Evaluation of breath biomarkers and serum haptoglobin concentration for diagnosis of bovine respiratory disease in heifers newly arrived at a feedlot

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Objective—To evaluate exhaled N2O (eN2O), exhaled CO (eCO), and serum haptoglobin concentrations as diagnostic criteria for bovine respiratory disease (BRD) and determine whether a combination of biomarkers would be useful for predicting health outcomes of heifer calves.

Animals—337 heifer calves newly arrived at a feedlot.

Procedures—Body weights, serum haptoglobin concentrations, and rumen temperatures were determined. Calves (n = 183) were randomly selected for breath sampling. Variables were compared among calves that remained healthy and those requiring treatment.

Results—Body weight at the time of first and second antimicrobial treatments did not differ from that at arrival, whereas body weight at the time of third antimicrobial treatment was lower. Temperature was lower at arrival, compared with that during antimicrobial treatment. Ratio of eN2O:eCO2 was lowest at arrival, intermediate at the first and second antimicrobial treatments, and greatest at the third antimicrobial treatment. Ratio of eCO:eCO2 was greater at times of antimicrobial treatment, compared with arrival. Concentration of serum haptoglobin was greatest at the time of the first antimicrobial treatment, lowest at the times of second and third treatments, and intermediate at arrival. Arrival ratios of eN2O:eCO2 and eCO:eCO2 and concentration of haptoglobin did not differ among heifers subsequently treated 1, 2, or 3 times.

Conclusions and Clinical Relevance—Although breath analysis was successfully implemented in a research feedlot, arrival rumen temperature, eN2O, eCO, and haptoglobin concentration were not accurate in predicting occurrence of BRD during a preconditioning program. However, these biomarkers might support the diagnosis of BRD. (Am J Vet Res 2009;270:1291–1298)

Bovine respiratory disease is the most economically important disease in the beef cattle industry, developing primarily in high-risk, recently weaned commingled cattle arriving at the feedlot. Despite the use of so-called new-generation antimicrobials, vaccines, and vaccination strategies in the past decade, the prevalence of BRD has been reported to increase. The economic losses associated with BRD include not only dead cattle and cost of antimicrobial treatments, but also decreased performance and carcass quality.

One of the greatest challenges for feedlot personnel is early and accurate diagnosis of BRD. In previous studies, it has been reported that 37% to 38.5% of steers that never receive a diagnosis of BRD during the finishing period have lung lesions at slaughter. In addition, only 55.4% of the cattle treated at least once for BRD have lung lesions. On the basis of those results,
it can be concluded that a high prevalence of lung lesions exists in cattle that are never identified with clinical BRD. These results suggest inaccuracy in diagnosis or differences in case definitions of BRD among evaluators and feedlots. In addition, the low prevalence of lung lesions in cattle that were identified as sick could indicate that cattle were identified as sick early in the disease and that appropriate antimicrobial treatment decreased the presence and severity of lung lesions. Because of these inconsistencies, there is a need for development of diagnostic tools that allow for accurate diagnosis of BRD.

Recently, several attempts have been made to improve the accuracy of BRD diagnosis. Serum concentrations of haptoglobin, an acute-phase protein produced by the liver in response to inflammation, increase in high-risk, stressed cattle. Haptoglobin has been used as a stress biomarker after transportation of bull calves and has been correlated with the number of antimicrobial treatments administered for BRD. When calves are intranasally challenged with a type 1b BVDV alone or in combination with Mannheimia haemolytica, they respond with an increase in haptoglobin concentrations in serum. In the experience of some of the authors (LOBR, BPH, DLS, and CRK), when healthy steers are exposed for 72 hours to steers PI with BVDV type 1b, steers seroconvert, but haptoglobin concentrations are not increased, compared with concentrations in control, nonexposed cattle. However, haptoglobin concentrations in these 2 groups are lower, compared with concentrations in cattle that are challenged with M haemolytica alone or in combination with 72 hours’ exposure to PI cattle. The discrepancy might indicate that not all pathogens involved in BRD increase serum haptoglobin concentrations, which might decrease the accuracy of haptoglobin for use alone in the diagnosis of this disease. In addition to the low specificity of haptoglobin as a BRD marker, analysis of this protein presently requires at least a 24-hour turnaround period, which might not be practical in a commercial feedlot setting.

In addition to the use of haptoglobin, attempts to use other technologies as aids in early and accurate diagnosis of BRD have been made. For example, infrared thermographic scanning of the orbital area in calves is an effective method for early and accurate diagnosis of undifferentiated fever in high-risk cattle arriving at the feedlot. Moreover, analysis of eNO in bovine breath as a biomarker by use of TDLAS to identify cattle with BRD has been reported. The TDLAS technology has been successfully implemented in the daily operations of a backgrounding facility to measure eNO and eCO, upon arrival and at BRD treatment time in sick cattle. Although numeric analyses suggested eNO may be useful in diagnosing and monitoring respiratory tract disease in cattle, present technology does not allow measurements at the precision required for a clinical test.

Bovine respiratory disease has multiple etiologies, which needs to be accounted for when considering the development of diagnostic tools. The objectives of the study reported here were to evaluate eN\textsubscript{2}O, eCO, and serum haptoglobin concentration as diagnostic tools for BRD, and to determine whether a combination of biomarkers and response variables would be useful for predicting health outcomes of feeder heifers upon arrival at a backgrounding facility.

Materials and Methods

On September 10 and September 12, 2007, a total of 360 crossbred beef heifer calves (mean ± SD weight, 238.9 ± 16.7 kg) were purchased by an order buyer and assembled at a commercial drylot in Marion, Ky. After 2 truckload-sized lots were assembled on each date, calves were individually identified and administered an electronic rumen temperature bolus, and a blood sample was collected via jugular venipuncture for obtaining serum. On September 11 (2 truckloads) and 13 (2 truckloads), calves were transported (964 km) to the Willard Sparks Beef Research Center at Oklahoma State University, Stillwater, Okla.

After arrival in Stillwater on day –1, calves were allowed to rest for approximately 5 hours without access to feed or water. Subsequently, calves were individually weighed, an ear-notch skin sample was collected for detection of cattle PI with BVDV, and a blood sample was collected via jugular venipuncture for obtaining serum. A subset of calves (n = 183) was randomly selected for breath sampling by use of TDLAS. Following initial procedures, calves were placed in holding pens and given ad libitum access to prairie hay and fresh water.

The following day (day 0), calves were vaccinated against viral respiratory pathogens by use of a 5-way modified-live viral respiratory vaccine, vaccinated against clostridial organisms, and dewormed. Calves received an implant with an estrogen-based growth promoter. Calves selected for the experiment (n = 337; mean ± SD weight, 241 ± 16.6 kg) were sorted into 12 pens (12 × 30.5 m with a 12.2-m concrete feed bunk and fence-line automatic water basin [6 pens/arrival date]). Pens contained a mean of 28 cattle (range, 18 to 34 cattle). Calves were fed a 65% concentrate growing ration formulated to meet or exceed nutrient requirements ad libitum twice daily (7 AM and 1 PM). On days 7, 14, 21, and 42, all calves were individually weighed, a blood sample was collected, and serum was harvested after centrifugation at 3,000 g for 20 minutes; serum was stored at −20°C for further analysis. In addition, the same subset of calves from which breath was sampled on day –1 was resampled on days 7, 14, 21, and 42.

Health assessment—Each morning, calves were evaluated by trained individuals according to facility protocol for clinical signs of BRD, including, but not limited to, signs of depression and lack of fill (apparent fullness of the rumen), compared with penmates; cough; nasal or ocular discharge; altered gait; and weakness. Calves with those signs were assigned a severity score of 1 through 4, where 1 was mild, 2 was moderate, 3 was severe, and 4 was moribund. All calves assigned a severity score were removed to the processing chute for rectal temperature evaluation and breath sampling. The fourth criterion for rectal temperature, an objective evaluation used to determine whether antimicrobial therapy was to be administered. Any calf subjectively identified as

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sick with a score of 1 or 2 and a rectal temperature ≥ 40°C received an antimicrobial agent, and breath was sampled. In situations where the evaluators assigned a severity score of 3 or 4 to an animal, antimicrobial therapy was administered and breath was sampled regardless of the animal’s rectal temperature. If a call had a severity score of 1 or 2 but did not meet the temperature criteria, no antimicrobial therapy was administered. After temperature evaluation and antimicrobial administration (if necessary) were performed, all calves were returned to their respective home pens. Calves with qualifying rectal temperature or severity score for the first time were administered 10 mg of tilmicosin/kg, SC, in the neck. Calves that were identified as sick at least 120 hours after the first treatment and met the antimicrobial treatment criteria were treated with 10 mg of enrofloxacin/kg, SC, in the neck. Calves that were identified as sick at least 48 hours after the second treatment and met the antimicrobial treatment criteria were treated with 2.2 mg of ceftiofur hydrochloride/kg, SC, in the neck, followed by a second dose 48 hours later. All medications were administered according to the manufacturer’s label directions.

Rectal and rumen temperature—For each animal that was removed from its pen for further evaluation, rectal temperature was recorded with a digital thermometer. In addition, every animal in the study was administered a rumen bolus for real-time rumen temperature recording. In a study of the temperature boluses, rumen temperature was highly correlated (r² = 0.78) with rectal temperature. In the present study, rumen temperature recorded on days that calves were weighed was used to evaluate changes in body temperature over time for all calves included in the experiment. However, we did not compare temperatures measured at the different locations in the same statistical model because of the potential for temperature variation measured at the different sites.

Breath analysis—For breath collection, a custom bovine mask with minor modifications was connected via a disposable bacterial-viral filter to the gas sampling line that continuously drew a sample into the measurement system consisting of a nondispersive infrared capnograph CO₂ sensor and a multi–optical–pass Herriot gas cell equipped with a tunable mid-infrared laser, detector, and associated electronics for measurement of CO, N₂O, and CO₂ as summarized elsewhere. The total breath test procedure lasted approximately 35 seconds. Data collection was initiated within 5 to 10 seconds of placing the mask on the muzzle (approx the sample tube clearance time) when the measured exhaled CO₂ concentration exceeded 0.8%. Data were collected continuously for 15 seconds, after which the mask was removed and an additional 15 seconds was allowed to evacuate the gas handling system. During each breath test, molecular concentrations of CO₂, N₂O, and CO were measured.

Because of a limited response time of the TDLAS system, a multibreath sample was collected to provide adequate gas volume for analysis. As a result, sample measured at any given time during the breath test was a mixture of exhaled breath and ambient gases. Although there is little change in the concentration of CO₂ in ambient air, concentrations of CO and N₂O can vary widely because of pollution, weather, and wind patterns, complicating data calculation. Ambient contamination was addressed by eliminating tests in which breath CO or N₂O concentration was > 20% greater than the ambient concentration measured prior to or following the breath sample, or if there was a > 5% difference in the absolute concentration of ambient CO or N₂O measured prior to and following the breath sample. Of the 1,433 individual tests, 83 tests failed because of ambient CO contamination from fossil fuel combustion, which originated from running engines such as skid-steer loaders, tractors, and mixers. Additionally, the assumption was made that each gas sample consisted of 75% breath and 25% ambient air according to the following calculation:

\[ C_{\text{Actual}} = \frac{\%_{\text{Breath}}}{\%_{\text{Breath}}} \times C_{\text{Breath}} + \frac{\%_{\text{Air}}}{\%_{\text{Air}}} \times C_{\text{Air}} \Rightarrow C_{\text{Breath}} = \frac{(C_{\text{Actual}} - \frac{\%_{\text{Air}}}{\%_{\text{Air}}} \times C_{\text{Air}})}{\%_{\text{Breath}}} \]

where C is concentration.

Given that there were multiple data points for each breath sample, we developed a method for determining a molecular score for each breath test by calculating the ratio of either biomolecule (CO or N₂O) to CO₂ at multiple noncontiguous points (10 data points, approx 3 seconds) with the highest CO₂ concentration during each breath sampling.

Serum haptoglobin concentration—After blood sample collection, samples were allowed to clot for 24 hours at 4°C and centrifuged at 3,000 × g at 4°C for 20 minutes. Serum was harvested in 2-mL centrifuge tubes and stored at −20°C until further analyses were performed. Once all serum samples were collected, a bovine haptoglobin ELISA was used to determine haptoglobin concentration of each serum sample. Prior to the analysis, serum samples were diluted 1:10,000 in Tris-buffered saline solution. The intra- and interassay coefficients of variation were < 5%.

Statistical analysis—All data were analyzed as a randomized complete block design by use of computer software. For a subset of calves (n = 42) that were never treated against BRD during the experiment, body weight, rumen temperature, concentration of haptoglobin, percentage of eCO₂, and ratios of eN₂O:eCO₂ and eCO₂:eCO₂ were analyzed as repeated measures. For this model, day of experiment was the fixed effect, load was assigned as a random effect, and a nonstructured covariance structure was used because of inequality of time across the different measurement days. A similar repeated-measures model was used for a subset of calves (n = 33) that were treated 3 times for BRD. For this analysis, number of antimicrobial treatments (1, 2, or 3) was the fixed effect and the remaining terms in the model were as described. For response variables measured in all calves at arrival and at the time of antimicrobial treatment, a model was used that included the number of antimicrobial treatments as a fixed effect and load was assigned as a random effect. In addition, body weight, rumen temperature, concentra-
tion of haptoglobin, percentage of eCO₂, and ratios of eN₂:O:eCO₂ and eCO₂:eCO₂ at arrival were analyzed by use of the health outcome (subsequent number of antimicrobial treatments) as the fixed effect and load as the random effect to determine whether data collected at arrival were different at the time of antimicrobial treatment. Health status at the end of the experiment was included as a fixed effect and load as a random effect in a model to determine differences in calves classified as healthy versus sick.

Partial Pearson coefficients were determined between arrival ratios of eN₂:O:eCO₂ and eCO₂:eCO₂, haptoglobin concentration, body weight, and rumen temperature, and multiple linear regression analysis was used to predict the number of days from arrival at the feedlot (ie, days on feed) to first antimicrobial treatment by use of software. For all comparisons, P < 0.05 was considered significant.

Results

Rates of morbidity and death attributed to BRD were high, with 222 of 337 (65.9%) calves requiring at least 1 antimicrobial treatment, 37 (11.0%) requiring only 2 treatments, and 41 (12.2%) requiring 3 treatments. In addition, 29 (8.6%) calves were euthanatized or died during the experiment, 27 of which were classified as BRD case fatalities.

In calves never treated for BRD, body weight increased (P < 0.001) as days on feed increased (Table 1). Ruminal temperature was significantly greater on day 7 compared with days –1, 14, and 42, and concentration of haptoglobin decreased (P < 0.001) as days of sampling increased.

For calves treated 3 times, means on days feed at treatment were 4.76, 9.58, and 13.9 for the first, second, and third antimicrobial treatments, respectively (Table 2). Body weight at the time of antimicrobial administration did not differ (P = 0.56) among treatments. Rectal temperature was significantly lower at the time of the third antimicrobial treatment, compared with antimicrobial treatments 1 and 2 in calves that were treated 3 times. The ratio of eN₂:O:eCO₂ was significantly lower during the second antimicrobial treatment, intermediate during the first antimicrobial treatment, and greatest during the third antimicrobial treatment. Number of antimicrobial treatments did not influence the ratio of eCO₂:eCO₂ (P = 0.59). Concentration of serum haptoglobin was significantly lower at the time of the third antimicrobial treatment, compared with the first antimicrobial treatment; concentration of haptoglobin at the time of the second antimicrobial treatment was intermediate.

In the population of calves treated for clinical signs of BRD, mean days on feed at treatment were 4.89, 11.2, and 14.9 for the first, second, and third antimicrobial treatments, respectively (P < 0.001; Table 3). Body weight at the time of the first and second antimicro-

Table 1—Effect of day of sampling on breath, blood, and body measurement variables (least squares means) in cattle never treated for BRD during a 42-day preconditioning program.

<table>
<thead>
<tr>
<th>Variable</th>
<th>–1</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>42</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>46</td>
<td>260</td>
<td>267</td>
<td>277</td>
<td>306</td>
<td>3.65</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Rumen temperature (°C)</td>
<td>39.2</td>
<td>39.8</td>
<td>39.0</td>
<td>39.9</td>
<td>38.7</td>
<td>0.10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>eCO₂ (%)</td>
<td>0.032</td>
<td>0.028</td>
<td>0.030</td>
<td>0.029</td>
<td>0.031</td>
<td>0.001</td>
<td>0.05</td>
</tr>
<tr>
<td>eN₂:O:eCO₂ ratio (vol/vol)</td>
<td>6.29</td>
<td>6.26</td>
<td>6.40</td>
<td>6.31</td>
<td>7.54</td>
<td>0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>eCO₂:eCO₂ ratio (vol/vol)</td>
<td>4.98c</td>
<td>4.14c</td>
<td>4.05c</td>
<td>3.62</td>
<td>4.06c</td>
<td>0.16</td>
<td>0.001</td>
</tr>
<tr>
<td>Haptoglobin (mg/100 mL)</td>
<td>3.01</td>
<td>1.27c</td>
<td>1.99</td>
<td>0.73c</td>
<td>0.17</td>
<td>0.35</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*Within a row, values with different superscripts are significantly (P < 0.05) different.

Table 2—Breath, blood, and body measurement variables (least squares means) across antimicrobial treatment frequency in 33 cattle that were treated 3 times against BRD during the first 21 days after arrival at a preconditioning facility.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treated once*</th>
<th>Treated twice†</th>
<th>Treated thrice‡</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days on feed at treatment</td>
<td>4.76c</td>
<td>9.58c</td>
<td>13.9c</td>
<td>0.98</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>236</td>
<td>223</td>
<td>231</td>
<td>3.75</td>
<td>0.56</td>
</tr>
<tr>
<td>Rumen temperature (°C)</td>
<td>41.2c</td>
<td>40.9c</td>
<td>40.6c</td>
<td>0.10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>eCO₂ (%)</td>
<td>0.025</td>
<td>0.025</td>
<td>0.023</td>
<td>0.001</td>
<td>0.11</td>
</tr>
<tr>
<td>eN₂:O:eCO₂ ratio (vol/vol)</td>
<td>7.64c</td>
<td>7.03c</td>
<td>8.12c</td>
<td>0.33</td>
<td>0.03</td>
</tr>
<tr>
<td>eCO₂:eCO₂ ratio (vol/vol)</td>
<td>7.62</td>
<td>7.80</td>
<td>7.32</td>
<td>0.38</td>
<td>0.59</td>
</tr>
<tr>
<td>Haptoglobin (mg/100 mL)</td>
<td>4.96c</td>
<td>3.84c</td>
<td>2.95c</td>
<td>0.55</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Cattle that were removed from their pen for evaluation of illness and met antimicrobial treatment criteria were treated with 10 mg of tilmicosin/kg, SC, administered in the neck. †Cattle that were identified as sick at least 120 hours after the first treatment and met antimicrobial treatment criteria were treated with 10 mg of enrofloxacin/kg, SC, administered in the neck. ‡Cattle that were identified as sick at least 48 hours after the second treatment and met antimicrobial treatment criteria were treated with 2.2 mg of cefotaxime hydrochloride/kg, SC, administered in the neck, followed by a second dose 48 hours later.

* Within a row, values with different superscripts are significantly (P < 0.05) different.
Although not significantly (weight was greater in healthy compared with sick calves, combined into categories of healthy versus sick, body weight did not differ (Table 5) among treatments, compared with calves that were never treated for clinical signs of BRD, body weight did not differ (P ≥ 0.12) among treatments. Ratios of eN\textsubscript{2}O:eCO\textsubscript{2} did not differ (P ≥ 0.12) among treatments. However, concentration of serum haptoglobin was greater (P = 0.02) in sick calves.

Days to first treatment were negatively correlated (P < 0.001) with rumen temperature (Table 6). The ratios of eN\textsubscript{2}O:eCO\textsubscript{2} and eCO\textsubscript{2}:eCO\textsubscript{2} were positively correlated (P < 0.001). Serum haptoglobin concentration at arrival was positively correlated (P = 0.01) with body weight at arrival. No other response variables were significantly correlated.

Multiple linear regression analysis revealed that the number of days on feed to first treatment could be estimated by use of the following equation:

\[
\text{Days to first treatment} = 315.205 - (0.0719 \times \text{body weight (kg)}) - (8.102 \times \text{days on feed}) + (0.366 \times \text{haptoglobin (mg/100 mL)}) - (0.516 \times \text{rumen temperature})
\]
Table 6—Results of Pearson product moment correlation of measurements obtained on arrival in a feedlot and subsequent days on feed (DOF) to first antimicrobial treatment during a 42-day preconditioning program in 73 calves.

<table>
<thead>
<tr>
<th>Variable</th>
<th>eN\textsubscript{2}:eCO\textsubscript{2}</th>
<th>eCO:eCO\textsubscript{2}</th>
<th>HP</th>
<th>Body weight</th>
<th>Rumen temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOF to first treatment</td>
<td>-0.022 (0.80)</td>
<td>-0.0126 (0.89)</td>
<td>0.0126</td>
<td>0.105 (0.24)</td>
<td>-0.384 (&lt; 0.001)</td>
</tr>
<tr>
<td>(P value)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eN\textsubscript{2}:eCO\textsubscript{2} ratio (P value)</td>
<td>NA</td>
<td>0.469 (&lt; 0.001)</td>
<td>-0.060</td>
<td>-0.137 (0.11)</td>
<td>0.038 (0.67)</td>
</tr>
<tr>
<td>eCO:eCO\textsubscript{2} ratio (P value)</td>
<td>NA</td>
<td>NA</td>
<td>-0.0278 (0.75)</td>
<td>-0.160 (0.06)</td>
<td>0.0388 (0.67)</td>
</tr>
<tr>
<td>HP (P value)</td>
<td>NA</td>
<td>NA</td>
<td>0.126</td>
<td>0.0680 (0.45)</td>
<td></td>
</tr>
<tr>
<td>Body weight (P value)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.0938 (0.30)</td>
</tr>
</tbody>
</table>

HP = Haptoglobin. \textit{See Table 3 for remainder of key.}

Discussion

In recent years, the search for clinically useful biomarkers to diagnose health status, disease, or outcome of pharmacological treatments has increased in human and veterinary medicine. In human medicine, breath tests have been used to investigate a broad range of diseases including cancer\textsuperscript{16} and asthma.\textsuperscript{20} In addition, the use of exhaled breath has potential as a diagnostic tool in veterinary medicine.\textsuperscript{21} Although more than 3,000 gases have been identified in samples of human breath,\textsuperscript{22} few have been associated with specific disease processes. For example, an increased concentration of NO\textsuperscript{23} and increased concentrations of NO and CO in the exhaled breath of asthmatic patients\textsuperscript{24,25} have been reported. Twenty-one volatile organic compounds in exhaled breath of cattle have been isolated by use of gas chromatography–mass spectrometry.\textsuperscript{26} In the present experiment, it was possible to measure eN\textsubscript{2}, eCO, and eCO\textsubscript{2} in the breath of newly received feeder calves by use of TDLAS technology. We first attempted to determine steady state of these molecules in cattle that were never identified as sick during the experiment. Interestingly, in the population of healthy calves, the ratio of eN\textsubscript{2}:eCO\textsubscript{2} remained constant during the first 21 days of the experiment (Table 1), which indicated that changes in this ratio might be useful to aid in the diagnosis of BRD. This idea was supported when considering the mean values of the eN\textsubscript{2}:eCO\textsubscript{2} ratio for the entire population of calves from which valid breath samples were collected (Table 3). Arrival values of the eN\textsubscript{2}:eCO\textsubscript{2} ratio were lower, compared with the mean eN\textsubscript{2}:eCO\textsubscript{2} ratio when calves required either a first, second, or third antimicrobial treatment, and the eN\textsubscript{2}:eCO\textsubscript{2} ratio increased as the number of antimicrobial treatments increased. Similar to the present results, it has been reported that eNO had potential to be used as a diagnostic tool for BRD; however, the detection limit of the instrument used for that experiment was not sensitive enough to provide conclusive results.\textsuperscript{16}

To the authors’ knowledge, there are no previous publications reporting the use of eN\textsubscript{2} as a biomarker of BRD. In the present study, eN\textsubscript{2} was selected instead of eNO because N\textsubscript{2} absorption is 2 orders of magnitude stronger than NO absorption on the TDLAS instrumentation. This makes measurement of eN\textsubscript{2} easier than eNO, which is a critical advantage in bovine breath analysis because of the lower concentrations of eNO. Although the exact origin of eN\textsubscript{2} in breath is unknown, we have 2 hypotheses regarding its origin: bacterial denitrification of NO\textsubscript{2} that produces N\textsubscript{2}O or activation of the immune system via inducible NO synthase plus l-arginine that produces l-citrulline plus NO, in which O + H is transformed to HNO (nitroxyl) and 2HNO results in N\textsubscript{2}O + H\textsubscript{2}O. In contrast to the promising use of the eN\textsubscript{2}:eCO\textsubscript{2} ratio as a diagnostic tool for BRD, comparison of the arrival value for eN\textsubscript{2}:eCO\textsubscript{2} relative to the number of antimicrobial treatments an animal would subsequently require revealed no difference (Table 4). In addition, when this population was classified as healthy versus sick, the ratio was not significant (Table 5). As indicated, no previous results have been published regarding N\textsubscript{2}O in exhaled breath and its relationship with respiratory tract disease, although the concentration of NO\textsubscript{2} has been correlated with respiratory tract inflammation in humans.\textsuperscript{27,28} However, it has also been reported that for the analysis of NO, breath samples must be collected during open-mouth breathing because of contamination or loss of NO in the upper portion of the respiratory tract.\textsuperscript{29} In the present experiment, the values of the eN\textsubscript{2}:eCO\textsubscript{2} ratio remained constant in calves that never required an antimicrobial treatment. This might be related to a normal respiration pattern with the breath sample coming mainly from the lungs and the upper portion of the respiratory tract. In contrast, values of the eN\textsubscript{2}:eCO\textsubscript{2} ratio in sick calves at the time of antimicrobial treatment might reflect more open-mouth breathing. One of the most common signs of BRD is an altered pattern of respiration and open-mouth breathing caused by decreased lung capacity from inflammation. If this potential pathological variation is accounted for, the breath sample collected in sick cattle might be diagnostic because of decreased contamination or loss of molecules that can act as biomarkers in the upper portion of the respiratory tract. In support of this conclusion, a 3-times increase in respiration rate in pigs infected with \textit{Chlamydia suis} with a subsequent increase in volume of air ventilated in sick animals, compared with nonchallenged pigs, has been reported.\textsuperscript{30} It appears from our data that eN\textsubscript{2} has value as a diagnostic marker, but not as a predictive marker of BRD.

Another breath molecule measured in the present experiment was CO. This molecule has been associated with an increased concentration of NO in conjunction with oxidative stress.\textsuperscript{14} In addition, concentrations of eCO are increased in clinically normal human subjects with infections of the up-
per and lower portions of the respiratory tract. In the present experiment, values of the eCO: eCO\textsubscript{2} ratio varied across time in calves that never required an antimicrobial treatment during the experiment (Table 1). However, the numeric values were lower compared with the population of calves that required 3 antimicrobial treatments during the experiment, which might suggest that differences exist between healthy and sick calves. In addition, when the eCO: eCO\textsubscript{2} ratio was compared across the entire population, values were lower on arrival, compared with the ratio at the time of first, second, or third antimicrobial treatment (Table 3). Similar to eN\textsubscript{2}O: eCO\textsubscript{2}, when the eCO:eCO\textsubscript{2} values at arrival were analyzed on the basis of the number of antimicrobial treatments subsequently required during the study, no differences were observed, suggesting that eCO: eCO\textsubscript{2} might be useful as a diagnostic tool but not as a predictive tool. The lack of predictive power of eN\textsubscript{2}O:eCO\textsubscript{2} and eCO:eCO\textsubscript{2} as markers for the onset of BRD might be attributed to the acute response of the immune system to the pathogens involved in the disease. In high-risk calves, the onset of BRD is typically during the first week after arrival to the feedlot facility when substantial social, metabolic, and physiological adaptations are occurring. In research of biomarkers that might be useful for the diagnosis and prognosis of BRD, acute-phase proteins have gained a lot of attention. For example, in healthy cattle, haptoglobin might be nondetectable or present in low concentrations ranging from 0.05 to 0.10 mg/L. However, haptoglobin concentration might increase 50 to 100 times in response to BRD, and analysis of this protein as an indication of microbial infection has been recommended. In the present study, even in calves that were never identified as sick during the experiment, haptoglobin concentration was greatest at arrival and decreased as days on feed increased. This decrease in haptoglobin concentration might suggest that this population of calves remained healthy during the study. In calves that were treated 3 times against BRD, haptoglobin concentration was greater at the time of first and second antimicrobial treatments, compared with the third antimicrobial treatment. These data are in agreement with reported data that indicate that when cattle are recovering from BRD, 7 days are required for the haptoglobin concentration to begin to decrease after an antimicrobial treatment. Although haptoglobin might be useful as a diagnostic tool, haptoglobin concentrations at the time of arrival was not effective to predict the number of antimicrobial treatments required in the present experiment. However, arrival haptoglobin concentration might be useful to predict whether an animal will require an antimicrobial treatment during a preconditioning period.

Arrival serum haptoglobin concentrations have been used to predict the number of antimicrobial treatments required during the preconditioning and finishing phase. In 1 study, an increased arrival haptoglobin concentration in calves requiring more than 1 antimicrobial treatment was reported \((r^2 = 0.36; P < 0.001)\). In contrast, a low correlation \((r^2 = 0.06; P < 0.001)\) between the arrival serum haptoglobin concentration and subsequent number of antimicrobial treatments has also been reported. Although haptoglobin concentration alone was not useful in the diagnosis of BRD in young dairy calves, an increased discriminative ability was obtained if, in addition, a second quantitative value (rectal temperature > 39.5°C) was included in the model. In the present experiment, we attempted to predict the number of days on feed to first antimicrobial treatment on the basis of arrival serum haptoglobin concentration, arrival rumen temperature, arrival body weight, and arrival eN\textsubscript{2}O:eCO\textsubscript{2} and eCO:eCO\textsubscript{2} ratio values. Multiple linear regression analysis revealed that the number of days on feed to first treatment was correlated with these arrival measurements and can be estimated by use of the following equation:

\[
\text{Days on feed to first treatment} = 315.205 - (0.0719 \times eN\textsubscript{2}O:eCO\textsubscript{2}) + (0.366 \times eCO:eCO\textsubscript{2}) - (0.516 \times \text{haptoglobin concentration}) + (0.0987 \times \text{body weight [kg]}) - (8.102 \times \text{arrival rumen temperature})
\]

On the basis of the equation, although a correlation existed between the measurements obtained at arrival, there was a lack of predictive power regarding the onset of BRD. Although predictive power was lacking, changes in eN\textsubscript{2}O and eCO and their relationship with CO\textsubscript{2}, rectal (or rumen) temperature, and haptoglobin concentration appeared to coincide with the appearance of clinical signs of BRD. As suggested previously, the lack of accuracy in visual observation for the diagnosis of BRD might open the window for the implementation of these technologies regarding the decision for treatment interventions for cattle identified as sick, and decrease the proportion of cattle with lung lesions at slaughter that were never identified as sick. This has potential to decrease the economic losses associated with decreased performance caused by BRD.

Although breath analysis by use of TDLAS was successfully implemented in a research feedlot setting, we conclude that eN\textsubscript{2}O, eCO, eCO\textsubscript{2}, and haptoglobin concentration were not accurate in predicting future BRD events during a preconditioning program. However, these markers may be useful for identifying BRD in cattle that are evaluated for illness via visual observation. These biomarkers might support the diagnosis of BRD and provide early treatment interventions to decrease economic losses associated with BRD. Development of a model in which these measurements are implemented in conjunction with other variables might increase the ability to predict the onset of BRD and implement management practices that can decrease the prevalence of this disease. A better understanding of the disease, physiological origin of biomarkers, physiological changes during the disease, and increased sensitivity of measuring devices might be useful in the development of more specific BRD biomarkers.

b. Eastern Livestock Cattle Co, Marion, Ky.
c. Livestock Market, Marion, Ky.
d. SmartStock LLC, Pawnee, Okla.
e. Clott activator, Benton Dickinson Vacutainer Systems, Franklin Lakes, NJ.
References