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) ReliOn® 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.

Key words: ketosis, dairy cattle, diagnostic test, electronic nose

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 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, 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, 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. Beta-hydroxybutyrate testing (KSVDL) was conducted using a colorimetric/spectrophotometric method kit 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 1) from the surrounding air across the senor array and out through the exhaust port (Figure 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 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 1.

Figure 1: Cyranose 320®* eNose with labeled features used for sample collection.

*Sensigent, Baldwin Park, CA

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 2 and 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 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 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.

Table 1: Cyranose 320®* eNose instrument settings for analysis of milk and urine headspace.

Flow operations (cubic centimeters/minute)	Time (sec)	Pump speed
Baseline purge:	10	Medium (120 cc/min)
Sample draw 1:	30	High (180 cc/min)
1st Air intake purge:	10	High (180 cc/min)
2nd Sample gas purge:	90	High (180 cc/min)

*Sensigent, Baldwin Park, CA

Table 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 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

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 4) and were evaluated for test sensitivity and specificity (Table 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 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 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

Table 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, CA

Table 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

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. 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 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 2) and the Precision Xtra produced the highest NPV (Figure 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% ($CI_{95\%}$: 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 2 & 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). 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 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 2, as prevalence increases, PPV increases regardless of the diagnostic tool. Inversely, as prevalence increases NPV decreases for all diagnostic tools (Figure 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™ 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

Table 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, IL

†Wal-Mart Stores, Inc., Bentonville, AR

‡Sensigent, Baldwin Park, CA

Table 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, IL

†Wal-Mart Stores, Inc., Bentonville, AR

‡ Sensigent, Baldwin Park, CA

calculated using the Equation 1.

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

R_t – Sensor response to sample

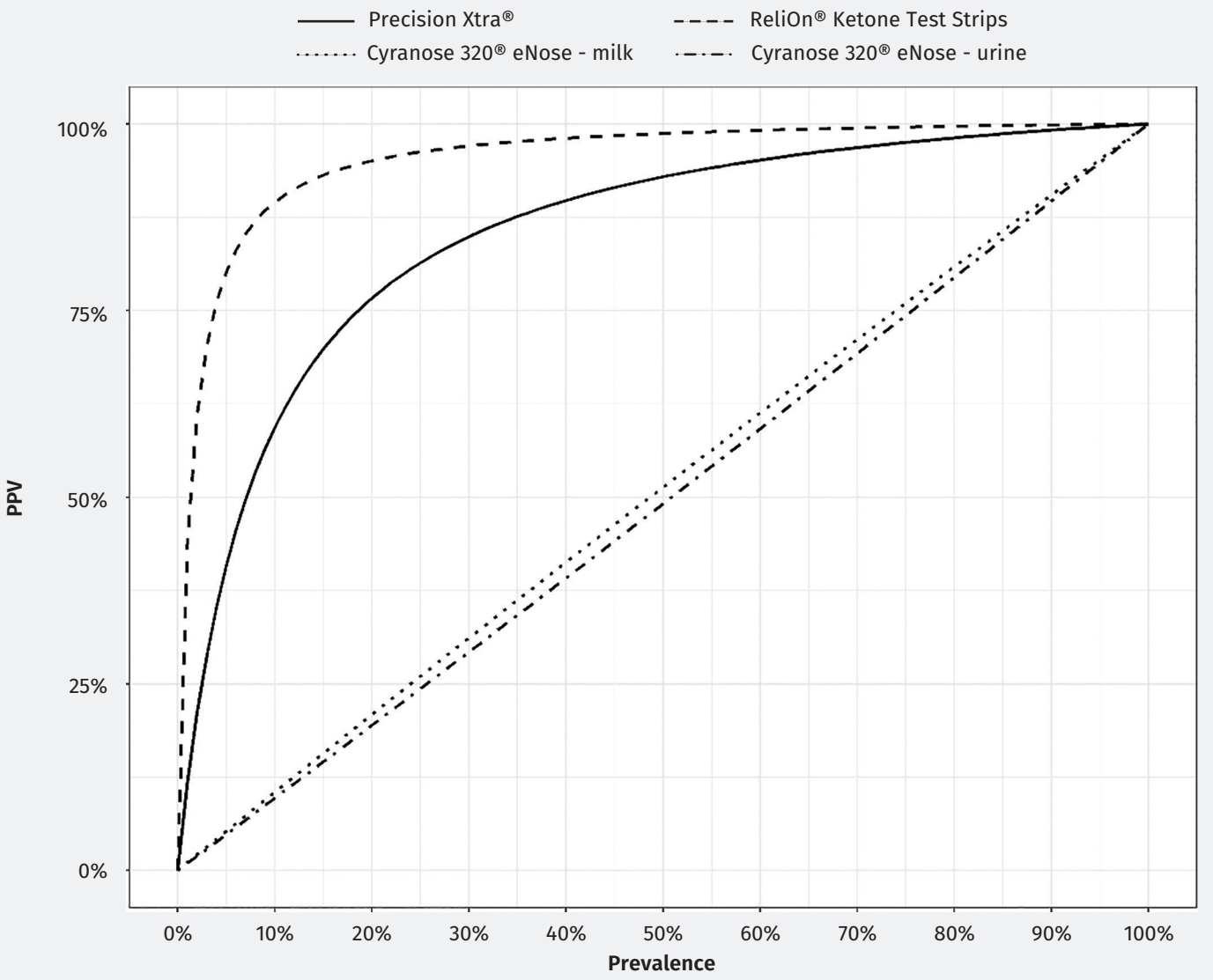
$R_{t=0}$ – Sensor response at baseline/purge

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

Instrument flow settings (Table 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 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

Figure 2: Diagnostic tool positive predictive value (PPV) distribution across all potential herd ketosis prevalence rates.

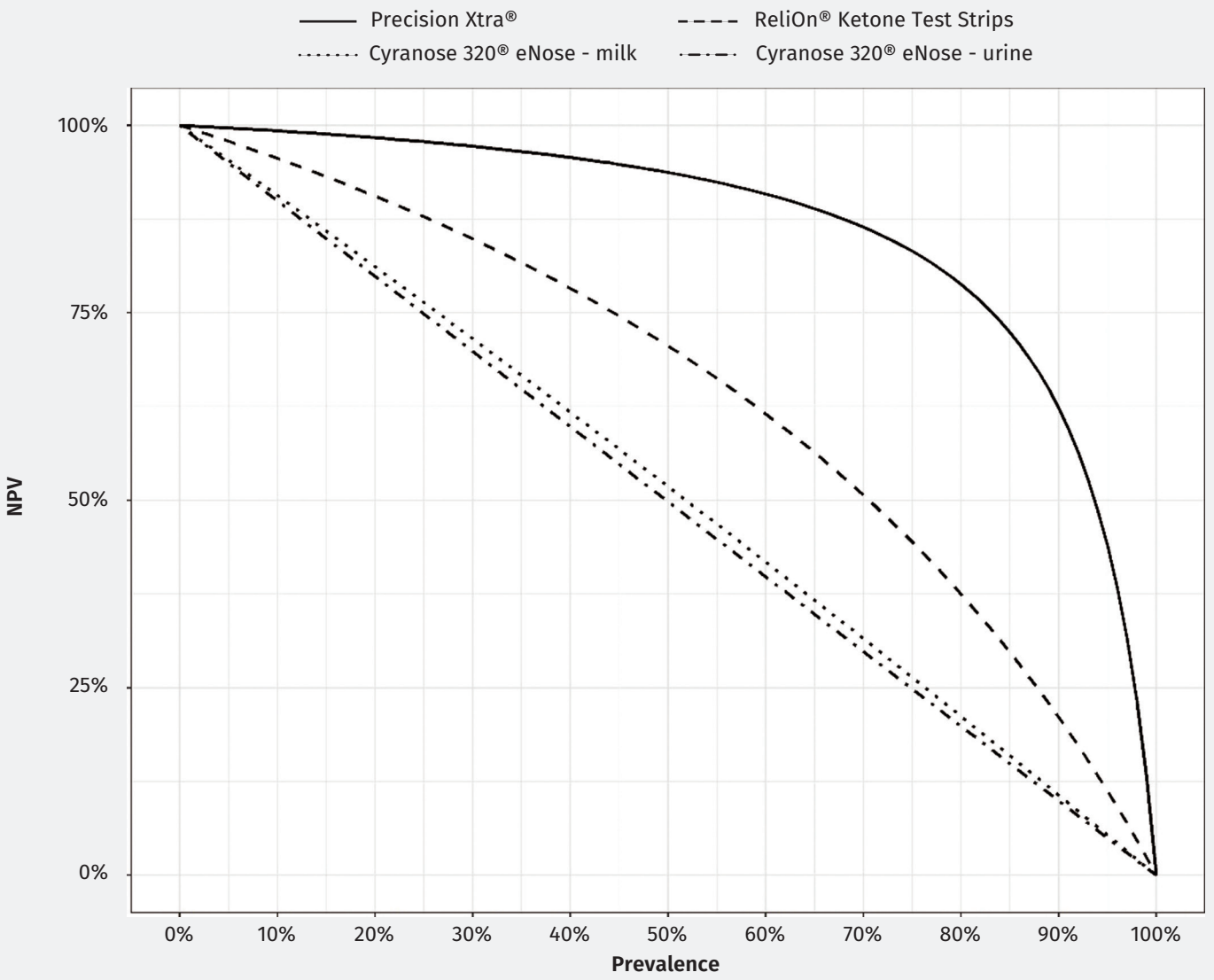


Precision Xtra® Abbott Laboratories, Abbott Park, IL
ReliOn® Ketone Test Strips, Wal-Mart Stores, Inc., Bentonville, AR
Cyranose 320® eNose, Sensigent, Baldwin Park, CA

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

Figure 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

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 time-frame 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

Acknowledgement
The authors would like to thank Jeff Stevenson and Mike Scheffel from Kansas State University Dairy Research and Teaching Unit and to Bailey Fritz for her assistance with sample collection.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Author contributions
CS designed the study, performed the sample collection, data analysis, and wrote the manuscript, MA reviewed and edited the manuscript, and BL conceived the idea, supervised the work and edited the manuscript.

Conflict of interest
Authors have no financial or non-financial conflicts of interest relative to the contents of the manuscript.

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

References
1. Enjalbert F, Nicot MC, Bayourthe C, et al. Ketone bodies in milk and blood of dairy cows: relationship between concentrations and utilization for detection of subclinical ketosis. *J Dairy Sci* 2001;84:583-589.

2. Herdt TH. Ruminant Adaptation to Negative Energy Balance: Influences on the Etiology of Ketosis and Fatty Liver. *Vet Clin North Am Food Anim Pract* 2000;16:215-230.

3. Geishauser T, Leslie K, Kelton D, et al. Evaluation of Five Cowside Tests for Use with Milk to Detect Subclinical Ketosis in Dairy Cows. *J Dairy Sci* 1998;81:438-443.

4. Ospina PA, Nydam DV, Stokol T, et al. Evaluation of nonesterified fatty acids and β-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. *J Dairy Sci* 2010;93:546-554.

5. Duffield TF, Lissemore KD, McBride BW, et al. Impact of hyperketonemia in early lactation dairy cows on health and production. *J Dairy Sci* 2009;92:571-580.

6. McArt JA, Nydam DV, Ospina PA, et al. A field trial on the effect of propylene glycol on milk yield and resolution of ketosis in fresh cows diagnosed with subclinical ketosis. *J Dairy Sci* 2011;94:6011-6020.

7. Duffield T. Subclinical ketosis in lactating dairy cattle. *Vet Clin North Am Food Anim Pract* 2000;16:231-253, v.

8. Steeneveld W, Amuta P, van Soest FJS, et al. Estimating the combined costs of clinical and subclinical ketosis in dairy cows. *PloS One* 2020;15:e0230448-e0230448.

9. McArt JAA, Nydam DV, Overton MW. Hyperketonemia in early lactation dairy cattle: A deterministic estimate of component and total cost per case. *J Dairy Sci* 2015;98:2043-2054.

10. Herman N, Bourgès-Abella N, Braun JP, et al. Urinalysis and determination of the urine protein-to-creatinine ratio reference interval in healthy cows. *J Vet Intern Med* 2019;33:999-1008.

11. Burke CM, Raphael W, Leslie KE, et al. Test Comparison of Precision Xtra® and Ketostix® for Ketosis in DairyCows. *Proc Am Assoc Bov Pract Conf 41st Annual Conference* Charlotte, NC, 2008;287.

12. Carrier J, Stewart S, Godden S, et al. Evaluation and use of three cowside tests for detection of subclinical ketosis in early postpartum cows. *J Dairy Sci* 2004;87:3725-3735.

13. Iwersen M, Falkenberg U, Voigtsberger R, et al. Evaluation of an electronic cowside test to detect subclinical ketosis in dairy cows. *J Dairy Sci* 2009;92:2618-2624.

14. Kupczyński R, Cupok A. Sensitivity and specificity of various tests determining β-hydroxybutyrate acid in diagnosis of ketosis in cows. *J Polish Ag Universities* 2007;10.

15. Panousis N, Kritsepi M, Karagiannis I, et al. Evaluation of Precision Xceed® for on-site monitoring of blood ß-hydroxybutyric acid and glucose in dairy cows. *J Hell Vet Med Soc* 2011;62.

16. Voyvoda H, Erdogan H. Use of a hand-held meter for detecting subclinical ketosis in dairy cows. *Res Vet Sci* 2010;89:344-351.

17. Tatone EH, Gordon JL, Hubbs J, et al. A systematic review and meta-analysis of the diagnostic accuracy of point-of-care tests for the detection of hyperketonemia in dairy cows. *Prev Vet Med* 2016;130:18-32.

18. Konkol K, Godden S, Rapnicki P, et al. Validation of a Rapid Cow-side Test for the Measurement of Blood Beta-Hydroxybutyrate in Fresh Cows. *Proc Am Assoc Bov Pract Conf 42nd Annual Conference* 2009;190.

19. Oetzel GR, McGuirk SM. Evaluation of a Hand-Held Meter for Cowside Evaluation of Blood BetaHydroxybutyrate and Glucose Concentrations in Dairy Cows. *Proc Am Assoc Bov Pract Conf 41st Annual Conference* 2008;234.

20. Galvão KN, Neto AV, Peña G, et al. Comparing the Urine Ketone Strip Test and the Handheld Ketone Meter to Diagnose Ketosis in Early Lactation Dairy Cows. UF College of Veterinary Medicine: University of Florida Extension, 2020.

21. Denis-Robichaud J, DesCoteaux L, Dubuc J. Accuracy of a new milk strip cow-side test for diagnosis of hyperketonemia. *Bov Pract* 2011;45:97-100.

22. Shire J, Gordon JL, Karcher EL. Short communication: The effect of temperature on performance of milk ketone test strips. *J Dairy Sci* 2013;96:1677-1680.

23. Wilson DJ, Goodell GM. Comparison of blood strips, milk strips and automated milk measurement of beta-hydroxybutyrate in periparturient dairy cattle and resultant diagnoses of ketosis. *J Vet Sci Technol* 2013;4.

24. Denis-Robichaud J, Dubuc J, Lefebvre D, et al. Accuracy of milk ketone bodies from flow-injection analysis for the diagnosis of hyperketonemia in dairy cows. *J Dairy Sci* 2014;97:3364-3370.

25. Magan N, Turner APF. Electronic noses and disease diagnostics. *Nat Rev Microbiol* 2004;2:161-166.

26. Burfeind O, Bruins M, Bos A, et al. Diagnosis of acute puerperal metritis by electronic nose device analysis of vaginal discharge in dairy cows. *Theriogenology* 2014;82:64-70.

27. Eriksson Å, Persson Waller K, Svennersten-Sjaunja K, et al. Detection of mastitic milk using a gas-sensor array system (electronic nose). *Int Dairy J* 2005;15:1193-1201.

28. Kauppinen R, Sairanen A, Hiltunen T, et al. Ion - mobility spectrometry based electronic nose - a promising tool for the evaluation of metabolic status of dairy cows. *Suomen Maataloustieteellisen Seuran Tiedote* 2006:1-5.

29. Mendoza Montoya JJ, Torregrosa Penalva G, Navarro EA, et al. Monitoring Diabetic Ketoacidosis by Urine Ketones Tracing Using an E-Nose. IEEE International Instrumentation and Measurement Technology Conference (I2MTC) 2020;1-5.

30. Esfahani S, Wicaksono A, Mozdiak E, et al. Non-invasive diagnosis of diabetes by volatile organic compounds in urine using FAIMs and FOX4000 electronic nose. *Biosensors (Basel)* 2018;8:121.

31. Rodríguez-Aguilar M, Díaz de León-Martínez L, Gorocica-Rosete P, et al. Identification of breath-prints for the COPD detection associated with smoking and household air pollution by electronic nose. *Resp Med* 2020;163:105901-105901.

32. Gut JM, Hegele A, Hofmann R, et al. Non-invasive detection of bladder tumors through volatile organic compounds: A pilot study with an electronic nose. *Anticancer Res* 2018;38:833-837.

33. Dutta R, Hines EL, Gardner JW, et al. Bacteria classification using Cyranose 320 electronic nose. *Biomedical Eng Online* 2002;1:4-4.

34. Cornell University College of Veterinary Medicine. Clinical Pathology Panels and Guidelines: ß-Hydroxybutyrate (BHB) Testing in Post-Partum Dairy Cows. 2021; **https://www.vet.cornell.edu/animal-health-diagnostic-center/testing/protocols/clinical-pathology**. Accessed 03 June, 2022.

35. American Medical Association. A Guide for Authors and Editors: Système International (SI) Conversion Table. 2021; 11:**https://www.amamanualofstyle.com/page/si-conversion-calculator**. Accessed 12 August, 2021.

36. Borkowf CB. Constructing binomial confidence intervals with near nominal coverage by adding a single imaginary failure or success. *Stat Med* 2006;25:3679-3695.

37. Mercaldo ND, Lau KF, Zhou XH. Confidence intervals for predictive values with an emphasis to case-control studies. *Stat Med* 2007;26:2170-2183

