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Agreement Among 4 Sampling Methods to Identify Respiratory Pathogens in Dairy Calves with Acute Bovine Respiratory Disease

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Background: Four sampling techniques commonly are used for antemortem identification of pathogens from cattle with bovine respiratory disease (BRD): the nasal swab (NS), guarded nasopharyngeal swab (NPS), bronchoalveolar lavage (BAL), and transtracheal wash (TTW). Agreement among these methods has not been well characterized.

Objective: To evaluate agreement among TTW and NS, NPS, or BAL for identification of viral and bacterial pathogens in dairy calves with BRD.

Animals: One hundred dairy calves with naturally acquired BRD.

Methods: Calves were sampled by all 4 methods. Viral agents were identified by real-time RT-PCR, bacteria were identified by aerobic culture, and *Mycoplasma bovis* (*M. bovis*) isolates were speciated by PCR. Agreement among TTW and NS, NPS, or BAL was evaluated by calculating the kappa statistic and percent positive agreement. McNemar's exact test was used to compare the proportions of positive results.

Results: Agreement among TTW and NS, TTW and NPS, and TTW and BAL, was very good for identification of *P. multocida, M. haemolytica, and M. bovis*. For bovine respiratory syncytial virus (BRSV), agreement with TTW was moderate for NS, good for NPS, and very good for BAL. For bovine coronavirus (BCV), agreement with TTW was moderate for NS and NPS, and good for BAL. McNemar's test was significant only for BCV, indicating that for this pathogen the proportion of positive results from NS and NPS could not be considered comparable to TTW.

Conclusions and Clinical Importance: This study provides guidance for veterinarians selecting diagnostic tests for antemortem identification of pathogens associated with BRD.

Key words: Bacteria; Diagnosis; Pneumonia; Virus.

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Abbreviations:

BAL	bronchoalveolar lavage
BCV	bovine coronavirus
BHV-1	bovine herpesvirus-1
BRD	bovine respiratory disease
BRSV	bovine respiratory syncytial virus
BVDV1 and BVDV2	bovine viral diarrhea virus types 1 and 2
NPS	guarded nasopharyngeal swab
NRC	National Research Council
NS	nasal swab
PI3V	parainfluenza type 3 virus
RT-PCR	reverse transcriptase polymerase chain reaction
TTW	transtracheal wash

Bovine respiratory disease (BRD) is a common cause of morbidity and mortality in dairy calves,[1] which has long-term consequences including decreased milk production, poor reproductive performance, and poor growth and longevity.[2–4] Although management practices such as commingling and group housing increase BRD risk, viral and bacterial pathogens cause the lesions characteristic of BRD. Monitoring and testing for pathogens associated with BRD may facilitate the development of appropriate, targeted vaccination programs. Moreover, recent identification of multidrug-resistant *M. haemolytica* from BRD cases[5] indicates that it may be informative for veterinarians to monitor cattle with BRD for the presence of resistant bacterial pathogens.

Four sampling methods commonly are used for antemortem identification of respiratory pathogens: the nasal swab (NS), guarded nasopharyngeal swab (NPS),

transtracheal wash (TTW), and bronchoalveolar lavage (BAL). However, each of these methods has limitations. Although the TTW allows collection of a sample from the lower airways while bypassing the normal flora of the nasopharynx, the procedure is invasive and time-consuming. In contrast, nasal swabs are simple to collect. However, the results of bacterial culture of nasal swabs may be difficult to interpret because of the potential for contamination by commensal organisms. The guarded NPS has been proposed to provide a more reliable sample of bacteria causing pneumonia,[6] but these samples can be unwieldy to collect, because the available guarded swabs are designed for mare uterine culture, making them longer than necessary for nasopharyngeal sampling; they also are relatively expensive. To our knowledge, no published study has compared the results of NS to guarded NPS in cattle with clinical BRD. Thus, it is not clear that NPS is sufficiently superior to NS to warrant the additional effort and expense they require. Furthermore, neither NS nor NPS provides a sample of the lower airways. The BAL has been proposed to provide a representative sample of the lower respiratory tract,[7] but the method of collection provides the possibility of upper airway commensal contamination of the sample. Moreover, because BAL samples only the bronchoalveolar unit distal to the wedged tube, it is possible to miss pathogens not evenly distributed throughout the lung.

Studies have compared bacterial culture results from guarded NPS to those from tracheal swabs or lung lavage in cattle with BRD.[6,8,9] These studies showed that the agreement between the results of NPS and a lower airway sample was sometimes, but not always, strong at the calf level, but generally was good at the group level. We are not aware of published reports comparing these 4 diagnostic tests for identification of pathogens associated with BRD in dairy calves. Therefore, the objective of our study was to evaluate the agreement among results obtained by TTW to those obtained by NS, guarded NPS, or BAL in calves clinically affected by BRD, by comparing pathogens isolated by each sampling method in the same calf. The TTW was chosen as the reference standard because it provides a sample from the lower airways while bypassing nasopharyngeal contamination.

Materials and Methods

Calf Management

Holstein and Holstein-cross bull calves housed on a calf-rearing facility in Tulare, California, were enrolled in the summer of 2014. The study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis. Calves arrived at the facility when they were between 2 and 24 hours of age. On arrival, all calves were vaccinated with a modified live intranasal vaccine containing bovine respiratory syncytial virus (BRSV), bovine herpesvirus-1 (BHV-1), and parainfluenza-3 virus (PI3V).[a] At arrival, calves were fed 2 L of commercial colostrum replacer by bottle and an additional 2 L of commercial colostrum replacer 12 and 24 hours later. Calves were grouped by age, housed in individual wooden hutches, and fed by bottle 2 L of milk replacer twice each day that was mixed by farm workers. Calves were weaned at approximately 60 days of age. Water and a farm-mixed calf starter formulated to meet National Research Council requirements for growing calves were provided free choice.

Enrollment Criteria

All bull calves >30 days of age that experienced morbidity due to primary, naturally occurring respiratory disease as defined by a score of ≥5 (University of Wisconsin Calf Respiratory Scoring Chart, www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_respiratory_scoring_chart.pdf) and with a rectal temperature of ≥39.4°C (103°F) were eligible for enrollment. One trained observer scored the calves for the entire duration of the study by evaluating the presence and severity of coughing, nasal discharge, ocular discharge, and head tilt or ear drooping. Calves with a score of ≥5 and a rectal temperature of ≥39.4°C were subjected to transthoracic ultrasound examination of their lungs to determine whether areas of consolidation were present. The lungs were examined with a portable ultrasound unit with a variable 5–8.5 MHz 66-mm linear probe;[b] the area from the 10th intercostal space to the 1st intercostal space on both sides of the thorax was imaged. Imaging was facilitated by clipping the hair and applying 70% ethanol to the surface of the skin. If an area of at least 2 cm^2 of consolidation[10] was observed in any lung lobe, the calf was eligible for enrollment. Any calf diagnosed and previously treated with antibiotics or flunixin meglumine for BRD or any other disease was excluded from study participation. Calves also were excluded if they had received any intranasal vaccine within the previous 30 days.

Sample Collection and Assessment

All calves enrolled in the study were sampled sequentially by NS, guarded NPS, TTW, and then BAL. For all procedures, calves were manually restrained in a standing position. For collection of NS, the calf's nares were wiped clean with a single-use paper towel. A 13-cm polyester nasal swab[c] then was inserted into the nostril to the full length of the swab, rubbed on the mucosa, and withdrawn. Next, the NPS sample was collected. The calf's nares again were wiped clean with a single-use paper towel, and a 59-cm guarded polyester swab[d] was advanced to a depth approximately 2 cm rostral to the medial canthus of the eye. The swab was advanced approximately 4 cm farther, rotated, and withdrawn into the sheath before removal. One NS and 1 NPS were collected from each nostril. The samples collected from the left nostril were submitted for viral multiplex RT-PCR, and the samples from the right nostril were submitted for bacterial culture. The swabs for viral RT-PCR were placed in viral transport media (Eagle's minimal essential media with HEPES and sodium bicarbonate; gentamicin was added at 50 mg/mL and amphotericin B was added at 250 mg/mL and pH was adjusted to 7.0–7.3), and the swabs for bacterial culture were stored in Brucella broth with 10% glycerol; media were obtained from the Biological Media Services at the University of California, Davis. After the NS and NPS were collected and a TTW was obtained, a 6 × 6 cm area over the ventral trachea was clipped, subjected to sterile preparation of alternating scrubs with chlorhexidine scrub followed by 70% isopropanol, repeated 3 times, and locally blocked with 2 mL of 2% lidocaine. A commercially available TTW kit with a 15-gauge cannula and a 48-cm catheter[e] was used for sample collection. After placement of the needle and catheter, 30 mL of sterile isotonic saline was instilled and withdrawn. A drop of the sample was removed from the retrieved volume of fluid with a plastic disposable serologic pipette for cytologic evaluation (cytology results to be reported elsewhere), and the remainder then was divided into equal aliquots

for bacterial culture and viral RT-PCR. After collection of the TTW, the BAL was obtained. The calf's nares again were wiped clean with a single-use paper towel. A BAL tube[f] was advanced through the nose until it wedged in a bronchus, and 100 mL of sterile isotonic saline was instilled and withdrawn. Retrieved fluid aliquots were pooled, and a drop of the sample was removed for cytologic evaluation (results to be reported elsewhere), and the remainder was divided into equal aliquots for bacterial culture and viral RT-PCR. One aliquot was subjected to aerobic bacterial culture for identification of *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and also *Mycoplasma* culture. Colonies identified as *M. haemolytica* were tested to confirm they were not *Bibersteinia trehalosi* or indole-positive *Mannheimia* spp., which can appear to be similar to *Mannheimia haemolytica*. Any isolate with morphology consistent with *Mycoplasma* spp. was subjected to digitonin testing to differentiate *Mycoplasma* spp. from *Acholeplasma* spp.[11] The second aliquot was subjected to viral multiplex real-time RT-PCR to identify the following viruses, with published primers and probes: BHV-1,[12] bovine viral diarrhea virus types 1 and 2 (BVDV1 and BVDV2),[g] BRSV,[13] and bovine coronavirus (BCV).[14] The samples were submitted to the California Animal Health & Food Safety Laboratory in Tulare, California, for aerobic culture; the California Animal Health & Food Safety Laboratory in Davis, California, for viral multiplex RT-PCR; and the Milk Quality Laboratory in Tulare, California, for *Mycoplasma* culture and digitonin testing.

Speciation of *Mycoplasma* isolates to identify *Mycoplasma bovis* was completed by PCR as previously described.[15] Briefly, DNA was extracted from *Mycoplasma* isolates with a commercially available kit[h] according to the manufacturer's directions. The PCRs were prepared in 25 μL volumes with *M. bovis*-specific primers described.[15] The PCR assays were performed with an initial cycle at 94°C for 5 minute; 35 cycles of denaturation at 94°C for 20 second, annealing at 52°C for 1 minute, and extension at 72°C for 1 minute; and extension at 72°C for 5 minute. Samples that did not contain *M. bovis* DNA were included for quality control purposes to monitor for DNA contamination during the processing of the PCR assay reactions. The specificity of the amplified band was confirmed on the basis of detection of a single band representing a 319-bp product.

Statistical Analysis

The primary outcome for the samples collected from each calf for each diagnostic test was the presence of any of the following agents*: M. haemolytica, P. multocida, H. somni, Mycoplasma* sp., *Mycoplasma bovis,* BHV-1, BVDV 1 or 2, BRSV, or BCV. The TTW was established as the reference standard for test comparison. Therefore, the pathogens detected by TTW for each calf were evaluated for agreement with the corresponding pathogen results for each of the other tests from the same calf: NS, NPS, and BAL. McNemar's exact test was used to compare the methods with respect to their marginal proportions of positive results, with significance set at $P < .05$. A sample size of 100 calves was used based on the availability of funding and a consideration of the power to detect a significant difference among methods with respect to the paired proportions of positive test results. It was determined that a sample size of 72 calves with a particular pathogen would provide a power of 80% to detect a difference of 15 percentage points in the proportions of positive test results, assuming a 5% type I error probability and discordant test results in 20% of the calves. Based on previous research by Lehenbauer et al. (personal communication), it was assumed that approximately 70% of the calves might be expected to have the most common pathogen; thus, a minimum enrollment of 100 calves was desired. The agreement among diagnostic tests then was evaluated by calculation of the kappa statistic and the percent positive agreement. Kappa values were interpreted to indicate strength of agreement as defined by Altman[16]: <0.20 = poor; 0.21–0.40 = fair; 0.41–0.6 = moderate; 0.61–0.80 = good, and 0.81–1.00 = very good. The percent positive agreement between 2 methods was calculated as 100 times the number of calves that were positive by both methods divided by the average number of calves that were positive by either method.[17] Analyses were performed by commercially available statistical software.[i]

Results

Prevalence of Pathogens Identified by Sampling Methods Tested

The average age of the 100 enrolled calves was 49 days (range, 31–74 days). The clinical and ultrasound findings are summarized in Table 1. The prevalence of agents identified by each method is presented in Table 2. *Histophilus somni*, *Bibersteinia trehalosi,* BHV-1, BVDV1, or BVDV2 were not identified in any sample.

Agreement among TTW and NS, NPS, or BAL for Individual Pathogens

The percent positive agreement, kappa statistic, and McNemar's test results for the NS, NPS, and BAL relative to the TTW were calculated for each pathogen, and the results are presented in Table 3. The NS, NPS, and BAL showed very good agreement relative to TTW for *M. haemolytica*, *P. multocida,* and *M. bovis*. For *Mycoplasma* spp., the percent positive agreement among sampling methods was high, but the kappa statistics were near zero. This outcome was attributable to the fact nearly all of the samples had a positive result by at least 1 of the methods, which resulted in a negatively biased kappa statistic, as has been discussed (Cunningham. "More than just the kappa coefficient: a program to fully characterize inter-rater reliability between two raters." SAS Global Forum 2009, Paper 242-2009, http://support.sas.com/resources/papers/proceedings09/242-2009.pdf). When BRSV was detected, the agreement

Table 1. Descriptive statistics for age, clinical signs, and pulmonary ultrasound findings in 100 calves diagnosed with bovine respiratory disease by clinical assessment and thoracic ultrasound and enrolled for sampling to identify respiratory pathogens by 4 different methods.

	Minimum	Maximum	Mean	Std. Dev.
Age (days)	31	74	48.6	10.7
Wisconsin respiratory score	7.0	12.0	9.8	0.8
Rectal temperature, °C (°F)	39.4 (103.0)	41.0 (105.8)	39.8 (103.7)	0.7
Pulmonary consolidation area (cm^2)	2.0	70.0	22.1	15.8

Table 2. Percent of samples positive for respiratory pathogens identified in 100 dairy calves diagnosed with bovine respiratory disease by clinical assessment and thoracic ultrasound, and sampled by nasal swab (NS), guarded nasopharyngeal swab (NPS), bronchoalveolar lavage (BAL), and transtracheal wash (TTW).

Pathogen	NS	NPS	BAL	TTW
BCV	15/96 (15.6%)	19/91 (20.9%)	13/91 (14.3%)	6/91 (6.6%)
BRSV	9/96 (9.4%)	13/94 (13.8%)	15/94 (16.0%)	17/98 (17.4%)
M. haemolytica	20/100 (20%)	17/100 (17%)	17/100 (17%)	16/100 (16%)
P. multocida	60/100 (60%)	61/100 (61%)	60/100 (60%)	59/100 (59%)
Mycoplasma sp.	88/92 (95.7%)	85/95 (89.5%)	87/88 (98.9%)	87/92 (94.6%)
Mycoplasma bovis	45/96 (46.9%)	47/90 (52.2%)	51/94 (54.3%)	48/91 (52.7%)

For some agents, the total number of samples is less than 100 because samples were not included if the laboratory reported them as contaminated or indeterminate. BCV, bovine coronavirus. BRSV, bovine respiratory syncytial virus.

Table 3. Agreement among the transtracheal wash (TTW) and each of nasal swab (NS), guarded nasopharyngeal swab (NPS) or bronchoalveolar lavage (BAL) for identification of respiratory pathogens in 100 preweaned Holstein calves with respiratory disease.

		[b]No. Calves with Each Combination of Results						
Pathogen	[a]Method	+/+	+/−	−/+	−/−	Percent Positive Agreement	Kappa (95% CI)	[c]*P*
Mannheimia haemolytica	NS	16	0	4	80	88.9 (78.1, 99.7)	0.86 (0.74, 0.99)	.125
	NPS	15	1	2	82	90.9 (80.7, 100)	0.89 (0.77, 1.00)	1.00
	BAL	15	1	2	82	90.9 (80.7, 100)	0.89 (0.77, 1.00)	1.00
Pasteurella multocida	NS	57	2	3	38	95.8 (92.1, 99.5)	0.90 (0.81, 0.99)	1.00
	NPS	58	1	3	38	96.7 (93.4, 99.9)	0.92 (0.84, 1.00)	.625
	BAL	57	2	3	38	95.8 (92.1, 99.5)	0.90 (0.81, 0.99)	1.00
Mycoplasma spp.	NS	81	3	5	0	95.3 (92.0, 98.6)	−0.04 (−0.10, 0.01)	.727
	NPS	78	8	5	0	92.3 (88.1, 96.5)	−0.07 (−0.14, −0.01)	.581
	BAL	81	1	5	0	96.4 (93.6, 99.3)	−0.02 (−0.06, 0.02)	.219
Mycoplasma bovis	NS	41	7	1	39	91.1 (85.0, 97.3)	0.82 (0.70, 0.94)	.070
	NPS	41	4	3	35	92.1 (86.3, 97.9)	0.83 (0.71, 0.95)	1.00
	BAL	45	1	2	40	96.8 (93.1, 100)	0.93 (0.86, 1.00)	1.00
Bovine respiratory syncytial virus	NS	7	7	2	78	60.9 (37.3, 84.4)	0.56 (0.30, 0.81)	.180
	NPS	11	4	2	76	78.6 (61.8, 95.3)	0.75 (0.56, 0.94)	.688
	BAL	13	0	2	78	92.9 (83.0, 100)	0.92 (0.80, 1.00)	.500
Bovine coronavirus	NS	6	0	7	76	63.2 (37.8, 88.5)	0.59 (0.33, 0.86)	.016
	NPS	6	0	9	70	57.1 (31.9, 82.4)	0.52 (0.27, 0.78)	.004
	BAL	6	0	3	77	80.0 (57.8, 100)	0.78 (0.55, 1.00)	.250

[a]NS, nasal swab; NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage.

[b]Dichotomous testing results for paired samples collected by TTW/and the comparison method, respectively. The total number of calves differs across pathogens due to culture contamination or indeterminate PCR results for at least sample of each pair compared.

[c]*P*-value for McNemar's exact test.

with TTW was moderate for NS, good for NPS, and very good for BAL. When BCV was detected, the agreement with TTW was moderate for NS and DNP, and good for BAL, although NS and DNP both yielded significantly higher proportions of positive results than did TTW.

Discussion

Antemortem sampling of cattle or calves sometimes is undertaken to identify microbial pathogens associated with outbreaks of BRD. The sampling methods evaluated in our study are commonly used, and each method has advantages and disadvantages. The results presented here add new information to the body of knowledge regarding the comparative value of these antemortem sampling techniques. To our knowledge, no previously published studies have compared the results of all 4 of these methods, although previous studies have compared NS or NPS to lower airway sampling.[6,8,9] The results of our study suggest that NS, NPS, or BAL are all similar in their ability to determine when *M. haemolytica, P. multocida,* or *M. bovis* are present in the lower airways of dairy calves with acute BRD, as identified by TTW. In contrast, for the viral agents identified in this study, agreement (as measured by percent positive agreement and also by the kappa statistic) was stronger for BAL and TTW than for NS or NPS and TTW. Moreover, for BCV, the significant McNemar's test indicated that the positive test

proportions for NS and NPS could not be considered comparable to the less common TTW results. Future work is warranted to determine whether the relative agreement among these diagnostic tests is the same for other classes of cattle with BRD, such as feedlot cattle or stocker cattle.

Anecdotal comments have indicated that NS is unreliable for diagnosis of bacterial BRD agents, because NS cultures often are overgrown by contaminants. Although growth of other agents was recognized on some NS cultures in our study, overgrowth of contaminants did not make it impossible to identify the bacterial agents of interest. The practice used in our study of wiping the nostrils of calves clean with a single-use paper towel before collecting the NS may have helped decrease the number of contaminating organisms picked up by the NS.

Other investigators have compared the results of guarded NPS with lung lavage or TTW. One study compared the bacteria isolated from guarded NPS to those isolated from *postmortem* lung lavage in 4- to 6-month-old beef calves with naturally occurring acute BRD.[6] With samples from 20 animals, these investigators found that identification of these pathogens by NPS was a reliable way to predict that they also would be present in lung lavage. Another study compared results of bacterial culture to identify *M. haemolytica, P. multocida,* and *H. somni* from guarded NPS to results of culture of samples collected by "guarded transtracheal swab."[9] These investigators found that when both samples were positive on bacterial culture, the same organism was present 96% of the time. Similar to previous findings,[6] when the 2 samples were not in agreement, it was more common for the NPS to be negative when the lower airway sample was positive than vice versa. Another study evaluated the bacterial pathogens isolated from guarded NPS and BAL by endoscopy in 59 feedlot cattle with signs of acute BRD and 60 matched control cattle.[8] Using data from both cases and controls, these investigators found that kappa ranged from 0.47–0.61 for *M. haemolytica, P. multocida,* and *M. bovis*. They concluded that NPS accurately represented the results of lower airway culture at the group level but not at the level of the individual animal. In our study, the proportion of samples found to be positive for *M. haemolytica, P. multocida, Mycoplasma* sp., or *Mycoplasma bovis* by NPS was not significantly different from the proportion found to be positive for these bacterial pathogens by TTW.

The relative agreement among NS, NPS, or BAL and TTW was not the same for the 2 viral pathogens identified (BRSV and BCV). For BRSV, kappa and the percent positive agreement were highest for BAL. None of the sampling methods differed significantly from TTW with respect to the proportion of animals identified as positive for BRSV. However, given that approximately half as many nasal swabs were positive for BRSV as compared to TTW, if a larger number calves positive for BRSV had been identified, the difference between NS and TTW might have reached statistical significance. In contrast, for BCV, the NS and NPS both yielded significantly higher proportions of positive results compared with TTW. Thus, in dairy calves with acute BRD sampled by all 4 methods, it was more common to find BCV with NS or NPS than with TTW, and it was more common to find BRSV with TTW than NS.

Dairy calves may shed BCV in the absence of clinical signs of disease[18,19] and it may be that BCV shedding identified by NS or NPS in the calves in our study was unrelated to their pneumonia. Alternatively, it may be that BCV contributes to respiratory disease primarily through effects related to replication in the upper respiratory tract, without the necessity of replication in the lower respiratory tract.

The relative prevalence of the various pathogens isolated in dairy calves with acute BRD in this study was consistent with that of previous reports. By methods similar to those used in our study, another study evaluated the microbiologic results obtained from guarded NPS of 1023 dairy calves with acute BRD and found that 10% of swabs were positive for BCV, 20% were positive for BRSV, 65% were positive for *Mycoplasma* sp., 21% were positive for *M. haemolytica*, 36% were positive for *P. multocida*, and 2% were positive for *H. somni*.[20] No swabs were positive for BHV-1 or BVDV1 or 2 (Lehenbauer and Aly, unpublished data). As in our study, the calves sampled in the previous study were housed at a calf-rearing facility in California, but at a different facility than the site where calves described here were housed. Other groups also have shown that, of the common bacterial respiratory pathogens, *P. multocida* and *Mycoplasma* spp. are relatively more prevalent than *M. haemolytica* or *H. somni* in dairy calves with acute BRD,[21,22] and of the common viral pathogens, BRSV is relatively more prevalent than BHV-1 or BVDV.[22–24]

Footnotes

[a] Inforce-3, Zoetis Inc., Florham Park, NJ
[b] Ibex Pro with L6.2 transducer, E.I. Medical Imaging, Loveland, CO
[c] NC9935507 Butler Schein sterile Dacron Swabs, Fisher Scientific Company LLC, Hanover Park, IL
[d] 07-801-5704, Culture Swab-Kalayjian, Patterson Veterinary Supply Inc., Effingham, IL
[e] 014910, Jor-Vet Tracheal Wash Kit, MWI Veterinary Supply, Boise, ID
[f] BAL-240, Large Animal Broncho-Alveolar Lavage Catheter, MILA International Inc., Florence, KY
[g] VetMAXTM Gold BVDV Detection Kit
[h] UltraClean Microbial DNA isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA
[i] Stata 13, StataCorp, College Station, TX

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Conflict of Interest Declaration: The authors declare no conflict of interest.

Off-label Antimicrobial Declaration: The authors declare no off-label use of antimicrobials.

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