selection was based on health information in an attempt to separate sensor responses of animals between respective classes. It was unknown prior to the study whether health information would play any role in separating classes. However, this method was pursued as this data was readily available on this operation and would be data that could be collected and used to create training sets on different operations. Several iterations of the training set, by removal and replacement of animals, were performed prior to deployment of the final training set to optimize class separation. M-distances between classes changed only minimally between the training set iterations denoting that there is much overlap in sensor responses between animals of different classes (data not provided). Other methods for selecting training animals should be explored in future studies with consideration given to their applicability for animal selection in other operations.

The same 3 machine learning algorithms (CDA, KNN, and SVM) were applied to both the 3-class and 2-class training sets to provide direct comparison. The eNose manufacturer recommends ≥ 90% cross validation for any algorithm type to reliably discriminate between classes. This was only achieved for the SVM algorithms. Low cross validation observed with the CDA and KNN indicates that the classes incorporated in the training set have overlap in individual training sample sensor responses making discrimination with those algorithms unreliable. Previous research has challenged this with lower cross validation (< 90%) producing satisfactory, and high cross validation (> 90%) producing unsatisfactory discrimination ability.[6,12] Understanding the dynamics of these algorithms can lead to a more streamlined selection process when the sensor data characteristics are known. CDA is a linear classification algorithm that projects sensor data to maximize separation between classes in the training set.[16] When there is significant overlap between classes in the training set, the predictive performance of CDA tends to be reduced.

KNN is a nonlinear instance-based classification algorithm that assigns a class to a sample based on the majority of training samples nearest in the training set.[17] If classes are not well separated and training samples near the predicted sample have mixed class labels, the algorithm struggles to make accurate distinctions. SVM is a supervised, non-linear algorithm effective for handling overlapping or poorly resolved classes.[18] This algorithm's performance tends to degrade when training samples are mislabeled, as this introduces noise that can mislead the model and reduce predictive accuracy. It was not surprising that SVM produced the highest cross validation in this study due to sensor overlap, however the performance of this algorithm was underwhelming. We believe that "mislabeling" of training samples might play a role in its overall poor discrimination ability. While we are confident that our training samples were accurately labeled based on their treatment outcome, these labels may not necessarily correspond to distinctly different VOC signatures from nasal secretions.

Other considerations for improving discrimination between training set classes and ultimately treatment outcomes could include disabling specific sensors on the eNose that contribute to noise or show low discriminatory power. Additionally, sensor data can be exported for use on other statistical or machine learning programs not provided with the Cyranose® 320 eNose. Finally, while nasal secretions have demonstrated success in discrimination between healthy and sick animals, other sample types such as breath should be explored as their VOC signature could capture different or more comprehensive aspects of the disease related to determining treatment response.

Conclusions

The use of eNose technology for prognosticating outcomes in feedlot cattle treated for BRD remains inconclusive. This study highlights the challenges associated with direct application of this tool in a commercial production setting. Although the eNose training sets and methodology developed in this study and expanded upon from previous work in this research field represent important progress, they currently lack the accuracy required to reliably discriminate between treatment outcomes relevant to production medicine. Future studies should address these limitations to improve the eNose's performance for prognostication purposes.

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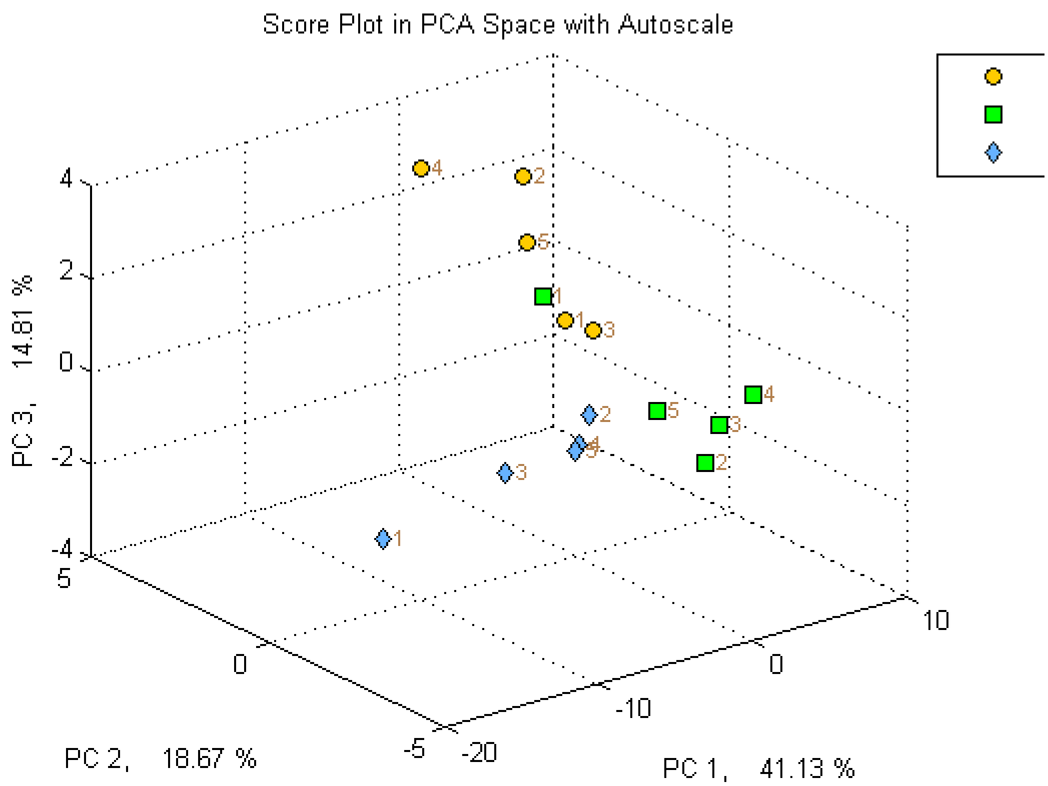

Figure 4.1. Principal component analysis (PCA) score plot from Cyranose® 320 eNose CDAnalysis™ software for the final 3-class training sets. Individual shapes represent a single animal included in one of the training set classes. Five animals were used to create a single class. The 3 classes consisted of: yellow circle = did not finish (DNF) class, green square = retreated (RET) class and blue diamond = first treatment success (FTS).

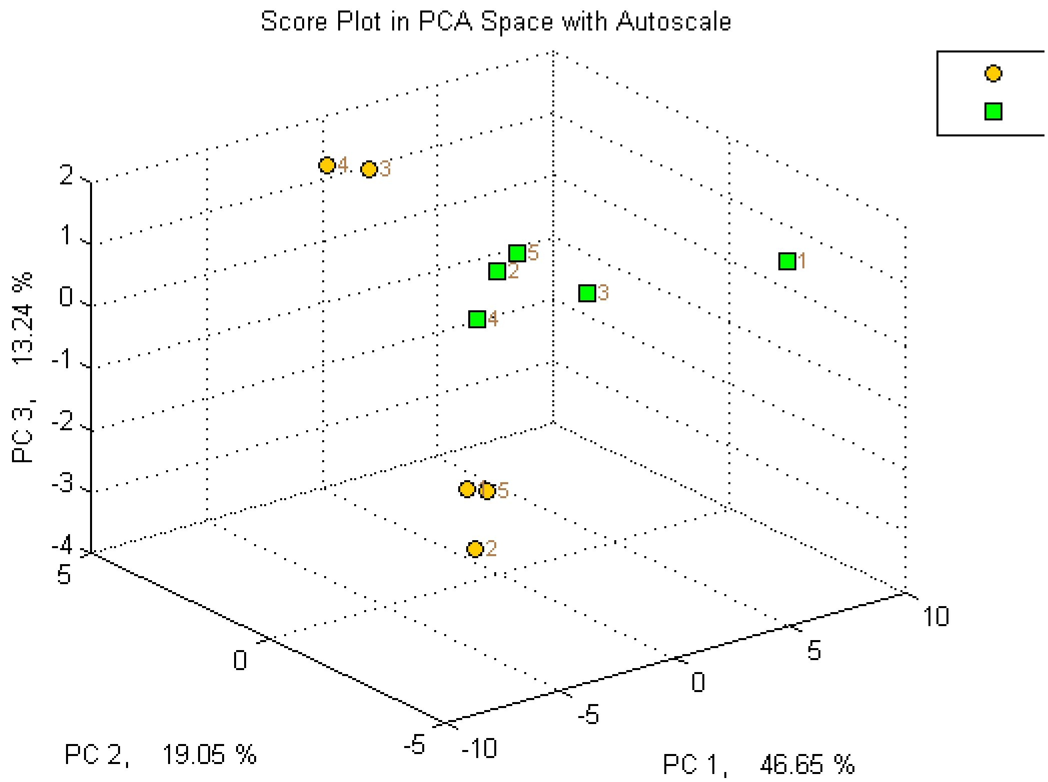

Figure 4.2. Principal component analysis (PCA) score plot from Cyranose® 320 eNose CDAnalysis™ software for the final 2-class training sets. Individual shapes represent a single animal included in one of the training set classes. Five animals were used to create a single class. The 2 classes consisted of: yellow circle = first treatment failure (FTF) and green square = first treatment success (FTS).

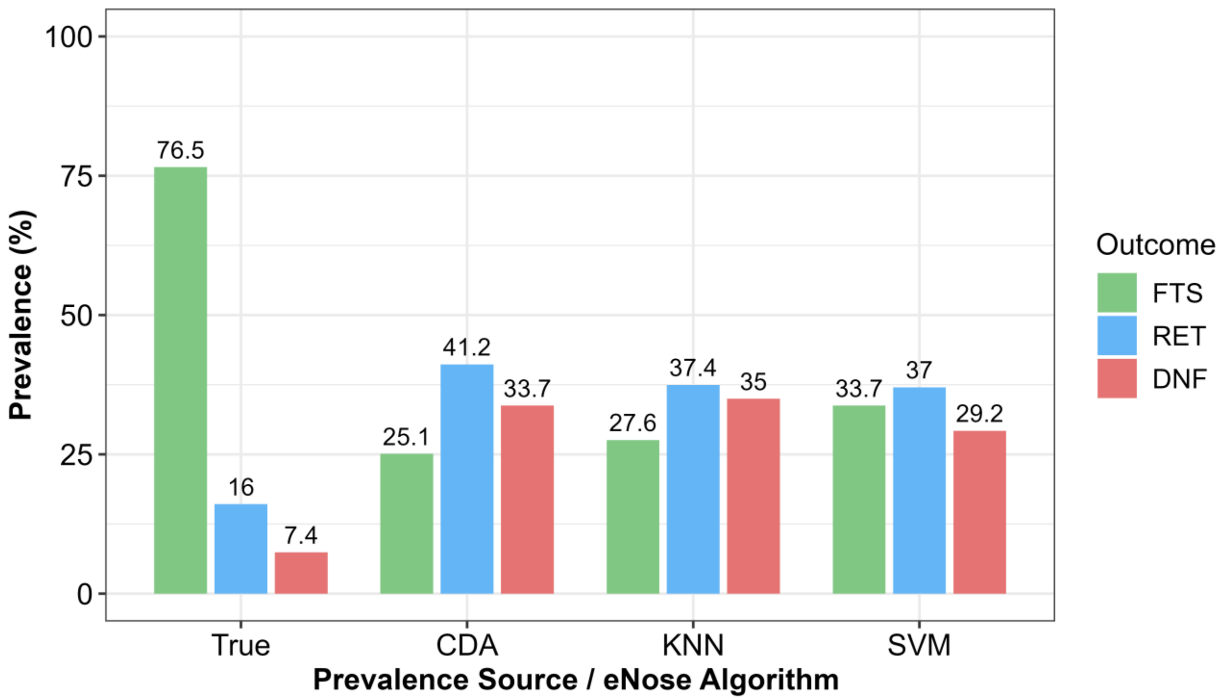

Figure 4.3. Comparison of 3-class outcome prevalence between true 60-day treatment outcomes and eNose predicted 60-day treatment outcomes across 3 machine learning algorithms (CDA, KNN, and SVM). Prevalence sources (n = 4) exclude animals used in creation of training set (n = 15).

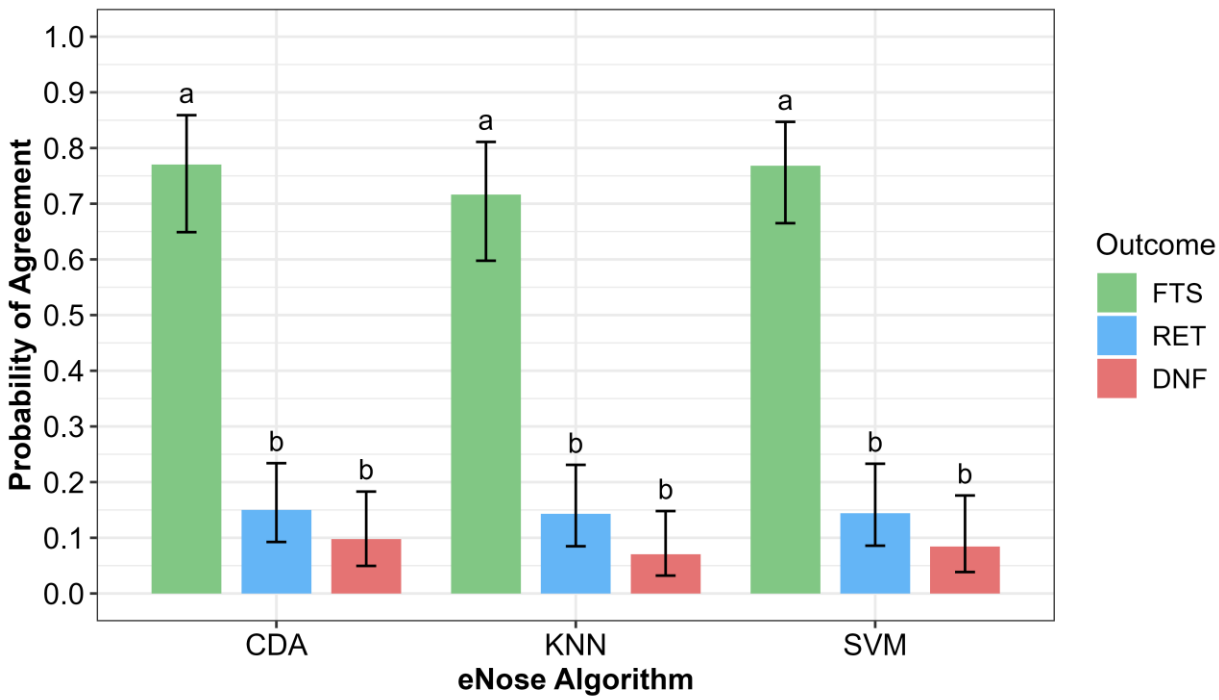

Figure 4.4. Model-estimated mean probability of agreement between 3-class true 60-day treatment outcomes and eNose predicted 60-day treatment across 3 machine learning algorithms. Error bars represent 95% confidence intervals. Statistical differences in agreement were assessed within each algorithm and not between algorithms. Different letters above bars indicate statistically significant differences in agreement level ($p < 0.05$) between outcome classes within algorithm only. Animals used to create the training set are excluded from the analysis ($n = 15$).

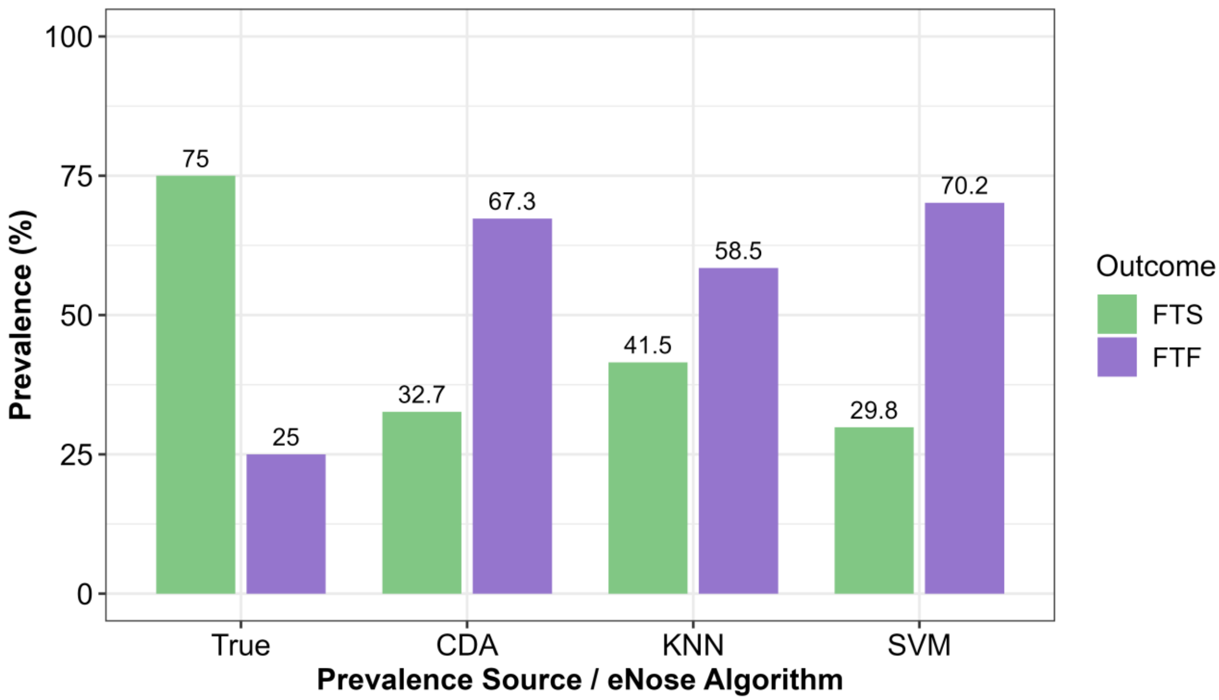

Figure 4.5. Comparison of 2-class outcome prevalence between true 60-day treatment outcomes and eNose predicted 60-day treatment outcomes across 3 machine learning algorithms (CDA, KNN, and SVM). Prevalence sources (n = 4) excludes animals used in creation of training set (n = 10).

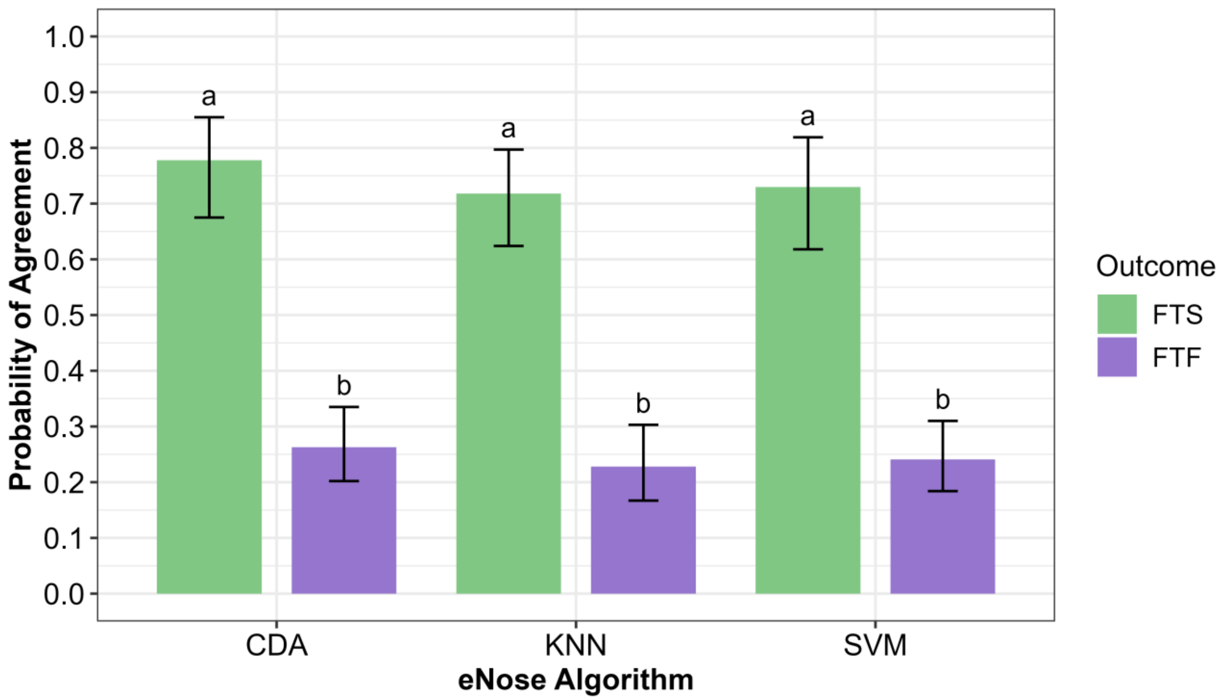

Figure 4.6. Model-estimated mean probability of agreement between 2-class true 60-day treatment outcomes and eNose predicted 60-day treatment across 3 machine learning algorithms. Error bars represent 95% confidence intervals. Statistical differences in agreement were assessed within each algorithm and not between algorithms. Different letters above bars indicate statistically significant differences in agreement level ($p < 0.05$) between outcome classes within algorithm only. Animals used to create the training set are excluded from the analysis ($n = 10$).

Table 4.1. Cattle demographic information collected at enrollment for all cattle included in the final data set (n = 258) and stratified by 3 and 2-class outcomes.

		3-Class Outcomes			2-Class Outcomes	
Category	**All Cattle**	**FTS**	**RET**	**DNF**	**FTS**	**FTF**
Count	258	191 (74.0)	44 (17.1)	23 (8.9)	191 (74.0)	67 (26.0)
Sex						
Heifer	131	92 (70.2)	23 (17.6)	16 (12.2)	92 (70.2)	39 (29.8)
Steer	127	99 (78.0)	21 (16.5)	7 (5.5)	99 (78.0)	28 (22.0)
Weight (kg)	369.1 ± 86.6	384.3 ± 86.2	324.2 ± 68.7	328.6 ± 80.3	384.3 ± 86.2	325.7 ± 72.3
Days-on-feed	28 (2 – 141)	35 (2 – 141)	19.5 (2 – 128)	18 (2 – 97)	35 (2 – 141)	18 (2 – 128)

Data are n (%), median (range) or mean ± SD.
FTS = first treatment success, RET = retreated, DNF = did not finish, FTF = first treatment failure.

Table 4.2. Health demographic information of animals used in eNose training set for 3-class and 2-class outcomes. Animals included in the 2-class outcome training set are identified with superscripts in the table.

Animal Identification	Lot BRD Morbidity (%)[d]	# of Additional BRD Treatments	Days to 2nd BRD Treatment from Enrollment	Days to Culling or Mortality from Enrollment
First Treatment Success (FTS)				
Animal #1[a]	4.7%	0	–	–
Animal #2[a]	6.1%	0	–	–
Animal #3[a]	4.0%	0	–	–
Animal #4[a]	4.9%	0	–	–
Animal #5[a]	4.7%	0	–	–
Retreated (RET)				
Animal #6[b]	61.2%	2	7	–
Animal #7	46.8%	2	2	–
Animal #8[b]	25.3%	2	6	–
Animal #9[b]	28%	2	7	–
Animal #10	30%	1	3	–
Did Not Finish (DNF)				
Animal #11	41.2%	1	6	7[c]
Animal #12[c]	3.4%	2	2	10
Animal #13	10%	2	5	7[c]
Animal #14	11.7%	2	4	14[c]
Animal #15[c]	16.9%	2	6	39

[a]Identifies animals used in FTS for 2-outcome group.
[b]Identifies animals used in FTF for 2-outcome group.
[c]Identifies animals that experienced mortality.
[d]Calculated as # of animals receiving 1st BRD treatment in the lot divided by all animals in the lot.

Table 4.3. Cyranose® 320 eNose data analysis configuration settings and associated cross validation for each machine learning algorithm used in the 3-class and 2-class training sets.

Algorithm	Preprocessing	Normalization	Identification quality	Cross Validation
3-Class Training Set				
CDA	Mean-centering	Normalization 1	Always Choose	20%
KNN	Auto-scaling	Normalization 1	Always Choose	40%
SVM	Auto-scaling	Normalization 1	Always Choose	95.6%
2-Class Training Set				
CDA	Auto-scaling	Normalization 1	Always Choose	40%
KNN	Auto-scaling	Normalization 1	Always Choose	40%
SVM	Auto-scaling	Normalization 1	Always Choose	90%

CDA = canonical discriminant analysis, KNN = k-nearest neighbors, SVM = support vector machine

Table 4.4. Contingency table and overall accuracy of eNose 3-class training set and machine learning algorithm combinations in predicting true 60-day treatment outcomes. Animals used to create the training set are excluded (n = 15).

		True Outcome				
eNose Algorithm	**eNose Predicted Outcome**	**FTS**	**RET**	**DNF**	**Total**	**Overall Accuracy**
CDA	FTS	47	10	4	61	28.81%
	RET	79	15	6	100	
	DNF	60	14	8	82	
KNN	FTS	48	13	6	67	27.57%
	RET	72	13	6	91	
	DNF	66	13	6	85	
SVM	FTS	63	14	5	82	33.74%
	RET	70	13	7	90	
	DNF	53	12	6	71	
	Total	186	39	18	243	

FTS = first treatment success, RET = retreated, DNF = did not finish,
CDA = canonical discriminant analysis, KNN = k-nearest neighbors, SVM = support vector machine.

Table 4.5. Contingency table and overall accuracy of eNose 2-class training set and machine learning algorithm combinations in predicting true 60-day treatment outcomes. Animals used to create the training set are excluded (n = 10).

		True Outcome			
eNose Algorithm	**eNose Predicted Outcome**	**FTS**	**FTF**	**Total**	**Overall Accuracy**
CDA	FTS	63	18	81	43.15%
	FTF	123	44	167	
KNN	FTS	74	29	103	43.15%
	FTF	112	33	145	
SVM	FTS	54	20	74	38.71%
	FTF	132	42	174	
	Total	186	62	248	

FTS = first treatment success, FTF = first treatment failure.
CDA = canonical discriminant analysis, KNN = k-nearest neighbors, SVM = support vector machine

Chapter 5 - Determination of Cardiac Troponin I Concentration Thresholds for Prognosticating Clinical Outcomes in Feedyard Cattle Treated Multiple Times for Bovine Respiratory Disease

Abstract

Bovine respiratory disease (BRD) remains the most significant health challenge affecting feedyard cattle in the United States. Despite its impact, few point-of-care (POC) testing methods are available to accurately predict clinical outcomes in cattle chronically affected by the disease. This study evaluated the prognostic potential of a commercially available POC blood analyzer (i-STAT®1) used to determine cardiac troponin I (cTnI) concentrations in cattle evaluated multiple times for BRD. Cattle (n = 84) previously treated for BRD were enrolled in the study at the time of 2nd or greater chute-side evaluation for BRD. Whole blood samples were collected for determination of cTnI concentrations, along with individual demographic information. Sixty-days post-enrollment, clinical outcomes were obtained to determine if an animal recovered or was culled or died related to BRD. Two cTnI thresholds (≥0.02 and ≥0.03 ng/mL) were independently associated with an increased probability of BRD related culling or death in logistical regression models controlling for demographic risk factors. These results suggest that cTnI concentrations obtained on the i-STAT®1 have the potential to help identity cattle at a higher risk of not finishing a 60-day follow-up period after multiple evaluations for BRD. However, careful consideration regarding the test's limitations and strategic implementation is essential for effective integration into feedyard BRD management practices.

Introduction

Bovine respiratory disease (BRD) poses a considerable health and economic burden to the U.S. cattle industry.[1] Although numerous tools are available for diagnosing the disease, few effective methods are in place for predicting clinical outcomes related to BRD.[2] The ability to determine animals that are likely to fail consecutive treatments for BRD and consequently be at a higher risk of culling or death, would be of immense value to feedyard operations. Such prognostic methods could enhance targeted animal health management through better treatment decisions, improve animal welfare, and decrease economic losses.

Practical implementation of prognostic testing for BRD-related outcomes depends on the availability of tools that are rapid, cost-effective, easy to use, and capable of delivering clinically relevant predictive value. Technologies in development or being evaluated with the potential of fulfilling these requirements include biomarker tests[3-5], acoustic-based diagnostic systems[6-9], wearable sensor systems that track behavior and physiology[10,11], and machine learning algorithms coupled with chute-side data capture.[12,13] These tools have the potential to offer real-time or near-real-time information regarding an animal's risk of treatment failure or death, thus facilitating earlier and better-informed management decisions.

One such biomarker approach was evaluated by Mancke, et al.[4] to assess serum cardiac troponin I (cTnI) concentration as a predictor of adverse outcomes in feedyard cattle at first BRD treatment. The assay had a 75% positive predictive value (PPV) for correctly identifying cattle at risk for not finishing the follow-up period because of respiratory disease. While results from the study were promising for guiding clinical decision making, the test required submission to an external laboratory and the lower limit of quantification for the assay resulted in detectable cTnI concentrations in only 2.5% of the animals sampled. These drawbacks identify the need to assess a point-of-care (POC) cTnI assay with a lower limit of detection and in a population with a greater prevalence of adverse outcomes for its potential as a prognostic tool for cattle suffering from BRD.

A POC analyzer (i-STAT®1) that can deliver near-real-time results chute-side is available commercially and has been used in cattle for evaluating cTnI in diseases such as BRD.[14,15] The i-STAT®1 was initially intended for the measurement of human cTnI levels, but because bovine cTnI is 96.4% homologous to the human metabolite, this analyzer can be sufficient for measuring cTnI levels from cattle.[16,17] Implementing this tool to target a population of animals with an increased probability of poor prognosis (i.e., animals that have previously failed treatment(s) for respiratory disease) might increase the applicability and usefulness of cTnI-based testing in commercial feedyard operations.

Therefore, the objective of this study was to determine if i-STAT®1 cTnI concentrations in feedyard cattle treated multiple times for BRD are predictive of mortality or culling within a 60-day follow-up period. A secondary objective was to determine the clinical usefulness of the i-STAT®1 cTnI device for prognostication of BRD related outcomes in a feedyard setting.

Materials and Methods

This prospective observational study was conducted on crossbred beef cattle housed in dry lot pens at a Kansas feedyard during the months of November and December 2024. The study protocol was approved by the Institutional Animal Care and Use Committee at Kansas State University (protocol #5060 approved 16 September 2024). Cattle were monitored daily by experienced feedyard personnel for signs of clinical respiratory illness. Cattle displaying one or more signs, such as depression, lethargy, nasal discharge, coughing or labored breathing, were removed from their pen for chute-side BRD evaluation by feedyard personnel trained in disease detection and treatment, following protocols established by a consulting veterinarian. Enrollment of study animals occurred on a rolling basis following chute-side evaluation for respiratory disease. Enrollment criteria consisted of animals who had received at least one prior treatment for respiratory disease, were currently being evaluated for respiratory disease, were not currently being treated for non-respiratory related diseases and were not currently enrolled in the study.

At enrollment, demographic information was collected from the feedyard's computer management software. Demographic information consisted of lot number, individual identification number, sex, number of respiratory disease treatments and evaluations, weight at evaluation, and days-on-feed at evaluation. Additionally, a single, whole blood sample was collected at enrollment from the coccygeal vein via a vacutainer with 18 G × 1.5" hypodermic needle into a 6 ml lithium heparin blood collection tube. After collecting, blood samples were stored on ice in a portable cooler maintained at 4°C.

After enrollment concluded on each collection day, the whole blood sample(s) were transported to an off-site laboratory where they were held in refrigeration (4°C) until analysis (same day) for cTnI concentration on the i-STAT®1 blood analyzer. The samples were analyzed between one to eight hours following collection. Before a blood sample was analyzed on the i-STAT®1, the single use cTnI i-STAT®1 cartridge and the whole blood tube were removed from refrigeration (4°C) and held at room temperature (20°C) for at least five minutes, according to manufacturer's instructions. The blood collection tube was then gently inverted 10 times before the tube stopper was removed. A 5 ml transfer pipette was used to remove 1 ml of whole blood and dispense into the cTnI cartridge sample well. The sample well was filled to an indicated mark (1 to 2 drops of blood) denoted on the cartridge. The sample well was then closed, and the cartridge was inserted into the i-STAT®1 analyzer port. cTnI concentrations were analyzed on the i-STAT®1 according to manufacturer's instructions for use. Blood cTnI results, reported in ng/mL of cTnI, were displayed on the main screen of the i-STAT®1 and recorded. Instrument run time was approximately 10 minutes after insertion of the cartridge.

Post-enrollment health outcomes were obtained from the feedyard's computer management software after all enrolled cattle completed the follow-up period. Individual animal records were reviewed to determine whether the animal (1) survived and remained at the feedyard, (2) was culled due to respiratory or non-respiratory related disease, (3) died at the feedyard due to respiratory or non-respiratory related disease, or (4) was shipped to harvest within the 60-day post-enrollment period. Cattle that were culled or died due to non-respiratory related disease or were shipped to harvest within the 60-day post-enrollment period were removed from the data set. Cattle that finished the 60-day post-enrollment period without being culled or died due to respiratory related disease were labeled "recovered", while cattle who died or were culled due to respiratory related disease during the 60-day post-enrollment period were labeled "did not finish" (DNF).

Statistical Analysis

All statistical analyses were conducted using R Studio® (R Studio®, version 2024.12.1.563; R Core Team). Descriptive statistics and data visualization techniques were used to examine differences in cTnI concentrations between cattle that recovered or DNF. To evaluate the prognostic performance of cTnI concentrations, a receiver operating characteristic (ROC) curve was generated using the "roc" function from the 'pROC' package in R, and the Youden Index (J statistic) was calculated to determine the optimal cTnI threshold(s) for 60-day post-enrollment outcomes. Diagnostic performance metrics, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), were calculated based on the selected threshold(s) against 60-day outcomes using the "epi.tests" function from the 'epiR' package in R Studio®. The threshold(s) were then dichotomized and incorporated as a fixed binary predictor in a multivariable logistic regression model to assess its association with the DNF outcome, while adjusting for demographic covariates, collected at enrollment, as additional fixed variables in the model. The model was fitted using the "glmer" function from the 'lme4' package in R studio® to account for the potential random effect of cattle lot. Model fit was evaluated using Akaike Information Criterion (AIC). Beta coefficients from the model were exponentiated to obtain odds ratios (OR), and corresponding predicted probabilities of DNF were calculated. An alpha level of 0.05 was used to determine statistical significance for all analyses.

Results

A total of 99 whole blood samples were collected from cattle over the course of the study. Fifteen animals were excluded from the final analysis for the following reasons: one due to a faulty i-STAT cTnI cartridge (n = 1), three were culled or died due to causes unrelated to respiratory disease (n = 3), and eleven were shipped to harvest prior to completing the 60-day post-enrollment period (n = 11). In total, 84 animals were included in the final analysis. Of these, 51 animals recovered from respiratory disease and completed the 60-day post-enrollment period, referred to as the recovered group, while 33 animals failed to complete the 60-day period due to being culled (n = 27) or died (n = 6) from respiratory-related causes, referred to as the DNF group. All animals included in the final data set received at least one BRD treatment prior to enrollment and at least a 2nd BRD treatment at enrollment. Figure 5.1 displays individual animal cardiac troponin I (cTnI) concentrations between the 60-day post-enrollment outcomes. Both the recovered and DNF groups exhibited a median cTnI concentration of 0.01 ng/mL. However, the DNF group showed a wider interquartile range and greater skewness towards higher cTnI concentrations compared to the recovered group. No clear threshold in cTnI concentration separating the 60-day outcome groups could be visually identified.

To further evaluate the prognostic performance of the i-STAT®1 analyzer across the observed cTnI concentration range in this study (0.00–0.09 ng/mL), a ROC curve was created (Figure 5.2), with a calculated area under the ROC curve (AUROC) of 0.535. The Youden index (J statistic), used to identify an optimal threshold, was the highest for cTnI concentrations of 0.02 ng/mL ($J = 0.12$) and 0.03 ng/mL ($J = 0.10$). Given the close proximity and J statistic values of these thresholds, both values were further assessed using diagnostic performance metrics and model-based evaluation via logistic regression.

Table 5.1 compares diagnostic performance metrics for cTnI thresholds of 0.02 ng/mL and 0.03 ng/mL. Metrics were calculated using a standard 2×2 contingency table format, where DNF was designated as the true positive and recovered as the true negative. Test-positive results were defined as cTnI concentrations ≥ 0.02 ng/mL or ≥ 0.03 ng/mL, and test-negative as concentrations below those respective thresholds. The 0.02 ng/mL threshold demonstrated higher sensitivity (0.36), whereas the 0.03 ng/mL threshold showed greater specificity (0.86). Predictive values were calculated using the study population's actual DNF prevalence of 39.3%. The 0.03 ng/mL threshold yielded a slightly higher positive predictive value (PPV) of 0.53 compared to the 0.02 ng/mL threshold of 0.50, while negative predictive values (NPVs) for both thresholds were nearly identical at approximately 0.65.

Table 5.2 presents a summary of demographic variables for the study population, both overall and by the two evaluated cTnI thresholds (≥ 0.02 ng/mL and ≥ 0.03 ng/mL). These demographic covariates were included as fixed effects in the multivariable logistic regression models alongside the cTnI thresholds. Model comparison using AIC indicated the best fit was achieved when all covariates were retained in both threshold models. Table 5.3 displays the model β coefficients and corresponding OR for each fixed effect for both cTnI threshold models. Both cTnI thresholds were significantly associated with a higher odds of DNF (≥ 0.02: $p = 0.04$; ≥ 0.03: $p = 0.03$). When these odds ratios were transformed into predicted probabilities (Figure 5.3), cattle with cTnI concentrations ≥ 0.02 ng/mL had an estimated probability of DNF of 67.2% (standard error [SE] = 0.13), compared to 32.0% (SE = 0.09) for those below the threshold, after adjusting for other covariates in the model. Similarly, animals with cTnI concentrations ≥ 0.03 ng/mL had an estimated DNF probability of 76.8% (SE = 0.14), compared to 35.1% (SE = 0.08) for those below 0.03 ng/mL, when adjusting for the same model covariates. Additionally, both cTnI models showed significant associations for the two heaviest weight categories (352-408 kg and > 408 kg) having a lower odds ratio of DNF compared to the lightest weight category (< 295 kg), when holding all other covariates in the model constant.

Discussion

Using cTnI to help predict respiratory disease related outcomes in cattle is based on the physiological link between severe respiratory illness and cardiac stress or injury.[18] cTnI is a highly specific protein released into the bloodstream at elevated levels when the cardiomyocytes undergo damage.[19] BRD pathology is not limited to the lungs, it also causes systemic inflammation, hypoxemia, acid-base imbalances, and endotoxemia in moderate to severe cases.[20] These systemic effects can comprise myocardial perfusion or directly injure cardiomyocytes leading to elevated cTnI levels.[21,22] Additionally, animals incorrectly diagnosed with BRD could have underlying heart disease (i.e., bovine congestive heart failure), due to some similarities in clinical signs, leading to elevated cTnI concentrations. Elevated cTnI concentrations are not well defined in the veterinary literature, and studies evaluating both healthy and BRD-affected dairy and beef cattle have shown mixed results on whether a significant difference exists. Reported median cTnI concentration for healthy cattle range from 0 to 0.04 ng/mL, while median concentrations from cattle with BRD range from 0.03 to 0.1 ng/mL.[14,15,23,24] This overlap indicates the difficulty in clearly defining a concentration threshold for determining elevated cTnI in BRD affected cattle. Even fewer data are available for what constitutes elevated cTnI concentrations indicative of an adverse clinical outcome for BRD.

Most studies evaluating cTnI concentrations in BRD-affected cattle have been done at the time of initial diagnosis. In contrast, the current study provides new insights into expected cTnI concentrations in feedyard cattle chronically affected by BRD. This may explain why the median cTnI concentration observed in this study (0.01 ng/mL) was lower than previously reported values for animals acutely affected with BRD. cTnI thresholds established in this study may not hold true for predicting clinical outcomes in cattle diagnosis with BRD for the first time. To date, only one other study, conducted in stocker cattle, has evaluated cTnI concentrations across multiple BRD treatments.[15] That study found no association between cTnI concentration and treatment number, consistent with the findings of the current study (data not shown). Also, stocker cattle that died from BRD tend to have higher concentrations than those that survived, not consistent with the results of the current study.

The i-STAT®1 blood analyzer used for determination of cTnI concentration has the potential, from a logistical standpoint, to be implemented into an existing on-farm diagnosis, treatment, and prognosis protocol. The device is equipped for in-field use as it is easy to administer and provides rapid results. However, the predictive abilities of the cTnI thresholds determined in this study are dependent on the context of their use. When evaluating the cTnI thresholds in this study as standalone tests, for any animal who enters the chute with unknown demographics and previously received a treatment for BRD, positive predictive ability (PPV in Table 5.1) is only mildly higher than the actual prevalence of DNF in this study (0.02ng/mL: 50%, 0.03ng/mL: 53% vs. DNF prevalence: 39.3%). This indicates the test provides minimal additional information related to the animal's probability of recovering or dying when no other demographic information is known. However, when accounting for demographic covariates that influence DNF in this population, the predictive ability of cTnI threshold is increased (0.02ng/mL: 67.2% and 0.03 ng/mL: 76.8%) improving the precision of the estimate when holding all other variables constant. Covariates included in the models were chosen based on availability of data provided by the feedyard along with known risk factors associated with treatment outcomes in feedyard cattle identified by Neal, et al.[25]

The current study builds upon the work by Mancke, et al.[4] who evaluated cTnI as a predictor of clinical outcomes in feedyard cattle. This study aimed to address the previous limitations related to assay lowest detection limit and targeting a population of cattle more likely to have negative outcomes related to BRD. Interestingly, in the present study no animals exhibited cTnI concentration above the lowest detection limit used in the previous study (0.1 ng/mL), making the previously established threshold untestable in this dataset. Further research is needed to determine the optimal sampling time for capturing peak cTnI concentrations in cattle with BRD and to assess whether those levels are associated with negative clinical outcomes.

When implementing prognostic tests, within a production system, it is important to distinguish whether the approach is oriented towards population-level diagnostic medicine versus precision (individual-level) diagnostic medicine. Viewing model generated predictive probabilities for a single predictor variable, such as cTnI threshold, offers a convenient way to assess population-level effects. However, they are not always directly interpretable for individual prognosis, especially when other demographic factors contributing to DNF are included in the model. For accurate individual-level risk assessment, the full set of β-coefficients found in Table 5.3 should be used to calculate an individual animal's predicted probability of DNF. In the population studied here, this method allows for a more accurate, animal-specific risk assessment and enables more informed management decisions at the individual level. If an operation aims to implement precision diagnostic medicine strategies, the demographic factors of individual animal not only improve the test performance, but they also provide context for refining the animal populations that would benefit most from testing. Furthermore, the applicability of the predictive equations derived from this study should be carefully evaluated before use in a different operation, as demographic factors influencing DNF may differ across populations, potentially limiting the direct transferability of these results.

Another important consideration for applying i-STAT®1 cTnI testing to predict BRD-related clinical outcomes in a commercial setting is the number of animals that must be tested to identify those exceeding the prognostic threshold. This becomes especially relevant when accounting for time and cost associated with administering each test. In this study, although 39.3% of the population were classified as DNF, only 28.6% and 17.9% had cTnI concentrations above the 0.02 and 0.03 ng/mL thresholds, respectively. Among those animals exceeding either threshold, approximately half were true DNFs. Depending on an operation's specific management goals, this rate of false positives (0.02ng/mL: 14% and 0.03ng/mL: 10%) or false negatives (0.02ng/mL: 25% and 0.03ng/mL: 30%) may be considered unacceptable, potentially resulting in inefficient use of time, labor, and financial resources. Additionally, the use of lower-cost, higher-sensitivity screening tools to identify animals at elevated risk of DNF prior to administering the i-STAT®1 analyzer may improve overall efficiency and reduce unnecessary testing.

Limitations of this research concern the potential application of cTnI testing as a chute-side prognostic tool. Because of logistical constraints experienced in this study, samples were not analyzed on the i-STAT®1 analyzer at the time of collection but anywhere from one to eight hours after collection. Although the findings remain pertinent to same-day testing, it is uncertain whether any biochemical processes occurred during that interval which could have altered cTnI concentrations. Additionally, the utilization of lithium heparin blood collection tubes provides a convenient sample collection methodology, in that no pre-processing is necessary before analysis using the i-STAT®1. Nevertheless, other studies have reported decreased recovery of measurable cTnI from heparinized plasma samples, which could influence assay sensitivity.[26,27] However, no specific data are available regarding cTnI loss with the i-STAT®1/heparinized whole blood combination compared to the i-STAT®/serum combination, therefore, this remains an important consideration for future work in this area. Future applications of this testing approach should take these factors into consideration when evaluating their feasibility and accuracy in real-time, chute-side settings.

Conclusions

The i-STAT®1 with associated cTnI thresholds identified in this study demonstrated its potential use as a standalone test or as a component of a battery of prognostic tools in determining clinical outcome among cattle treated multiple times for BRD. Values at or above the cTnI thresholds (0.02 and 0.03 ng/mL) evaluated throughout this study resulted in significantly higher odds of not finishing the 60-day post enrollment period and could help guide clinical decision making during a chute-side evaluation. These results also indicate that cTnI concentrations should be interpreted in light of other individual animal demographic factors that contribute to an animal's probability of DNF to promote better-informed treatment and management decisions.

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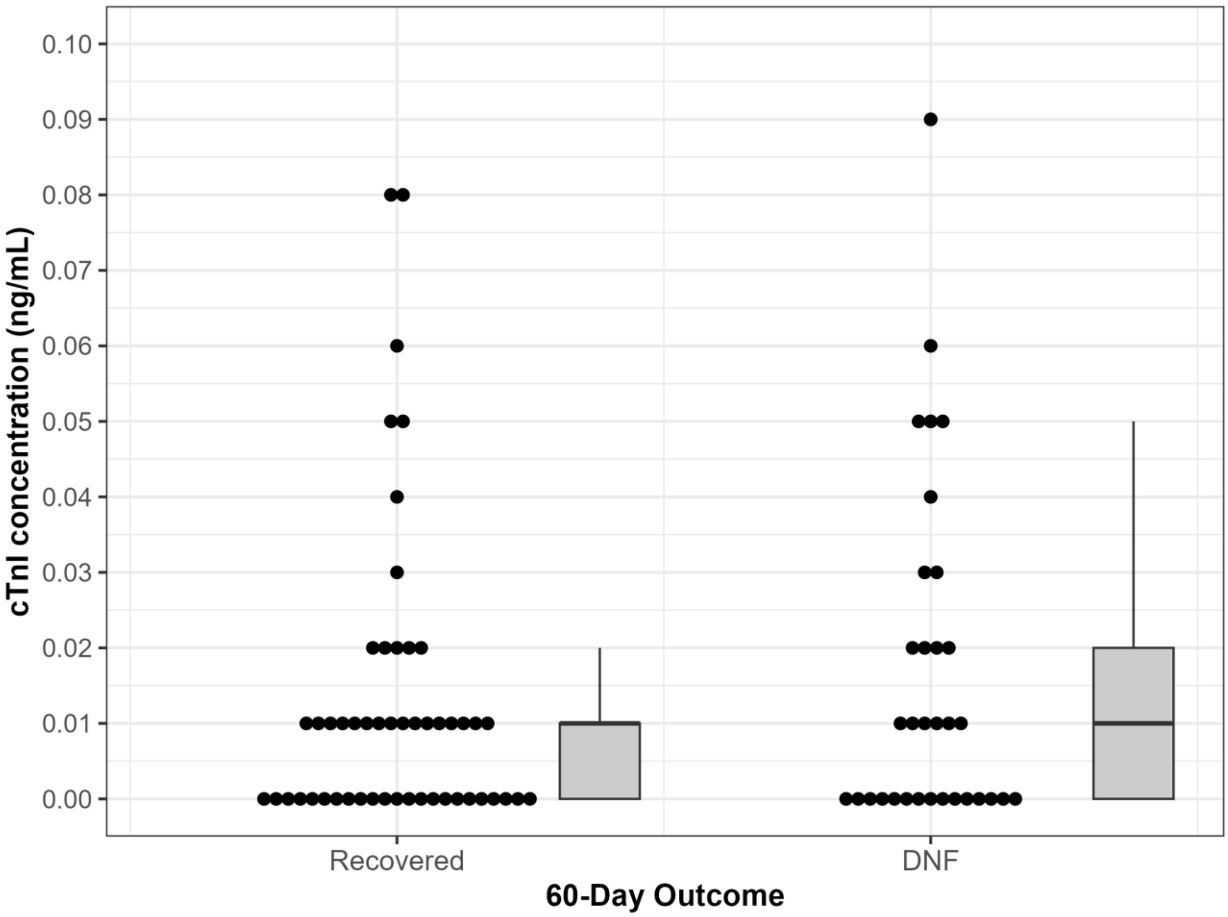

Figure 5.1. Distribution of cardiac troponin I (cTnI) concentrations by 60-day clinical outcome status. Black circles represent individual animal cTnI concentrations. Boxplots represent cTnI distributions for each 60-day outcome.

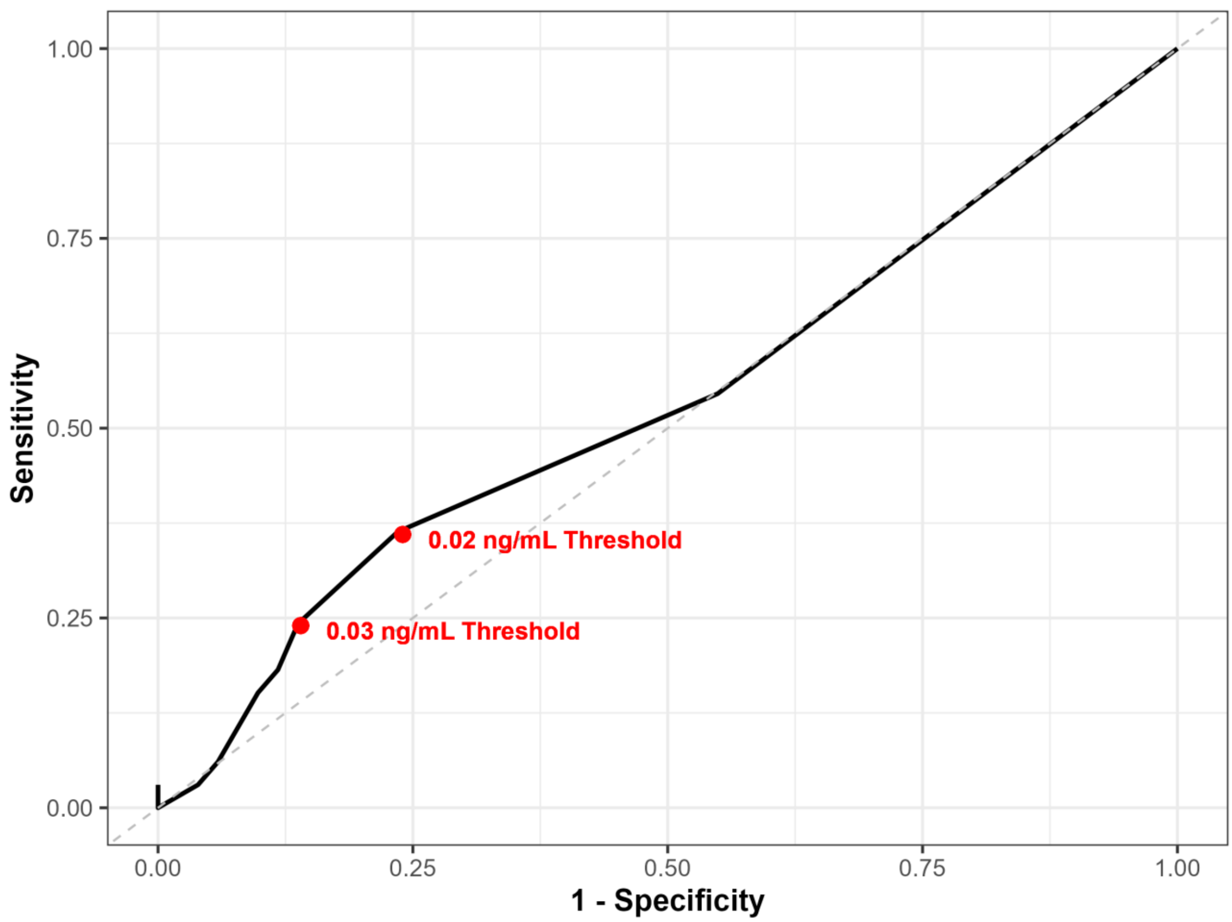

Figure 5.2. Receiver operating characteristic (ROC) curve evaluating cTnI thresholds for predicting 60-day did not finish (DNF) outcome. The 0.02 ng/ml and 0.03 ng/ml thresholds, identified as having the highest Youden index values, are marked in red for comparison.

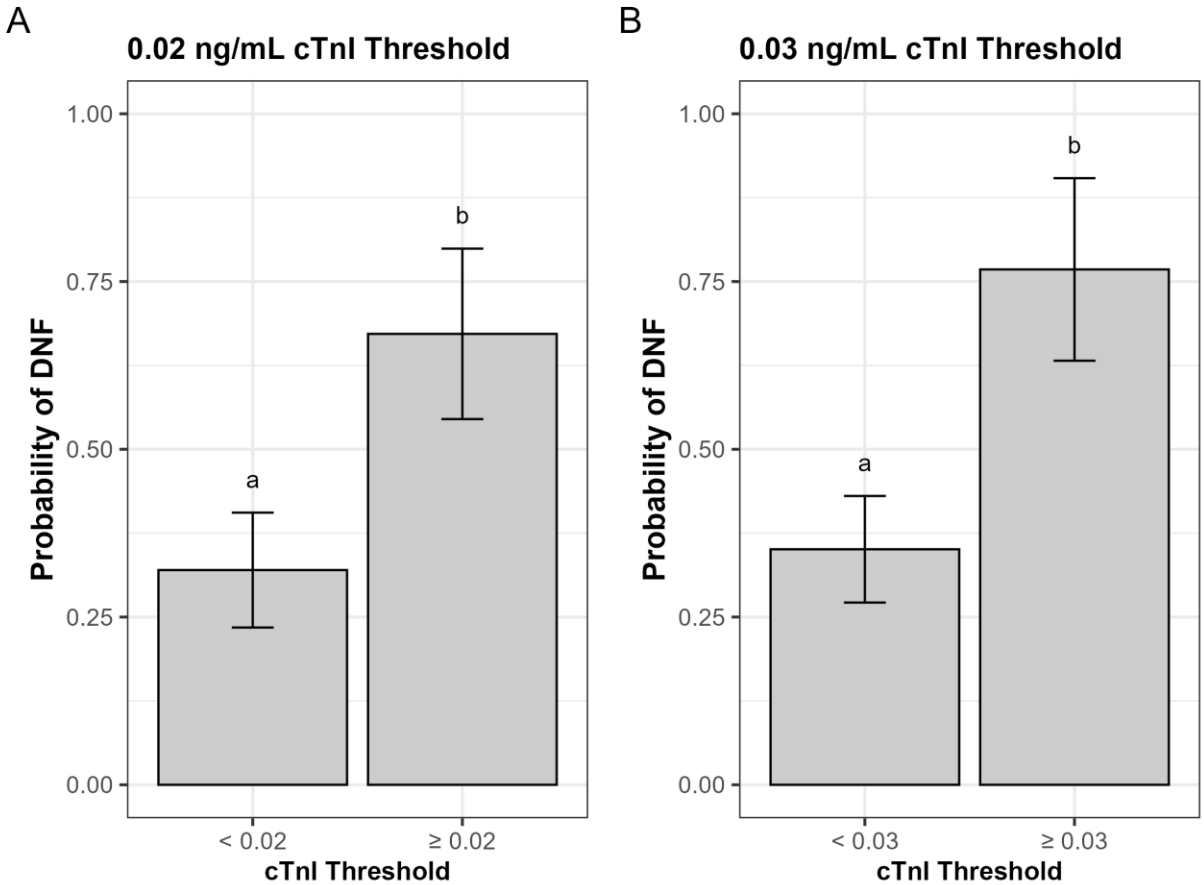

Figure 5.3. Multivariable logistic regression model predicted probabilities (± SE) of 60-day did not finish (DNF) status for animals with cardiac troponin I (cTnI) concentrations below and above two thresholds: (A) 0.02 ng/mL and (B) 0.03 ng/mL. Bars labeled with different letters (a, b) indicate statistically significant differences ($p < 0.05$) between groups.

Table 5.1. Comparison of diagnostic metrics for cardiac troponin I (cTnI) thresholds of 0.02 ng/mL and 0.03 ng/mL in predicting 60-days did not finish (DNF) outcome.

	0.02 ng/mL cTnI Threshold		**0.03 ng/mL cTnI Threshold**	
Metric	**Estimate**	**95% CI[a]**	**Estimate**	**95% CI[a]**
Sensitivity	0.36	(0.20 – 0.55)	0.24	(0.11 – 0.42)
Specificity	0.76	(0.63 – 0.87)	0.86	(0.74 – 0.94)
Positive Predictive Value	0.50	(0.29 – 0.71)	0.53	(0.27 – 0.79)
Negative Predictive Value	0.65	(0.52 – 0.77)	0.64	(0.51 – 0.75)

[a]95% CI calculated using exact confidence interval

Table 5.2. Demographic characteristics of cattle stratified by cTnI thresholds (0.02 ng/mL and 0.03 ng/mL) presented as counts with overall study percentages in parentheses.

		0.02 ng/ml cTnI Threshold		**0.03 ng/ml cTnI Threshold**	
Demographic	**Study Population (n = 84)**	**< 0.02 ng/ml (n = 60)**	**≥ 0.02 ng/ml (n = 24)**	**< 0.03 ng/ml (n = 69)**	**≥ 0.03 ng/ml (n = 15)**
60-Day Outcome					
Recovered	51 (60.7%)	39	12	44	8
DNF	33 (39.3%)	21	12	25	7
Sex[a]					
Heifer	54 (64.3%)	36	18	42	12
Steer	30 (35.7%)	24	6	27	3
Weight, lb[a]					
< 295	20 (23.8%)	16	4	18	2
295 – 351	22 (26.2%)	14	8	18	4
352 – 408	26 (31.0%)	20	6	22	4
> 408	16 (19.0%)	10	6	11	5
Days-on-feed[a]					
0 – 60	55 (65.5%)	38	17	44	11
> 60	29 (34.5%)	22	7	25	4
BRD Evaluations[a]					
2	49 (58.3%)	38	11	40	9
≥ 3	35 (41.7%)	22	13	29	6

[a]Indiviudal animal demographic information collected at enrollment

Table 5.3. Multivariable logistic regression model summary evaluating association between cTnI thresholds (0.02 ng/mL and 0.03 ng/mL) and 60-day did not finish (DNF) outcome.

	0.02 ng/ml cTnI Threshold				0.03 ng/ml cTnI Threshold			
Variable	β coefficients	Odds Ratio	95% CI	p-value	β coefficients	Odds Ratio	95% CI	p-value
Intercept	0.033	–	–	–	0.066	–	–	–
cTnI Threshold								
< Threshold	–	Reference	–	–	–	Reference	–	–
≥ Threshold	1.473	4.36	(1.08 – 17.56)	0.04	1.808	6.10	(1.18 – 31.61)	0.03
Sex								
Heifer	–	Reference			–	Reference	–	–
Steer	1.253	3.50	(0.84 – 14.5)	0.08	1.324	3.76	(0.89 – 15.88)	0.07
Weight (kg)								
< 295	–	Reference	–	–	–	Reference	–	–
295 – 351	-1.611	0.20	(0.04 – 1.10)	0.06	-1.548	0.21	(0.04 – 1.13)	0.07
352 – 408	-2.130	0.12	(0.02 – 0.89)	0.04	-2.212	0.11	(0.02 – 0.78)	0.03
> 408	-3.379	0.03	(0.00 – 0.41)	0.01	-3.518	0.03	(0.00 – 0.35)	0.01
Days-on-feed								
0 – 60	–	Reference	–	–	–	Reference	–	–
> 60	1.398	4.04	(0.84 – 19.5)	0.08	1.373	3.95	(0.90 – 17.41)	0.07
Pull Count								
2	–	Reference	–	–	–	Reference	–	–
≥ 3	-0.666	0.51	(0.14 – 1.82)	0.30	-0.417	0.66	(0.20 – 2.15)	0.49

Chapter 6 - Comparison of Electronic Nose and Conventional Cow-Side Diagnostic Tools for Detection of Ketosis in Early Lactation Dairy Cows

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Abstract

Rapid diagnosis of ketosis in dairy cows is imperative for treatment and managing economic losses. Cow-side ketosis diagnostic tools are greatly needed. The objective of this study was to compare three tools for the detection of ketosis, using serum β-hydroxybutyrate (BHB) as the gold standard. The diagnostic tools tested were: (1) Precision Xtra® handheld blood ketone meter, (2) ReliOnR urine ketone test strip, and (3) Cyranose 320® electronic nose (eNose) for use on milk and urine. Dairy cows (n = 60) were sampled immediately post-calving.

Whole blood, serum, milk, and urine were collected and analyzed on the same day. Each modality was compared to BHB to determine sensitivity and specificity. Positive predictive value (PPV) and negative predictive value (NPV) were calculated across a range of ketosis, consistent with reported prevalence (10-35%) in U.S. dairy operations. Urine ketone strips provided the highest specificity (99.2%), yet low sensitivity (58.6%). The Precision Xtra demonstrated adequate sensitivity (93.8%) and specificity (92.9%). The eNose had substandard sensitivity and specificity when used on milk (Sn: 58.8%; Sp: 44.3%) and urine (Sn: 18.8%; Sp: 80.6%) compared to the other modalities. Although the actual predictive values change with prevalence, urine ketone test strips had the highest PPV (89.5-97.6%) and Precision Xtra had the highest NPV (99.3-96.5%) across all simulated ketosis prevalences. Both urine ketone test strips and the Precision Xtra are adequate cow-side ketosis detection tools. Further optimization of the eNose is needed before deployment as a field diagnostic tool.

Introduction

The use of diagnostic tools for rapid detection of disease in food animal veterinary species is of increasing importance. Rapid disease diagnosis allows livestock producers and veterinarians to initiate therapy early and decrease illness duration. Ketosis is a metabolic disease commonly observed in early lactation, high producing dairy cows characterized by a negative energy balance.[1] Clinical and subclinical ketosis both result in increased concentrations of ketone bodies, acetoacetate (AcAc), acetone (Ac) and beta hydroxybutyrate (BHB), that are released into the blood, milk and urine of cows.[2,3] A definitive ketosis threshold has not been established, with various sources using values of 1.0 mmol/L[4] to 1.4 mmol/L[5] for classification of subclinical ketosis and values near 3.0 mmol/ L[6] for classification of clinical ketosis. Estimates of the in-herd prevalence of ketosis also vary with subclinical ketosis being reported as far more prevalent

(8.9% to 34%) compared to clinical ketosis (2% to 15%).[7] While ketosis does not directly cause mortality, its presence in a dairy herd is associated with decreased milk production and reproductive performance, as well as, increased culling rates, development of other diseases, and treatment costs.[3,8] When accounting for these factors, the estimated total cost of subclinical ketosis is nearly $300 per case.[9]

Cow-side diagnostic tools for the detection of ketosis are important for managing both individual animals and for herd level screening. The gold standard for ketosis determination in the dairy industry is a diagnostic laboratory serum or plasma BHB test. The gold standard laboratory BHB test is validated, widely accepted and provides the most accurate results for a dairy cow's ketosis state. However, serum or plasma BHB testing requires transport to a diagnostic laboratory, results are routinely returned in 24-48 hours and sample submission is relatively expensive compared to currently used cow-side tests.

Due to the testing delays associated with laboratory BHB testing, dairy producers primarily rely on single-use urine ketone test strips. Ketone test strips detect AcAc through the reaction of sodium nitroprusside with ketone bodies in the urine. The test strips, designed for human use, traditionally have served as one of the most useful diagnostic tools for detection of ketosis as they are cost effective, readily available, and provide results in less than 15 seconds.[10] Ketone test strips used in the dairy industry have proven to provide accurate diagnosis of ketosis when BHB concentrations, determined by diagnostic laboratory assay, are greater than 1.4 mmol/L.[11,12] Unfortunately, urine collection in dairy cows can be challenging and inconsistent which leads to the need for additional rapid detection tools that can be used on more reliably collected biological samples, such as blood or milk.

Use of the Precision Xtra® handheld ketone meter[a] is increasing on dairy operations due to its success in ketosis detection as demonstrated by prior research studies.[13-16] The Precision Xtra determines blood BHB concentrations, the same ketone detected by the diagnostic laboratory BHB test. This electronic ketone meter requires a single drop of blood and provides results in 10 seconds. The feasibility of the Precision Xtra was outlined in a systematic review and meta-analysis by Tatone et al.[17] as a point-of-care test for the detection of ketosis in dairy cattle evaluating its use as an index test against the blood/serum laboratory BHB test[11,13,18,19] and blood plasma laboratory BHB test.[16] The Precision Xtra accurately diagnosed both clinical and subclinical ketosis, with a higher sensitivity and specificity than milk and urine ketone test strips.[13] Similarly, a number of studies represented in the meta-analysis utilized the Precision Xtra as a reference test for evaluation of urine ketone test strips (Ketostix®[20]), milk ketone test strips (PortaBHB®[21] and Keto-Test®[22,23]), along with a flow-injection analysis for BHB and acetone in milk[24] and the Fossomatic™ milk analyzer.[23]

Electronic nose (eNose) technology is gaining attention for medical applications due its noninvasive, rapid disease diagnostic capabilities. Although the upfront instrument purchase carries a substantial investment, the ongoing/consumable costs are negligible per test. This technology operates on the premise that different biological compounds or biomarkers are generated in diseased versus non-diseased states.[25] Biomarkers can range from volatile chemical compounds produced by the diseased animal themselves to chemical compounds produced as byproducts of bacterial fermentation. eNose instruments consist of a bank of sensors (of varying number and composition depending on the specific instrument) that sample the air above a biological sample (sample headspace) and classify individual samples based on their unique composition or “smellprint™”.[25] Samples with similar smellprints can then be grouped qualitatively or semi-qualitatively and differentiated by computer algorithms.

eNose technology has been previously studied in the dairy industry for the detection of metritis and mastitis. Burfeind et al.[26] classified specific bacterial species from the vaginal discharge of dairy cows with acute puerperal metritis. Eriksson et al.[27] were able to differentiate cows with acute clinical mastitis from healthy cows using an eNose on milk samples. When applied to ketosis, the production of ketones in either milk or urine could potentially serve as a detectable biomarker for determining disease state. A study conducted by Kauppinen et al.[28] compared cows with induced ketosis to control cows to evaluate a marine gasoline fume detector as an eNose using expired air and milk samples. That study demonstrated that this technology is capable of correctly classifying samples of milk from ketotic and non-ketotic cows; however, it failed to accurately classify animals using expired air samples. More recently, human medical researchers have explored the use of eNose technology in diagnosing individuals with diabetic ketoacidosis, a disease marked by increased ketone concentrations similar to ketosis in dairy cattle. Mendoza Montoya et al.[29] demonstrated a metal oxide gas eNose could be used to determine ketone concentrations in synthetic- urine dimethyl ketone dissolution samples. Additionally, Esfahani et al.[30] demonstrated that a different metal oxide gas eNose could discriminate diabetic from control urine samples with a sensitivity and specificity over 90%. The application of eNose technology may potentially serve as a new, non-invasive, rapid method for the detection of ketosis in early lactation dairy cows.

The current study utilized the commercially available Cyranose 320® eNose[b], a portable handheld conducting-polymer sensor eNose. This instrument comes equipped with a 32-sensor array capable of detecting a wide range of volatile compounds to low parts per million (ppm) levels. The instrument operates by use of an internal pump that pulls in or "sniffs" the sample headspace, exposing it to the 32-sensor array. Within the sensor array, each sensor responds differently to the headspace analyzed creating the response pattern or smellprint. Each sensor is composed of a thin-film carbon black polymer composition chemoresistor with a conductive pathway through its length. The sensors function by absorbing the volatile compounds from the headspace creating resistance in the conductive pathway. The device then uses pattern matching algorithms to classify sample smellprints into distinct predetermined classes. Prior to instrument use on samples with an unknown disease status, the Cyranose 320 eNose must be trained on samples associated with a known disease status, such as biological samples from cows already diagnosed as positive or negative for ketosis, to create appropriate classifications for instrument usage. The Cyranose 320 has been used extensively in human medicine for detection of a variety of conditions including chronic obstructive pulmonary disease (COPD) from breath samples,[31] bladder tumors from urine,[32] as well as bacterial classification,[33] but limited research is available for its use in veterinary medicine, especially related to disease diagnosis in dairy cattle.

The objective of this study was to compare ReliOn® urine ketone test strips[c], the Precision Xtra handheld ketone meter, and the Cyranose 320 eNose for diagnosis of ketosis in dairy cattle using a laboratory BHB test as a gold standard. The secondary objective was to evaluate the clinical utility of each diagnostic tool across a simulated range of ketosis prevalence values typically seen in U.S. dairy operations.

Materials and Methods

This study was conducted at the Kansas State University Dairy Research and Teaching herd from May to August 2021. Samples of blood, milk and urine were collected for cows that freshened during the monitoring period. Samples were collected on Monday, Wednesday and

Friday, and each animal was sampled up to 3 times between day 0 and day 8 post-freshening. Samples were collected before morning feeding (blood, urine) and during morning milking (milk). One-hundred seventy-two (n = 172) individual samples of milk and blood and 160 individual samples of urine were collected on 60 primiparous and multiparous Holstein cows over the monitoring period. Urine samples were not obtained from every freshened cow at every sampling timepoint due to difficulty of the collection method to induce urination. Study procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC #4328).

All biological samples were transported to the laboratory for analysis at Kansas State University College of Veterinary Medicine. Blood serum samples were submitted and analyzed at the Kansas State Veterinary Diagnostic Lab (KSVDL) on the day of collection. Analysis of whole blood (Precision Xtra) and urine (ReliOn ketone test strips) samples were performed within 2 hours after arrival from the dairy, while milk and urine samples were analyzed on the eNose within 8 hours after arrival. All samples were held at room temperature between sample collection and analysis.

Urine collection and testing

Urine was collected by spontaneous urination or induced urination by manual stimulation of the perineum into 120 milliliter (mL) specimen containers. Urine ReliOn ketone test strips were used according to manufacturer's instructions, with semi-quantitative results determined by test strip color change relating to five levels of increasing concentration of AcAc, indicated by the color chart provided on the test bottle. Results were recorded as negative (0 mg/dL), trace (5 mg/dL), small (15 mg/dL), moderate (40 mg/dL), or large (≥ 80 mg/dL). For this study, urine ketone test strip results in the small (15 mg/dL), moderate (40 mg/dL), or large (≥ 80 mg/dL) categories were classified as positive, while samples in the negative (0 mg/dL) and trace (5 mg/dL) categories were classified as negative.

Blood collection and testing

Blood (13 mL) was obtained from the coccygeal vein using a 20 ga x 1" needle. Specimens were collected into preservative free and potassium EDTA vacutainer tubes[d]. Beta-hydroxybutyrate testing (KSVDL) was conducted using a colorimetric/spectrophotometric method kit[e] used according to the manufacturer's instructions. Serum BHB results were reported as the concentration of BHB in milligrams per deciliter (mg/dL). Cows with serum BHB concentrations ≥ 10 mg/dL on the diagnostic laboratory test were classified as ketosis positive for this study. [4,34]

Whole blood was used for the Precision Xtra handheld ketone meter analysis of BHB concentrations, following manufacturer's instructions, using the disposable blood β-ketone test strip. Results obtained from the Precision Xtra were reported in mmol/L and were converted to mg/dL by multiplying the measurement in mmol/L by 10.4.[35]

Milk collection

Composite milk samples from each cow were collected in milk sampler bottles directly from the in-line automated milking apparatus in the milking parlor. Milk was then transferred from the sampler bottles to 120 mL specimen containers prior to transport to the laboratory for analysis.

eNose procedure

Milk and urine were analyzed by the eNose for ketosis detection according to the manufacturer's general recommendations for use. Milk and urine samples (10 mL of each) were separately transferred to 15 mL centrifuge tubes for analysis. Each day prior to sample analysis, the instrument underwent a conditioning phase consisting of a 6-minute purge cycle followed by 3 pre-sniffs. The purge cycle pulls vapors through the instrument's purge inlet (Figure 6.1) from the surrounding air across the senor array and out through the exhaust port (Figure 6.1). This process serves to establish a baseline exposure to the surrounding environment prior to its use on samples. Pre-sniffs are performed on sample gas headspace, but without analyzing the smellprint, in order to sensitize the sensors to either milk or urine depending on which sample type is to be analyzed.

For sample analysis, a 16 ga x ¾" needle was penetrated through the cap of the centrifuge tube into the sample headspace area. The needle was attached to a 76 cm intravenous extension set which connected directly to the sample inlet (Figure 6.1) of the eNose. A vent hole was created in the cap of the centrifuge tube by insertion of a second needle prior to the sample run to prevent vacuum build-up. Instrument settings for sample analysis are detailed in Table 6.1.

eNose training and data processing

Prior to eNose use for identification of unknown ketosis status samples, the device was trained on samples of known ketosis status. The training set uses samples of known ketosis status ("ground truths" by diagnostic serum BHB assay). The training sets created in this study consisted of 2 predetermined classes (ketosis positive and ketosis negative) constructed separately for milk and urine matrices. Each training set consisted of 5 ketosis negative and 5 ketosis positive test results (Tables 6.2 and 6.3). As the training sets were established in real-time, ketosis status of an individual sample was first predicted by the Precision Xtra and then confirmed by the diagnostic laboratory BHB assay. Any samples for which the classification of ketosis positive/negative (*a priori* criteria referenced above) by the Precision Xtra and laboratory BHB assay were discrepant were removed from the training set. The milk training set (Table 6.2) included test exposures with BHB concentrations for "negative" ranging from 6.49-8.88 mg/dL and test exposures for "positive" ranging from 11.31-12.44 mg/dL. Similarly, the urine training set (Table 6.3) had exposures with BHB concentrations as "negative" between 5.40-8.88 mg/dL and "positive" ranging from 11.15-12.44 mg/dL.

Training sets were optimized by changing data processing parameters including algorithm, normalization, preprocessing and identification quality within the software provided. The appropriate combination of these data processing parameters was determined by uploading the training set to the Chemometric Data Analysis Program (CDAnalysis™) software[g] provided. Within the CDAnalysis software, cross validation was performed on each combination of data processing parameters for both the milk and urine training sets. Cross validation was used to determine the ability of each data parameter combination to correctly classify samples within the specified training classes. Cross validation values of ≥ 90% correct prediction were used for sample identification on the eNose. Algorithms and normalization were the only 2 data processing parameters which differed between training sets. Preprocessing and identification quality were held constant among each training set. Therefore, eNose training sets for milk and urine are referred to as eNose training set algorithm and normalization combinations.

Statistical analysis

Results from each cow-side diagnostic tool and the laboratory BHB test were assigned either a positive or negative classification as described above. The three cow-side diagnostic tools were then compared individually to the laboratory BHB assay (reference test) to determine individual test sensitivity and specificity. Confidence intervals (95%) for test sensitivity and specificity were calculated using the Clopper-Pearson method.[36] All 5 eNose training set algorithm and normalization combinations were evaluated for sensitivity and specificity, however only 1 milk (Canonical Discriminant Analysis [CDA] [Norm1]) and 1 urine (CDA [Norm1]) training set was selected for comparison with other cow-side diagnostic tools. The singular milk and urine eNose training set selection was determined by optimization of sensitivity and specificity calculated as the smallest difference between sensitivity and specificity for the training set algorithm and normalization combinations. Test sensitivity and specificity of each cow-side diagnostic tool was modeled over various prevalence estimates to determine positive predictive value (PPV) and negative predictive value (NPV) across the range of ketosis prevalence potentially encountered in clinical practice. PPV and NPV 95% confidence intervals were calculated using the standard logit confidence intervals.[37]

Results

Cross validation of the eNose training set algorithm and normalization combinations were performed, 3 separate milk training set algorithm and normalization combinations and 2 urine algorithm and normalization combinations produced ≥ 90% correct prediction (Table 6.4) and were evaluated for test sensitivity and specificity (Table 6.5). Discrepancy between the total number of samples collected (Milk: n = 172; Urine: n = 160) and the number of samples used to determine sensitivity and specificity for each eNose algorithm and normalization combination are outlined in Table 6.5. Due to real time development of milk and urine eNose training sets during the course of the study, samples were collected until 5 positive and 5 negative ketosis samples were obtained to finalize the training set. Once a training set was finalized, all additional samples collected during the study were used to evaluate the performance of the eNose. Differences between the total number of samples used for training set development are reflective of when the training sets were determined to have a cross validation of ≥ 90% correct prediction and then subsequently employed on the remainder of the study samples. Additionally, samples classified as “unknown” were eliminated from the analysis for the training sets.

When evaluating the eNose training set algorithm and normalization combinations in Table 6.5, the highest sensitivity was obtained with Milk – Support Vector Machine (SVM) (Norm1) representing 100% ($CI_{95\%}$: 81.5-100) and the lowest sensitivity with Urine – SVM (Norm1) at 5.6% ($CI_{95\%}$: 0.1-27.3). The eNose training set algorithm and normalization combination of Urine – SVM (Norm1) had the highest specificity at 97.4% ($CI_{95\%}$: 91.0-99.7) while Milk – SVM (Norm1) had the lowest specificity at 6.1% ($CI_{95\%}$: 2.0-13.7). Optimization of the eNose training set algorithm and normalization combinations were determined as the smallest difference between sensitivity and specificity of the test. Milk – CDA (Norm1) was the best optimized combination for the eNose milk training set with a sensitivity of 58.8% ($CI_{95\%}$: 32.9-81.6) and a specificity of 44.3% ($CI_{95\%}$: 33.1-55.9). The best training set combination for urine was CDA (Norm1) which had a sensitivity of 18.8% ($CI_{95\%}$: 4.1-45.7) and a specificity of 80.6% ($CI_{95\%}$: 69.1-89.2). Both the Milk – CDA (Norm1) and the Urine – CDA (Norm1) were selected for comparison with the Precision Xtra and the urine ketone test strips.

Test sensitivity and specificity comparison of the eNose and conventional cow-side diagnostic tools are shown in Table 6.6. The Precision Xtra provided the highest sensitivity (93.8%; $CI_{95\%}$: 79.2-99.2) among the tools with a specificity of 92.9% ($CI_{95\%}$: 87.3-96.5). The urine ketone test strips had the highest specificity (99.2%; $CI_{95\%}$: 95.8-100), although displayed a much lower sensitivity (58.6%; $CI_{95\%}$: 38.9-76.5). eNose – Urine displayed the lowest sensitivity (18.8%; $CI_{95\%}$: 4.1-45.7) across all evaluated tools while the eNose – Milk had the lowest specificity at 44.3% ($CI_{95\%}$: 33.1-55.9).

In the modeling component of the study (Table 6.7), ketosis prevalence estimates of 10%, 20% and 35% were chosen to demonstrate the impact on PPV and NPV for the 4 tests across a range of herd ketosis rates potentially encountered within dairy operations. Across all prevalences, the urine ketone test strips produced the highest PPV (Figure 6.2) and the Precision Xtra produced the highest NPV (Figure 6.3). When ketosis prevalence was low (10%), the urine ketone test strips produced the highest PPV (89.5%; $CI_{95\%}$: 54.2-98.4), and the Precision Xtra had the highest NPV (99.3%; $CI_{95\%}$: 97.2-99.8). When the hypothetical ketosis prevalence was increased to 20% and 35%, the same trend among devices was observed with the highest PPV recorded at 95% ($CI_{95\%}$: 72.7-99.3) and 97.6% (CI95%: 85.1-99.7), respectively, for the urine ketone test strips. NPV behaved in a similar manner with the Precision Xtra having the highest NPV at 20% prevalence (98.3%; $CI_{95\%}$: 93.9-99.6) and 35% prevalence (96.5%; $CI_{95\%}$: 87.8-99.1). The eNose – Urine provided the lowest PPV and NPV across all prevalences with the eNose – Milk producing similarly low results (Figures 6.2 & 6.3).

Discussion

While all diagnostic tools evaluated in this study provided rapid results, the ability to correctly determine true disease status varied greatly. When analyzing the intrinsic properties (sensitivity and specificity) of the diagnostic tools, the Precision Xtra and the urine ketone test strips performed similarly to other published research trials assessing their use in early lactation dairy cattle for detection of subclinical ketosis. The Precision Xtra in the current study utilized a lower ketone cutoff (10 mg/dL - reference test; 1.0 mmol/L - Precision Xtra) than described in Tatone et al. systematic review and meta-analysis (~12-14 mg/dL - reference test; 1.2-1.4 mmol/L – Precision Xtra).[17] The Precision Xtra in this study demonstrated a sensitivity of 93.8% and a specificity of 92.9%, comparable to other studies conducted on the early lactation dairy cows having a sensitivity ranging from 75% to 100% and a specificity ranging from 91% to 100%.[17] The urine ketone test strips in the current study demonstrated a sensitivity of 58.6% and a specificity of 99.2%, similar to other studies described in systematic review and meta-analysis by Tatone et al. utilizing urine test strips (Ketostix) with the same ketone cutoff (Small – 15 mg/dL/1.5 mmol/L) but a different reference test ketone cutoff (1.2-1.4 mmol/L/~12-14 mg/dL) that reported a sensitivity ranging from 59% to 78% and a specificity of 95% to 96%.[17]

The high sensitivity of the Precision Xtra was best able to minimize the total number of false negatives (n = 2) amongst the cow-side tests in the current study, whereas the urine ketone test strips best minimize false positives (n = 1) (Table 6.6). The Precision Xtra is the tool of choice for ruling-out ketosis as those cattle which test negative with the Precision Xtra have the highest probability of being true negatives when compared to other tools utilized in this study. The urine ketone test strips are best used for ruling-in ketosis as those cattle which test positive with the urine ketone test strips have the highest probability of being true positives compared to the other tools in this study.

The clinical utility of a diagnostic tool, however, is predicated on the PPV and NPV, which is dependent on test sensitivity and specificity, as well as disease prevalence within the population. Table 6.7 shows the PPV and NPV for the cow-side tools tested in this study for simulated subclinical ketosis prevalences of 10%, 20% and 35%. Disease prevalence for the study herd calculated from serum laboratory BHB results was 18.6%. Across all prevalence ranges modeled in Figure 6.2, as prevalence increases, PPV increases regardless of the diagnostic tool. Inversely, as prevalence increases NPV decreases for all diagnostic tools (Figure 6.3). The Precision Xtra had the highest NPV across all modeled prevalence ranges and was best able to determine true negative cows (highest true negative rate). When evaluating the eNose on both samples of milk and urine, the PPV and NPV are much less reliable compared to the other 2 diagnostic tools.

No device provided both the highest PPV and NPV, so correct application of the diagnostic tool should match the clinical objective, whether it be predicting true disease or non-disease status. Applying an individual treatment for a case of ketosis is effective, relatively inexpensive and has minimal adverse effects. With this in mind, the Precision Xtra is best able to determine cows which are truly negative or will not require treatment. Those which test negative on the Precision Xtra are either unlikely to benefit from treatment or should be evaluated for other disease syndromes.

Although the current study demonstrates the first published use of the commercially available Cyranose 320 eNose for detection of ketosis in dairy cattle, these results indicate that the instrument is not ready for field application at this time as testing on milk and urine had much greater false positive and false negative rates than either the Precision Xtra or the urine ketone test strips. Several areas of research could be explored to optimize the Cyranose 320 for detection of ketosis and/or evaluate its potential use as a cow-side diagnostic tool. One area of investigation would be to optimize the test methodology. Acetone incurred samples were tested prior to the current study (data not shown) to evaluate the ability of the instrument to detect ketones in headspace samples of milk with varying total sample volume and sample-to-headspace ratios before a final, standardized test method was determined. Test method evaluations were performed by running sample method combinations on the eNose and evaluating sensor output in the PCnose™[f] application through the scrolling strip chart feature. Individual sensor response was evaluated in the scrolling strip chart and the method combination which provided the highest combined sensor response across all 32 sensors was utilized for the study. Quantitative sensor response was calculated using the Equation 1.

Equation 1: $((R_t - R_{t=0}) / R_{t=0} * 10^6)$

R_t – Sensor response to sample

$R_{t=0}$ – Sensor response at baseline/purge

However, qualitative visualization of the sensor responses via the scrolling strip chart guided selection of the appropriate test methodology.

Instrument flow settings (Table 6.1) can be modified including the time and pump speed. Optimal flow settings are dependent on the application of use and sample type tested. The final time determination for sample pull in this study was selected based on when all sensors have reached a maximum sensor response (highest peak) via scrolling strip chart feature in PCnose for the sampling method utilized. High pump speed 180 cubic centimeters per minute was used for sample testing in this study.

Data processing parameters (algorithm, normalization, preprocessing and identification quality) were largely determined by importing the created training set into the CDAnalysis™ software and evaluating the calculated cross validations from the training set algorithm and normalization combinations. While a ≥ 90% cross validation was the determined cutoff for eNose training set algorithm and normalization combination selection, a greater cross validation did not necessarily mean better outcomes in ketosis classification. As observed in Table 6.5, Milk – SVM (None) (Cross Validation: 100%) had a sensitivity of 82.4% and a specificity of 13.9%. Milk – CDA (Norm1) (Cross Validation: 90%) better optimized sensitivity (58.8%) and specificity (44.3%) with fewer total false positives (n = 44) when compared to Milk – SVM (None) (n = 68). One parameter that accounted for variation in the total number of samples was the identification quality. Because identification quality was set to “high” some samples were not classified into a training set category (ketosis positive or ketosis negative) and were reported as “unknown”. Applying a stricter criterion may intuitively seem beneficial in accurately diagnosing disease status, however, further studies should evaluate the use of a forced classification option especially when reporting results which are binary in nature.

Although instrument/method optimization was not fully explored in the current study, it is possible that performance of the Cyranose 320 could be improved. One limitation of the current study is that we only used one sensor material (carbon-black polymer). There are other sensor materials available that could be tested to improve detection of ketosis. With regard to optimizing the electronic nose for detection of ketosis, another limitation of this study is that no post-collection sample manipulations were conducted. While heating or dehydrating samples after collection may improve the performance of electronic noses for detection of ketosis, these procedures limit the utility of the instrument as a cow-side diagnostic tool. Future studies using this instrument as a diagnostic tool should consider the complexity of the matrix samples, pre-testing sample preparation, sensor materials employed, alternative data analysis methods and the certainty of “ground truth” samples for training to potentially improve the functionality of this instrument. Although these factors may optimize the electronic nose for detection of ketosis, one of the inherent limitations of this instrument is that it is designed to detect different smellprints, but not necessarily differentiate the intensity of two similar smellprints. As low levels of ketones are present in the milk of healthy cows, it may be that the instrument is simply not able to discriminate the signal intensity as well as other diagnostic tools that are designed to quantify ketone concentrations. A final limitation to consider is the window of time between sample collection and sample analysis on the eNose. In the current study, samples were analyzed on the eNose within 8 hours after collection. It is unknown what the impact of sample holding time could do to the utility of the sample for analysis. While extending the time between sample collection and analysis is thought to degrade/deteriorate the sample, this might possibly work in favor for production of volatile compounds to accumulate in the sample headspace. To eliminate the possibility of inter-sample variability with the time after collection, all samples should be analyzed on the eNose within a reasonable confined window. Should these limitations be addressed in subsequent research, the instrument will require testing to evaluate timeframe of analysis, performance under field conditions and determine the effects of air quality and intensive use, as well as to determine the longevity and stability of classification algorithms and sensor materials.

Urine ketone test strips and the Precision Xtra are adequate cow-side ketosis detection tools. For timely herd-level interventions, a high test sensitivity will minimize false negative results; thus, the handheld ketone meter is the optimal tool for this use while still providing a reasonable specificity. Further optimization of the eNose is needed before deployment as a field diagnostic tool.

Endnotes

[a]Precision Xtra®, Abbott Laboratories, Abbott Park, IL

[b]Cyranose 320®, Sensigent, Baldwin Park, CA

[c]ReliOn®, Wal-Mart Stores, Inc., Bentonville, AR

[d]Vacutainer, BD Diagnostics, Franklin Lakes, NJ

[e]Beta-Hydroxybutyrate LiquiColor®, Stanbio Laboratory,Boerne, TX

[f]PCnose™, Sensigent, Baldwin Park, CA

[g]CDAnalysis™, Version 11.2, Sensigent, Baldwin Park, CA

Abbreviations

BHB	Beta hydroxybutyrate
CDA	Canonical discriminant analysis
eNose	Electronic nose
NPV	Negative predictive value
PPV	Positive predictive value
SVM	Support vector machine

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Figure 6.1. Cyranose 320®* eNose with labeled features.

*Sensigent, Baldwin Park, California

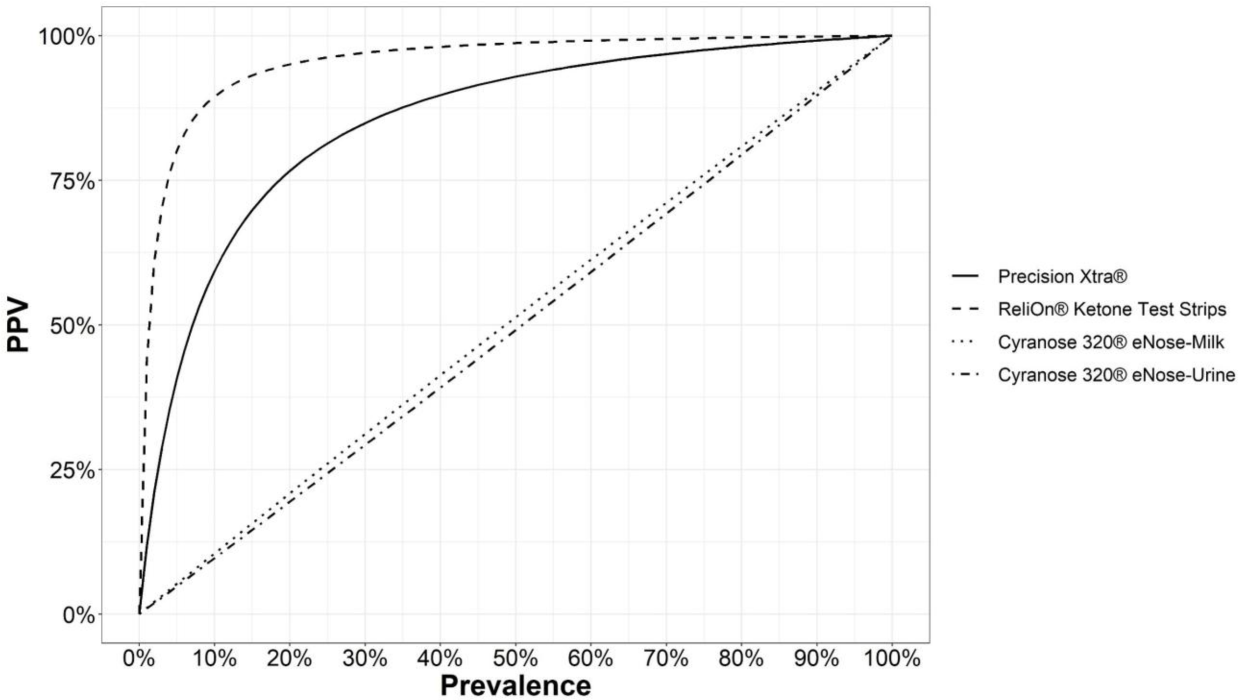

Figure 6.2. Diagnostic tool positive predictive value (PPV) distribution across all potential herd ketosis prevalence rates.

Precision Xtra® Abbott Laboratories, Abbott Park, IL
ReliOn® Ketone Test Strips, Wal-Mart Stores, Inc., Bentonville, AR
Cyranose 320® eNose, Sensigent, Baldwin Park, CA

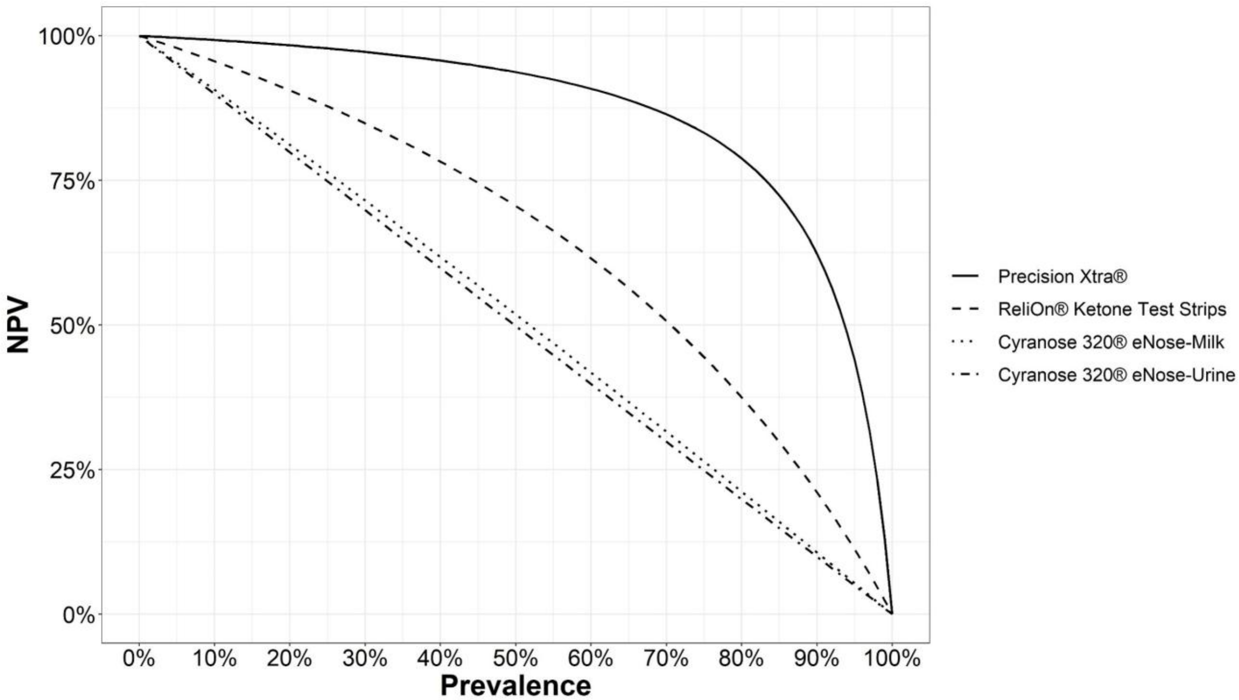

Figure 6.3. Diagnostic tool negative predictive value (NPV) distribution across all potential herd ketosis prevalence rates.

Precision Xtra® Abbott Laboratories, Abbott Park, IL
ReliOn® Ketone Test Strips, Wal-Mart Stores, Inc., Bentonville, AR
Cyranose 320® eNose, Sensigent, Baldwin Park, CA

Table 6.1. Cyranose 320®* eNose instrument settings for analysis of milk and urine headspace.

Flow Operations	**Time (sec)**	**Pump Speed (cubic-centimeters/minute)**
Baseline Purge:	10	Medium (120 cc/min)
Sample Draw 1:	30	High (180 cc/min)
1^{st} Air Intake Purge:	10	High (180 cc/min)
2^{nd} Sample Gas Purge:	90	High (180 cc/min)

*Sensigent, Baldwin Park, California

Table 6.2. eNose training set for milk and the respective serum β-hydroxybutyrate (BHB) result obtained by laboratory assay.

Negative Class		Positive Class	
Exposure #	**BHB Result (mg/dL)**	**Exposure #**	**BHB Result (mg/dL)**
1	7.90	1	12.44
2	8.88	2	12.44*
3	6.64	3	11.82*
4	8.32	4	11.31†
5	6.49	5	11.56†

**Exposures from same cow with samples collected on different days

† Exposures from same cow with samples collected on different days

Table 6.3. eNose training set for urine and the respective serum β-hydroxybutyrate (BHB) result obtained by laboratory assay.

Negative Class		Positive Class	
Exposure #	**Lab BHB Result (mg/dL)**	**Exposure #**	**Lab BHB Result (mg/dL)**
1	7.90	1	11.15*
2	8.88	2	11.15*
3	6.64	3	12.44
4	5.40	4	11.82†
5	6.49	5	11.82†

*Exposures from same cow with samples collected on different days

† Exposures from same cow with samples collected on different days

Table 6.4. eNose cross-validation results for various training set sample type/algorithm/normalization combinations performed on CDAnalysis™* software.

Sample Type – Algorithm (Normalization) Combinations	**Cross Validation (Percent Correct Prediction)**
Milk – CDA (Norm1)	90.0%
Milk – SVM (Norm1)	90.0%
Milk – SVM (None)	100.0%
Urine – CDA (Norm1)	90.0%
Urine – SVM (Norm1)	90.0%

CDA – Canonical Discriminant Analysis; SVM – Support Vector Machine
*Sensigent, Baldwin Park, California

Table 6.5. Test result comparison for 5 eNose training set algorithm and normalization combinations testing milk and urine headspace.

Sample Type – Algorithm (Normalization) Combinations	Number of samples for training set development	TP	FP	FN	TN	Number of samples in testing set	Samples classified as unknown	Sensitivity		Specificity	
								Estimate	95% CI	Estimate	95% CI
Milk – CDA (Norm1)	72	10	44	7	35	96	4	58.8%	32.9-81.6%	44.3%	33.1-55.9%
Milk – SVM (Norm1)	72	18	77	0	5	100	0	100%	81.5-100%	6.1%	2.0-13.7%
Milk – SVM (None)	76	14	68	3	11	96	0	82.4%	56.6-96.2%	13.9%	7.2-23.6%
Urine – CDA (Norm1)	68	3	13	13	54	83	9	18.8%	4.1-45.7%	80.6%	69.1-89.2%
Urine – SVM (Norm1)	64	1	2	17	76	96	0	5.6%	0.1-27.3%	97.4%	91.0-99.7%

TP – True positive; FP – False positive; FN – False negative; TN – True negative; CI – Confidence interval; CDA – Canonical Discriminant Analysis; SVM – Support Vector Machine

Table 6.6. Test sensitivity and specificity comparison for 3 cow-side diagnostic tests for detection ketosis in early lactation dairy cows.

Diagnostic Tools	Sample Type	TP	FP	FN	TN	Total	Sensitivity		Specificity	
							Estimate	95% CI	Estimate	95% CI
Precision Xtra®*	Blood	30	10	2	130	172	93.8%	79.2-99.2%	92.9%	87.3%-96.5%
ReliOn® Ketone Test Strips †	Urine	17	1	12	130	160	58.6%	38.9-76.5%	99.2%	95.8-100%
Cyranose 320® eNose‡	Milk	10	44	7	35	96	58.8%	32.9-81.6%	44.3%	33.1-55.9%
Cyranose 320® eNose‡	Urine	3	13	13	54	83	18.8%	4.1-45.7%	80.6%	69.1-89.2%

TP – True positive; FP – False positive; FN – False negative; TN – True negative; CI – Confidence interval
*Abbott Laboratories, Abbott Park, Illinois
†Wal-Mart Stores, Inc., Bentonville, Arkansas
‡Sensigent, Baldwin Park, California

Table 6.7. Diagnostic tool positive predictive value (PPV) and negative predictive value (NPV) distribution across selected herd ketosis prevalence estimates.

Diagnostic Tools	Sample Type	10% Prevalence		20% Prevalence		35% Prevalence	
		PPV (95% CI)	NPV (95% CI)	PPV (95% CI)	NPV (95% CI)	PPV (95% CI)	NPV (95% CI)
Precision Xtra®*	Blood	59.3% (44.4-72.7%)	99.3% (97.2-99.8%)	76.6% (64.2-85.7%)	98.3% (93.9-99.6%)	87.6% (79.4-92.8%)	96.5% (87.8-99.1%)
ReliOn® Ketone Test Strips †	Urine	89.5% (54.2-98.4%)	95.6% (93.3-97.1%)	95.0% (72.7-99.3%)	90.6% (86.1-93.7%)	97.6% (85.1-99.7%)	81.7% (74.3-87.3%)
Cyranose 320® eNose‡	Milk	10.5% (7.0-15.5%)	90.6% (83.9-94.7%)	20.9% (14.5-29.2%)	81.1% (69.8-88.9%)	36.3% (26.7-47.0%)	66.6% (51.8-78.8%)
Cyranose 320® eNose‡	Urine	9.7% (3.3-25.0%)	90.0% (87.3-92.1%)	19.5% (7.2-42.8%)	79.9% (75.3-83.8%)	34.2% (14.4-61.7%)	64.8% (58.6-70.6%)

PPV – Positive predictive value; NPV – Negative predictive value; CI – Confidence interval
*Abbott Laboratories, Abbott Park, Illinois
†Wal-Mart Stores, Inc., Bentonville, Arkansas
‡ Sensigent, Baldwin Park, California

Chapter 7 - Considerations and Future Direction for the Use of Volatile Organic Compound (VOC)-Based Diagnostics in Detecting Cattle Diseases

Many of the studies identified in the literature review (Chapter 1), along with those conducted as part of this dissertation (Chapters 2, 3, 4 and 6), reported encouraging results for VOC-based detection methods in identifying a variety of cattle diseases. Despite these results, the body of literature on these VOC-based detection methods remains relatively limited and is characterized by considerable variation in study population, designs, and methodologies. This heterogeneity makes it challenging to directly compare findings or to assess the broader applicability of reported results. Nevertheless, several themes emerge across the evaluation and undertaking of these studies that have the potential to provide valuable guidance for future research and for potential implementation of VOC-based diagnostics in cattle production settings.

In vitro and *in vivo* disease models were assessed throughout. While *in vitro* models provide highly controlled environments for determining if a VOC-based detection method can identify a single pathogen, they rarely simulate real-world samples from diseased cattle where multiple pathogens and potentially interfering VOCs can be present. This is where *in vivo* infection studies, both experimentally induced and naturally occurring, provide a much higher level of external validity. They allow the detection and differentiation of multiple pathogens, leading to a much more robust diagnostic test. When considering the most appropriate disease model for studying the diagnostic performance of these VOC-based tools, it’s important to identify the current research progress with each VOC-based detection method and the overall study goal. *In vitro* models, such as sampling bacterial cultures, are ideal for determining early on if a pathogen can be identified or differentiated from other potential pathogens or commensals. This also makes a favorable environment for testing multiple device methodologies. If designing studies based on established device methodologies, *in vivo* studies should be pursued. The use of experimental infection *in vivo* models helps reduce the need for expensive or elaborate gold standard diagnostic tests to determine the true disease state of the animal. Experimental infection models are usually done on a limited number of animals, which allows for multiple sample types and sampling procedures to be tested. However, if results are favorable in an experimental infection model, they should be followed up with natural infection models using the developed methodologies before application as a standard diagnostic method.

Before application of these VOC-based detection methods for disease detection they must first be trained to detect a specific disease. The selection of training samples is an important determination for diagnostic success. If applicable, applying current gold standard diagnostics for differentiating diseased and non-diseased animals is imperative for animals with naturally occurring disease. Animals with disease states that are inconclusive should be removed from consideration as a training sample. For diseases where a gold standard testing method is unavailable, multiple diagnostic tests should be applied and interpreted together to determine an animal's true disease status before inclusion as a training sample. However, both positive and negative disease status are highly important when considering samples for training set inclusion. Consideration should also be given to determining if negative animals have concurrent diseases unrelated to the disease under study and how that disease status could affect diagnostic reliability if included as a training sample.

Currently, little is known if canines or instrumentation with associated pattern matching algorithms trained on *in vitro* or experimental infection models can provide similar diagnostic performance when tested on natural infection models. Studies tend to retrain the VOC-based detection methods on the population under study instead of applying the originally trained detection method in a new study. The advantage of training VOC-based detection methods on *in vitro* or experimental infection models and then applying them to natural infection models stems from the reliability in knowing the true disease status in the animals or samples used for training the method. Again, it can be difficult to determine the true disease status in a natural infection model, and this approach could lessen that burden on identifying truly diseased or healthy animals included in training of the VOC-based detection method. Using these previously trained devices also enhances their applicability for practical use as they do not require retraining by the end user. Additionally, using *in vitro* or experimental infection models for training and then testing them on natural infection models could potentially lead to better diagnostic performance. This could be especially true when attempting to identify a single pathogen or just a few specific VOCs associated with a disease. Presenting these detection methods with a more controlled odor environment at training, with the goal of reducing background VOC noise, as compared to *in vivo* natural infection samples that could harbor complex microbial and VOC populations with heterogeneous and overlapping odor profiles could prove advantageous.

Sample size is among the largest limitations within this field of study. For adoption of a VOC-based detection method as a standard diagnostic for a particular disease it must demonstrate repeatable and reliable results. Setting a single sample size threshold for all studies testing VOC-based detection methods is not realistic given the diversity in study type and disease along with varying disease prevalences. *In vitro* and experimental infection models routinely have a limited number of animals or samples tested. When only limited numbers are available, thought should be given to the number needed to train the detection method. Designing studies where the majority of samples are used to train the method limits the samples available for instrument testing. This in turn reduces the diagnostic reliability of such results. Additional consideration should be given to the reliability of the results when samples used to train the method are also tested on the method. This approach may inflate the diagnostic performance. Alternatively, when limited animals are available, studies tend to sample the same animal multiple times. Collection of samples from an individual over time can allow for determination of the ideal post infection sampling period. However, when those repeated measures are compiled into a single statistic, without accounting for the lack of independence or uniqueness, reported diagnostic reliability for that study should be questioned. Sampling of the same animals may introduce bias toward consistent diagnoses across the study period. When using natural infection models, reliable results are best achieved by testing a sufficiently large sample size to capture population variability. Once that number is established, sampling each animal a single time can provide an accurate representation of disease dynamics within the population, thereby supporting the development of a robust diagnostic method.

Among these studies, little work has extended the existing methodologies and trained VOC-based detection methods to larger or more diverse cattle populations. When designing studies for the application of VOC-based detection methods the approach of either optimizing the detection method for a single population of cattle or for universal application should be evaluated. It is largely unknown if devices trained on one cattle population can be applied to others. It would be advantageous to determine if a single method already trained on one cohort could reliably and repeatably produce adequate diagnostic performance for a particular disease regardless of the cohort it is used on. Future studies should aim to apply already developed methodologies and trained instruments on new cattle populations. Finally, it should be evaluated whether optimization to a specific cattle operation will improve diagnostic performance or whether broadly trained methods can achieve generalizable accuracy.