AJVR

AVMA

Agreement of antimicrobial susceptibility testing of *Pasteurella multocida* and *Mannheimia haemolytica* isolates from preweaned dairy calves with bovine respiratory disease

Brent C. Credille, DVM, PhD, DACVIM[1*]; Sarah F. Capik, DVM, PhD, DACVPM[2,3]; Allison Credille, BSPH[1]; Beate M. Crossley, DVM, MPVM, PhD[4]; Pat Blanchard, DVM, PhD, DACVP[4]; Amelia R. Woolums, DVM, MVSc, PhD, DACVIM, DACVIM[5]; Terry W. Lehenbauer, DVM, MPVM, PhD, DACVPM[6,7]

[1]Food Animal Health and Management Program, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA
[2]Texas A&M AgriLife Research, Texas A&M University System, Amarillo, TX
[3]Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX
[4]California Animal Health and Food Safety Laboratory System, University of California-Davis, Davis, CA
[5]Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS
[6]Veterinary Medicine Teaching and Research Center, School of Veterinary Medicine, University of California-Davis, Tulare, CA
[7]Department of Population Health and Reproduction, School of Veterinary Medicine, University of California-Davis, Davis, CA

*Corresponding author: Dr. Credille (bc24@uga.edu)

Received June 28, 2023
Accepted August 1, 2023

doi.org/10.2460/ajvr.23.06.0140

OBJECTIVE
Evaluate agreement among the antimicrobial susceptibility profiles of *Mannheimia haemolytica* or *Pasteurella multocida* obtained by transtracheal wash, nasal swab, nasopharyngeal swab, and bronchoalveolar lavage.

ANIMALS
100 Holstein and Holstein-cross bull calves with bovine respiratory disease.

METHODS
Calves > 30 days old with naturally occurring bovine respiratory disease were sampled sequentially by nasal swab, nasopharyngeal swab, transtracheal wash, and then bronchoalveolar lavage. Samples were cultured, and for each antimicrobial, the MIC of 50% and 90% of isolates was calculated, and isolates were categorized as susceptible or not. Categorical discrepancies were recorded. Percent positive agreement and kappa values were calculated between isolates for each of the sampling methods.

RESULTS
Antimicrobial susceptibility varied by pathogen and resistance to enrofloxacin, florfenicol, tilmicosin, and spectinomycin was detected. Minor discrepancies were seen in up to 29% of classifications, with enrofloxacin, penicillin, and florfenicol more frequently represented than other drugs. Very major and major discrepancies were seen when comparing florfenicol (1.9%) and tulathromycin (3.8 to 4.9%) across sampling methods. Some variability was seen in agreement for enrofloxacin for several comparisons (8.3 to 18.4%).

CLINICAL RELEVANCE
Susceptibility testing of isolates from 1 location of the respiratory tract can reliably represent susceptibility in other locations. Nevertheless, the potential for imperfect agreement between sampling methods does exist. The level of restraint available, the skill level of the person performing the sampling, the age and size of the animal, disease status, and treatment history all must be factored into which test is most appropriate for a given situation.

Keywords: antimicrobial resistance, pneumonia, nasal swab, nasopharyngeal swab, transtracheal wash, bronchoalveolar lavage

© 2023 THE AUTHORS. Published by the American Veterinary Medical Association as an Open Access article under Creative Commons CCBY-NC license.

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC

Bovine respiratory disease (BRD) is 1 of the most common causes of morbidity and mortality in preweaned dairy calves and is second only to neonatal calf diarrhea as the primary disease syndrome affecting this population.[1] Consequences of BRD during the preweaning phase include a decreased rate of gain, as well as delayed time to first service, delayed age at first calving, higher culling risk, and, potentially, lower future milk production in replacement heifers.[2–4] Recent economic analyses have shown that a case of BRD within the first 120 days of life cost producers between $252 and $282 per affected animal.[2,3]

While multiple factors play a role in the development of BRD, bacteria, particularly *Pasteurella multocida* and *Mannheimia haemolytica*, are often associated with the characteristic clinical signs and pathologic lesions of BRD.[5,6] Consequently, antimicrobials have historically been and remain a mainstay of most therapeutic interventions. Unfortunately, the identification of *P multocida* and *M haemolytica* isolates in cattle with BRD classified as extensively drug resistant is becoming more commonplace.[5,7–10] Therefore, monitoring the susceptibility of bacteria commonly isolated from the respiratory tract of calves with BRD may help veterinarians when designing antimicrobial treatment protocols for dairy operations.

Multiple sampling methods are used to identify the various bacterial respiratory pathogens associated with BRD, with the nasal swab (NS), deep nasopharyngeal swab (DNP), transtracheal wash (TTW), and bronchoalveolar lavage (BAL) being most common.[11] Each sampling method has advantages and disadvantages, but research shows that agreement among the different methods for the detection of bacterial pathogens in dairy calves with BRD is generally good.[11] Nevertheless, there are limited data available for comparing the agreement of antimicrobial susceptibilities (AS) among bacterial isolates collected by the 4 common sampling methods. A study[12] performed in auction-market derived beef calves with naturally occurring BRD found that, with some exceptions, the AS of *M haemolytica* isolated from DNP and tracheal aspirates varied little by sampling site and that samples from the upper airway could be used to facilitate antimicrobial treatment decisions. Further research[13] evaluating multiple swab methods in beef calves indicated that NS was comparable to DNP in terms of culture, PCR, and 16S sequencing for *M haemolytica*. Another study[14] involving weaned beef calves found that gamithromycin susceptibility can vary depending on whether the sample was collected from the upper (DNP) or lower respiratory tract (BAL).

Unfortunately, there are few published data available comparing the AS of bacterial isolates obtained from dairy calves using each of the 4 diagnostic tests. Therefore, the objective of this study was to evaluate the agreement among the results of AS testing from bacterial isolates obtained by TTW to those obtained by NS, DNP, or BAL in preweaned dairy calves with BRD, by comparing susceptibilities from *P multocida* and *M haemolytica* isolates cultured from calves positive to all 4 sampling methods.

Methods

Calf management and selection

This prospective cross-sectional study was approved by the IACUC at the University of California, Davis and performed on Holstein and Holstein-cross bull calves housed in a rearing facility near Tulare, CA. Calves arrived at the rearing facility between 2 and 24 hours of age and were managed as described previously.[11] Briefly, calves were fed 2 liters of a commercial colostrum replacer by bottle at arrival and then again 12 and 24 h later. Additionally, calves were given ad libitum access to water and a farm-mixed calf starter formulated to meet National Research Council requirements for growing calves. All calves were weaned from milk at 60 days of age. Calves were observed daily for signs consistent with BRD by 1 trained observer for the duration of the study using the Wisconsin Calf Respiratory Scoring Chart.[15] Calves that scored ≥ 5 on the Wisconsin Calf Respiratory Scoring Chart and had a rectal temperature ≥ 39.4 °C (103 °F) were then subjected to transthoracic ultrasound of their lungs to determine if areas of consolidation were present. If an area of at least 2 cm^2 of consolidation was present in any lung lobe, the calf was declared a clinical case and enrolled in the study. Calves previously diagnosed with BRD or treated with antimicrobials or anti-inflammatory medications for any reason were excluded from study participation. The average age of the calves enrolled was 49 days with a range of 31 to 74 days.

Once declared a case, calves were sampled sequentially by NS, DNP, TTW, and then BAL as described by Doyle et al.[11] Briefly, for collection of the NS, the calf's nares were wiped clean with a single-use paper towel and a 13 cm polyester nasal swab (Butler Schein Sterile Dacron Swabs; Fisher Scientific Company LLC) was inserted into the nostril to the full length of the swab, rubbed on the mucosa, and withdrawn. For collection of the DNP, the calf's nares were again wiped clean, and a 59-cm guarded polyester swab (Double Guarded Uterine Culture Swab; Jorgensen Laboratories Inc) was advanced to a point just rostral to the level of the medial canthus of the eye. The swab was then advanced approximately 4 cm further, rotated, and withdrawn into the sheath before removal. Both the NS and DNP were stored in Brucella broth with 10% glycerol. After collection of the NS and DNP, a TTW was obtained using a commercially available kit (Jor-Vet Tracheal Wash Kit; MWI Veterinary Supply). After placement of the needle and catheter, 30 mL of sterile isotonic saline was instilled and withdrawn. For BAL, a commercial tube (Large Animal Broncho-Alveolar Lavage Catheter; MILA International Inc) was advanced through the nose until wedged in a bronchus, and 100 mL of sterile isotonic saline was instilled and withdrawn. All samples were subjected to aerobic bacterial culture at the California Animal Health and Food Safety Laboratory, an American Association of Veterinary Laboratory Diagnosticians (AAVLD) accredited laboratory, in Tulare, CA, to determine the presence of *M haemolytica*, *P multocida*, and *Histophilus somni*.

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC

All colonies identified as *M haemolytica* were subjected to additional biochemical testing to confirm they were not *Bibersteinia trehalosi* or indole positive *Mannheimia* spp, as they can appear morphologically similar to *M haemolytica*.

All bacteria were maintained as frozen stabilates at −80 °C until shipped for antimicrobial susceptibility testing at the Oklahoma Animal Disease Diagnostic Laboratory, also an AAVLD-accredited laboratory. All susceptibility testing was performed according to standard laboratory methods based on Clinical and Laboratory Standards Institute (CLSI) recommendations. Briefly, the selected isolates were removed from the freezer, thawed at room temperature, streaked on blood agar, and grown overnight. A broth dilution was inoculated on a standard 96-well susceptibility plate using an automated inoculation system in a single replicate. All isolates grew adequately on all culture media following removal from the freezer, and all plates were read using a manual system following 18 to 24 hours of incubation. Because not all antimicrobials included on the susceptibility plate had validated susceptibility breakpoints for *M haemolytica* and *P multocida* at the time of study completion, only those for which CLSI-approved breakpoints for BRD caused by these pathogens were included in this study. The antimicrobials evaluated for *P multocida* were florfenicol, ceftiofur, enrofloxacin, tulathromycin, spectinomycin, and penicillin. The antimicrobials evaluated for *M haemolytica* were ceftiofur, enrofloxacin, danofloxacin, florfenicol, tulathromycin, tilmicosin, spectinomycin, and penicillin.

Statistical Analysis

For each antimicrobial agent, the MIC of 50 and 90% of isolates was calculated, and the results of testing obtained by the 4 different sampling methods were converted to qualitative categories (susceptible, intermediate, and resistant) per CLSI breakpoints. Discrepancies between sampling methods were recorded as minor (intermediate result obtained by only 1 of the methods compared), major (isolate classified as susceptible by reference method and interpreted as resistant by the comparator), and very major (isolate classified as resistant by reference method categorized as susceptible by the comparator) errors. Very major discrepancies are worse in this instance because they could lead to the use of an antimicrobial that is ineffective, resulting in an increased risk of treatment failure. To evaluate the agreement between AS obtained from TTW and AS obtained by other sampling methods, a McNemar's test was used to compare methods with respect to their marginal proportions of positive results. Then, agreement among diagnostic tests was evaluated

Table 1—Proportion of *Pasteurella multocida* isolates classified as susceptible, intermediate, or resistant to specific antimicrobials based on Clinical and Laboratory Standards Institute-approved breakpoints and MIC_{50} and MIC_{90} for each organism by sampling method.

Pasteurella multocida

Antimicrobial/method	Susceptible (%)	Intermediate (%)	Resistant (%)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Ceftiofur					
TTW (n = 39)	100	0	0	≤ 0.25	≤ 0.25
BAL (n = 39)	100	0	0	≤ 0.25	≤ 0.25
DNP (n = 38)	100	0	0	≤ 0.25	≤ 0.25
NS (n = 38)	100	0	0	≤ 0.25	≤ 0.25
Enrofloxacin					
TTW (n = 39)	15	18	67	> 2	> 2
BAL (n = 39)	18	13	69	> 2	> 2
DNP (n = 39)	16	11	73	> 2	> 2
NS (n = 39)	16	16	68	> 2	> 2
Florfenicol					
TTW (n = 39)	18	36	46	4	≥ 8
BAL (n = 39)	15	28	57	8	≥ 8
DNP (n = 38)	16	39	45	4	≥ 8
NS (n = 38)	16	21	63	4	≥ 8
Tulathromycin					
TTW (n = 26)	100	0	0	4	8
BAL (n = 26)	92	4	4	8	32
DNP (n = 24)	100	0	0	4	8
NS (n = 24)	96	0	4	4	8
Spectinomycin					
TTW (n = 29)	97	0	3	16	16
BAL (n = 39)	97	0	3	16	16
DNP (n = 28)	100	0	0	16	16
NS (n = 28)	100	0	0	16	16
Penicillin					
TTW (n = 39)	69	31	0	≤ 0.12	0.25
BAL (n = 39)	62	36	2	≤ 0.12	0.25
DNP (n = 38)	76	24	0	≤ 0.12	0.25
NS (n = 38)	61	39	0	≤ 0.12	0.25

BAL = Bronchoalveolar lavage. DNP = Deep nasopharyngeal swab. NS = Nasal swab. TTW = Transtracheal wash.

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC

by calculation of the kappa statistic and the percent positive agreement (PPA). PPA 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.[11] All comparisons were made using TTW as the reference method, as described previously.[11]

To evaluate pairwise comparisons between respiratory tract sampling methods (NS vs DNP, NS vs BAL, NS vs TTW, DNP vs BAL, DNP vs TTW, and BAL vs TTW), AS results for *M haemolytica* and *P multocida* were categorized as either susceptible or not susceptible (intermediate or resistant) using CLSI breakpoints. Two by 2 tables were created for the susceptibility results within each pathogen and drug combination. The kappa statistic was calculated to estimate the level of agreement beyond chance when susceptibility results from different sample types were compared within each pathogen and each drug. For each comparison, calves for which any of the samples were missing were excluded from that comparison. The probability that a certain kappa value would fall within 1 of the benchmark kappa categories[16] was calculated, and the first benchmark interval for which the cumulative probability exceeded the 95% threshold was reported. All kappa values were interpreted to indicate the strength of agreement as defined previously: < 0.20 = poor; 0.21 to 0.40 = fair; 0.41 to 0.6 = moderate; 0.61 to 0.8 = good; and 0.81 to 1.00 = very good. Analyses were performed with commercially available statistical software (Stata 15; StataCorp).

Results

Of the 100 calves initially enrolled in the study and sampled as described previously,[11] only 13 were positive in all 4 samples for *M haemolytica* and 43 were

Table 2—Proportion of *Mannheimia haemolytica* isolates classified as susceptible, intermediate, or resistant to specific antimicrobials based on Clinical and Laboratory Standards Institute approved breakpoints and MIC_{50} and MIC_{90} for each organism by sampling method.

Mannheimia haemolytica

Antimicrobial/method	Susceptible (%)	Intermediate (%)	Resistant (%)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Ceftiofur					
TTW (n = 12)	100	0	0	≤ 0.25	≤ 0.25
BAL (n = 12)	100	0	0	≤ 0.25	≤ 0.25
DNP (n = 12)	100	0	0	≤ 0.25	≤ 0.25
NS (n = 12)	100	0	0	≤ 0.25	≤ 0.25
Enrofloxacin					
TTW (n = 12)	91	9	0	0.12	1
BAL (n = 12)	91	9	0	0.12	1
DNP (n = 12)	91	9	0	0.12	1
NS (n = 10)	91	9	0	0.12	1
Danofloxacin					
TTW (n = 9)	100	0	0	0.12	0.12
BAL (n = 9)	100	0	0	0.12	0.12
DNP (n = 9)	100	0	0	0.12	0.12
NS (n = 7)	100	0	0	0.12	0.12
Florfenicol					
TTW (n = 12)	100	0	0	1	≥ 8
BAL (n = 12)	92	8	0	1	4
DNP (n = 12)	92	0	8	1	≥ 8
NS (n = 10)	90	0	10	1	≥ 8
Tulathromycin					
TTW (n = 10)	90	0	10	4	≥ 64
BAL (n = 10)	90	0	10	4	≥ 64
DNP (n = 10)	90	0	10	4	≥ 64
NS (n = 9)	88	0	12	2	≥ 64
Tilmicosin					
TTW (n = 10)	70	0	30	4	≥ 64
BAL (n = 10)	70	0	30	4	≥ 64
DNP (n = 8)	70	0	30	4	≥ 64
NS (n = 10)	66	0	34	4	≥ 64
Spectinomycin					
TTW (n = 10)	20	10	70	≥ 64	≥ 64
BAL (n = 10)	20	20	60	≥ 64	≥ 64
DNP (n = 10)	20	10	70	≥ 64	≥ 64
NS (n = 8)	50	0	50	≥ 64	≥ 64
Penicillin					
TTW (n = 12)	100	0	0	≤ 0.12	0.25
BAL (n = 12)	92	8	0	≤ 0.12	0.25
DNP (n = 12)	100	0	0	≤ 0.12	≤ 0.12
NS (n = 10)	90	10	0	≤ 0.12	0.25

See Table 1 for key.

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC

positive in all 4 samples for *P multocida* Of those calves, only 1 was positive in all 4 sample locations for both bacteria, and *H somni* was not isolated from any calf.

A summary of AS as determined by broth microdilution is presented **(Tables 1 and 2)**. For *P multocida*, isolates were routinely susceptible to ceftiofur, spectinomycin, and tulathromycin. In contrast, susceptibility to penicillin was inconsistent (24 to 39% intermediate across sampling methods), and isolates were routinely resistant to enrofloxacin and florfenicol. For *M haemolytica*, isolates were routinely susceptible to penicillin, ceftiofur, enrofloxacin, danofloxacin, florfenicol, and tulathromycin. In contrast, the susceptibility of *M haemolytica* to tilmicosin was inconsistent (≥ 30% resistant across sampling methods), and isolates were routinely resistant to spectinomycin (≥ 50% resistant).

Categorical discrepancies between sampling methods are presented **(Table 3)**. For *P multocida*, there were no discrepancies detected for ceftiofur, while minor discrepancies were seen in up to 18.4% of classifications for enrofloxacin, 29.3% of classifications for florfenicol, 23.7% of classifications for penicillin, and 3.8% of classifications for tulathromycin. Additionally, major discrepancies were seen when comparing florfenicol and tulathromycin susceptibilities across sampling methods. For *M haemolytica*, there were no discrepancies detected for ceftiofur, tilmicosin, or tulathromycin, while minor errors were seen in up to 10% of classifications for enrofloxacin, 8.3% of classifications for florfenicol and penicillin, and 10% of classifications for spectinomycin. Additionally, both very major and major discrepancies were seen when comparing spectinomycin across sampling methods.

The PPA, kappa statistic, and McNemar's test results for the AS obtained from NS, DNP, and BAL relative to TTW were calculated for each pathogen and are presented **(Tables 4 and 5)**. Overall, agreement among AS results obtained by NS, BAL,

Table 3—Proportion of very major (defined as an isolate classified as resistant by reference method categorized as susceptible by the comparator), major (defined as an isolate classified as susceptible by reference method and interpreted as resistant by the comparator), and minor (defined as an intermediate result obtained by only 1 of the methods compared) antimicrobial susceptibility testing errors by sampling method for *Pasteurella multocida* and *Mannheimia haemolytica* isolates collected from dairy calves with acute respiratory disease.

	Pasteurella multocida			***Mannheimia haemolytica***		
	Error Type			**Error Type**		
Antimicrobial/method	**Very Major (%)**	**Major (%)**	**Minor (%)**	**Very Major (%)**	**Major (%)**	**Minor (%)**
Ceftiofur						
BAL	0	0	0	0	0	0
DNP	0	0	0	0	0	0
NS	0	0	0	0	0	0
Enrofloxacin						
BAL	0	0	15.3	0	0	8.3
DNP	0	0	13.2	0	0	8.3
NS	0	0	18.4	0	0	10
Danofloxacin						
BAL	N/A	N/A	N/A	0	0	0
DNP	N/A	N/A	N/A	0	0	0
NS	N/A	N/A	N/A	0	0	0
Florfenicol						
BAL	0	2.6	18.2	0	0	8.3
DNP	1.9	0	18.4	0	0	0
NS	1.9	1.9	29.3	0	0	0
Penicillin						
BAL	0	2.6	20.5	0	0	0
DNP	0	0	23.7	0	0	8.3
NS	0	0	18.4	0	0	0
Spectinomycin						
BAL	0	0	0	10	20	10
DNP	0	0	0	10	10	10
NS	0	0	0	20	10	10
Tilmicosin						
BAL	N/A	N/A	N/A	0	0	0
DNP	N/A	N/A	N/A	0	0	0
NS	N/A	N/A	N/A	0	0	0
Tulathromycin						
BAL	0	3.8	3.8	0	0	0
DNP	0	0	0	0	0	0
NS	0	4.2	0	0	0	0

Transtracheal wash was used as the reference method when determining error type.
N/A = Not available.
See Table 1 for remainder of key.

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC

Table 4—Agreement among TTW and each of NS, DNP, and BAL for antimicrobial susceptibility testing of *Pasteurella multocida* isolated from preweaned Holstein calves with respiratory disease.

	No. of Calves with Each Combination of Results											
Antimicrobial/method	S/S	S/I	S/R	I/S	I/I	I/R	R/S	R/I	R/R	PPA (%)	Kappa	*P**
Ceftiofur												
NS	38	0	0	0	0	0	0	0	0	100	1.00	1.00
BAL	39	0	0	0	0	0	0	0	0	100	1.00	1.00
DNP	38	0	0	0	0	0	0	0	0	100	1.00	1.00
Enrofloxacin												
NS	5	0	0	1	3	3	0	3	23	90.8	0.729	1.00
BAL	6	0	0	1	3	3	0	2	24	92.1	0.785	1.00
DNP	5	0	0	1	3	3	0	1	25	93.4	0.801	.625
Florfenicol												
NS	5	0	1	0	6	8	1	2	15	81.2	0.523	.109
BAL	6	0	1	0	7	7	0	4	14	83.3	0.574	.549
DNP	5	1	0	0	11	3	1	3	14	88.2	0.690	1.00
Penicillin												
NS	21	5	0	2	10	0	0	0	0	76.9	0.601	.453
BAL	21	5	1	3	9	0	0	0	0	75.9	0.467	.726
DNP	23	3	0	6	6	0	0	0	0	79.9	0.414	.507
Tulathromycin												
NS	23	0	1	0	0	0	0	0	0	95.8	0.850	1.00
BAL	24	1	1	0	0	0	0	0	0	94.2	0.820	1.00
DNP	24	0	0	0	0	0	0	0	0	100	1.00	1.00
Spectinomycin												
NS	28	0	0	0	0	0	0	0	0	100	1.00	1.00
BAL	28	0	0	0	0	0	0	0	1	100	1.00	1.00
DNP	28	0	0	0	0	0	0	0	0	100	1.00	1.00

PPA = Percent positive agreement.
**P* value for exact test of marginal homogeneity.
See Table 1 for reminder of key.

Table 5—Agreement among TTW and each of NS, DNP, and BAL for antimicrobial susceptibility testing of *Mannheimia haemolytica* isolated from preweaned Holstein calves with respiratory disease.

	No. of Calves with Each Combination of Results											
Antimicrobial/method	S/S	S/I	S/R	I/S	I/I	I/R	R/S	R/I	R/R	PPA (%)	Kappa	*P**
Ceftiofur												
NS	12	0	0	0	0	0	0	0	0	100	1.00	1.00
BAL	12	0	0	0	0	0	0	0	0	100	1.00	1.00
DNP	12	0	0	0	0	0	0	0	0	100	1.00	1.00
Enrofloxacin												
NS	8	0	0	1	1	0	0	0	0	95.0	0.615	1.00
BAL	10	0	0	1	1	0	0	0	0	95.8	0.625	1.00
DNP	10	0	0	1	1	0	0	0	0	95.8	0.625	1.00
Florfenicol												
NS	9	0	0	0	0	0	0	0	1	100	1.00	1.00
BAL	11	0	0	0	0	0	0	1	0	95.8	0.647	1.00
DNP	11	0	0	0	0	0	0	0	1	100	1.00	1.00
Penicillin												
NS	9	0	0	0	1	0	0	0	0	100	1.00	1.00
BAL	11	0	0	0	1	0	0	0	0	100	1.00	1.00
DNP	11	0	0	1	0	0	0	0	0	95.8	1.00	1.00
Tulathromycin												
NS	7	0	0	0	0	0	0	0	1	100	1.00	1.00
BAL	9	0	0	0	0	0	0	0	1	100	1.00	1.00
DNP	9	0	0	0	0	0	0	0	1	100	1.00	1.00
Spectinomycin												
NS	1	0	1	1	0	0	2	0	3	56.3	0.125	1.00
BAL	1	0	1	0	1	0	1	1	5	75.0	0.359	1.00
DNP	1	0	1	0	0	1	1	1	5	70.0	0.189	1.00
Danofloxacin												
NS	7	0	0	0	0	0	0	0	0	100	1.00	1.00
BAL	9	0	0	0	0	0	0	0	0	100	1.00	1.00
DNP	9	0	0	0	0	0	0	0	0	100	1.00	1.00
Tilmicosin												
NS	7	0	0	0	0	0	0	0	3	100	1.00	1.00
BAL	7	0	0	0	0	0	0	0	3	100	1.00	1.00
DNP	6	0	0	0	0	0	0	0	2	100	1.00	1.00

I/I = Intermediate/intermediate. I/R = Intermediate/resistant. I/S = Intermediate/susceptible. R/I = Resistant/intermediate. R/R = Resistant/resistant. R/S = Resistant/susceptible. S/I; Susceptible/intermediate. S/R = Susceptible/resistant. S/S = Susceptible/susceptible.
See Tables 1 and 3 for key.

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC

and DNP relative to the TTW was fair to excellent, depending on the antimicrobial-pathogen combination being evaluated. In the case of *P multocida*, the PPA and kappa value were lowest for both florfenicol and penicillin, and these values were associated with a relatively high proportion of minor discrepancies in the results of AS. Similar results were seen with the result of AS for enrofloxacin. In the case of *M haemolytica*, PPA and kappa were lowest for spectinomycin and enrofloxacin. In the case of spectinomycin, these misclassifications were associated with a high proportion of both minor and very major discrepancies.

For the evaluation of pairwise comparisons between respiratory tract sampling methods (NS vs DNP, NS vs BAL, NS vs TTW, DNP vs BAL, DNP vs TTW, and BAL vs TTW), In the case of *P multocida*, complete susceptibility data were available for 40 animals that were culture positive for that organism. Overall, there was good to very good agreement across most of the drugs when comparing the results of AS across sampling methods for *P multocida* **(Supplementary Table S1)**. Complete susceptibility data for all 4 samples and all 8 drugs was only obtained from 13 animals that were culture positive for *M haemolytica*. Within those *M haemolytica* positive animals, only 3 had samples that yielded identical susceptibility interpretations across all 4 samples for all drugs considered. There was overall good agreement across most of the drugs when comparing the results of AS across sampling methods for both pathogens. However, spectinomycin had fair to poor agreement among the sampling locations **(Supplementary Table S2)**. Given the relatively small number of paired samples that had culturable *M haemolytica* and *P multocida* and the limited variability in results, kappa values were not able to be calculated for ceftiofur for either organism or danofloxacin for *M haemolytica* (there are no CLSI-approved breakpoints for danofloxacin for *P multocida*). In many instances, all samples agreed in terms of susceptible or nonsusceptible within a single organism and a single drug in which case kappa values were equal to 1 with no variability.

Discussion

There is considerable debate among researchers and clinicians regarding the optimal sample for BRD diagnostics. In the end, the best sampling method will depend on several factors. The level of restraint available, the skill level of the person performing the sampling, the age and size of the animal, the disease status and treatment history of the animal, the type of diagnostics being performed on the sample, and the intended use of the results (screening, diagnosis, protocol development, treatment decisions, etc) all impact the choice of sampling method.[14] No 1 sampling method will be perfect in all situations and sufficient to answer all questions. In this study, TTW was chosen as the reference method because it provides a sample from the lower airway and bypasses potential contaminants in the nasopharynx.[11] Other antemortem sampling methods have been reviewed elsewhere in greater detail.[14]

Previously published research using the same samples evaluated the agreement of NS, DNP, and BAL with TTW via culture at the organismal level and found relatively high (though imperfect) agreement beyond chance for both *M haemolytica* and *P multocida*.[11] While knowing whether basic sampling methods agree in terms of the pathogens found is helpful for interpretation and planning diagnostics, it is also important in some instances to understand whether organisms in different samples might have different AS. For example, if a pathogen in the lungs is resistant to a clinician's drug of choice, but an upper respiratory sample is obtained that is either culture negative or susceptible, there may be a disconnect in the expected treatment outcome versus the actual outcome after treatment. Therefore, further analysis of a subset of that dataset was performed in this study to specifically explore AS agreement of the sampling methods within 2 major BRD-related bacterial species.

Although *M haemolytica* can be found throughout the nasal mucosa of clinically healthy cattle,[17] the utility of upper respiratory tract samples to indicate what is in the lungs remains controversial, and agreement between sampling methods as well as between bilateral samples taken from the same calves at the same time can vary when culture is used.[14,18–22] In this study, out of 100 calves sampled via all 4 methods, a relatively small number had consistent culture results across all 4 methods for *M haemolytica* (13%) or *P multocida* (43%) and only 1 calf (1%) was positive for both organisms in all 4 samples. This inconsistency in culture results between samples is frustrating yet not completely surprising, as previous work[20] has noted that paired samples taken from the left and right nasopharyngeal regions at the same time could yield different culture results. Often, it is tempting to conclude that a negative culture result means the organism is not present, but that assumption can prove dangerous, as repeating upper respiratory tract cultures on the same side 15 minutes apart has shown that *M haemolytica*– and *P multocida*–negative calves can become culture positive and vice versa.[20] In this study, NS, DNP, and BAL samples were collected sequentially after TTW. It is possible that this sampling scheme could have resulted in cross-contamination and altered the results of our study. Overall, there are many reasons for potentially inconsistent culture results,[14,23] and additional research regarding the optimal sampling location and the impact of cross-contamination with the use of different sampling methods for evaluation of organismal AS is needed.

Although in many comparisons we found there was complete observed agreement within a single organism and a single drug, this level of observed agreement may not be repeatable in other populations of animals including those with different feeding and housing practices, as well as those who have received previous antimicrobial therapy, other classes of animals in different production systems, or animals at other stages of production. We observed the potential for inconsistent AS results

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC

and imperfect agreement between sampling methods for both organisms. Additionally, the potential for inconsistency in antimicrobial susceptibility profiles of *M haemolytica*[13] and *P multocida* obtained from different sampling methods has been observed previously (personal communication, SFC). Different genetic subtypes of *M haemolytica*[24] have been found within nasopharyngeal samples, and gamithromycin susceptibility has been found to vary even within isolates of the same subtype.[14] Therefore, it is becoming clear that, even within a given bacterial species in a single animal, there can be genetic and phenotypic variation that has the potential to influence clinical outcome or decision-making. The ability of a bacterial species to show diversity in terms of phenotype and genotype adds an additional layer of complexity to the already complex BRD situation and may explain part of the disconnect sometimes seen in clinical outcomes when a diagnostic test indicated the organism should have been susceptible.

While the results of this study are informative, several limitations exist. It would have been ideal to compare treatment success or failure rates with the AS data on these calves; those data were not available in this study. Despite sampling 100 calves in the original study[11] from which this dataset was obtained, the number of calves with culturable *M haemolytica* or *P multocida* in multiple samples was relatively few, a factor that limited our ability to statistically compare AS within antimicrobials. This small sample size also limits our ability to draw conclusions related to the actual prevalence of resistance in *M haemolytica* in this population. While kappa statistics can be useful, they also have limitations, especially when based on small samples sizes, and can vary based on underlying prevalence,[25] which makes it difficult to compare kappa values between populations. This gives rise to another limitation of this dataset; in that we chose to focus on preweaned dairy calves with clinical disease. The same level of agreement may not be repeatable in different types of animals or production settings where the prevalence of the bacteria or the prevalence of AMR varies. Additionally, just because the "arbitrary" descriptive scale outlined by Landis and Koch[16] indicates a certain level of agreement beyond chance is "substantial" does not mean that it is truly good enough in all diagnostic settings. There are instances where an "almost perfect" agreement of 0.81[16] would indeed be insufficient for a given patient and more certainty would be warranted. Additionally, the limitations of culture must be kept in mind.[14] Recent research has focused on exploring the microbial ecology of the respiratory tract with advanced sequencing methodologies in an attempt to better understand the impact of the entire microbiome on the presence of pathogens of interest.[26] Others have explored different respiratory swab types via culture, PCR, and 16S sequencing for detection of *M haemolytica* and found that nasal swabs were comparable to deep nasopharyngeal swabs taken on the same side in feedlot animals 14 days postarrival but not perfectly concordant.[13] Additional research in this area is needed in different subpopulations of cattle to help further assist clinicians and diagnosticians choose and interpret diagnostic tests.

Overall, this study reflects the complexity of the respiratory tract microbiome,[27] the dynamic nature of the microbial ecology of the bovine respiratory tract, and our incomplete understanding of the pathogenesis of BRD.

Acknowledgments

The authors thank Danielle Doyle for sampling calves and the staff of the California Animal Health and Food Safety Laboratory at Tulare, CA, for the original isolation of the *Mannheimia haemolytica* and *Pasteurella multocida* isolates.

Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

Funding

The authors have nothing to disclose.

References

1. National Animal Health Monitoring System (NAHMS). In: US Animal and Plant Health Inspection Service, ed. *Dairy 2007 Part III: Reference of Dairy Cattle Health and Management Practices in the United States*; 2007.
2. Overton MW. Economics of respiratory disease in dairy replacement heifers. *Anim Health Res Rev.* 2020;21(2):143–148. doi:10.1017/S1466252320000250
3. Dubrovsky SA, Van Eenennaam AL, Aly SS, et al. Preweaning cost of bovine respiratory disease (BRD) and cost-benefit of implementation of preventative measures in calves on California dairies: the BRD 10K study. *J Dairy Sci.* 2020;103(2):1583–1597. doi:10.3168/jds.2018-15501
4. Abuelo A, Cullens F, Brester JL. Effect of preweaning disease on the reproductive performance and first-lactation milk production of heifers in a large dairy herd. *J Dairy Sci.* 2021;104(6):7008–7017. doi:10.3168/jds.2020-19791
5. Holschbach CL, Aulik N, Poulsen K, Ollivett TL. Prevalence and temporal trends in antimicrobial resistance of bovine respiratory disease pathogen isolates submitted to the Wisconsin Veterinary Diagnostic Laboratory: 2008–2017. *J Dairy Sci.* 2020;103(10):9464–9472. doi:10.3168/jds.2019-17570
6. Deepak, Aly SS, Love WJ, et al. Etiology and risk factors for bovine respiratory disease in pre-weaned calves on California dairies and calf ranches. *Prev Vet Med.* 2021;197:105506. doi:10.1016/j.prevetmed.2021.105506
7. Sweeney MT, Lubbers BV, Schwarz S, Watts JL. Applying definitions for multidrug resistance, extensive drug resistance and pandrug resistance to clinically significant livestock and companion animal bacterial pathogens. *J Antimicro Chemother.* 2018;73(6):1460–1463. doi:10.1093/jac/dky043
8. Lubbers BV, Hanzlicek GA. Antimicrobial multidrug resistance and coresistance patterns of Mannheimia haemolytica isolated from bovine respiratory disease cases–a three-year (2009–2011) retrospective analysis. *J Vet Diagn Invest.* 2013;25(2):413–417. doi:10.1177/1040638713485227
9. Snyder E, Credille B, Berghaus R, Giguère S. Prevalence of multi drug antimicrobial resistance in isolated from high-risk stocker cattle at arrival and two weeks

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC

after processing. *J Anim Sci.* 2017;95(3):1124–1131. doi:10.2527/jas2016.1110
10. Crosby S, Credille B, Giguère S, Berghaus R. Comparative efficacy of enrofloxacin to that of tulathromycin for the control of bovine respiratory disease and prevalence of antimicrobial resistance in Mannheimia haemolytica in calves at high risk of developing bovine respiratory disease. *J Anim Sci.* 2018;96(4):1259–1267. doi:10.1093/jas/sky054
11. Doyle D, Credille B, Lehenbauer TW, et al. Agreement among 4 sampling methods to identify respiratory pathogens in dairy calves with acute bovine respiratory disease. *J Vet Intern Med.* 2017;31(3):954–959. doi:10.1111/jvim.14683
12. Hirsch C, Timsit E, Uddin MS, Guan LL, Alexander TW. Comparison of pathogenic bacteria in the upper and lower respiratory tracts of cattle either directly transported to a feedlot or co-mingled at auction markets prior to feedlot placement. *Front Vet Sci.* 2022;9:1026470. doi:10.3389/fvets.2022.1026470
13. Crosby WB, Pinnell LJ, Richeson JT, et al. Does swab type matter? Comparing methods for Mannheimia haemolytica recovery and upper respiratory microbiome characterization in feedlot cattle. *Anim Microbiome.* 2022;4(1):49. doi:10.1186/s42523-022-00197-6
14. Capik SF, White BJ, Lubbers BV, et al. Comparison of the diagnostic performance of bacterial culture of nasopharyngeal swab and bronchoalveolar lavage fluid samples obtained from calves with bovine respiratory disease. *Am J Vet Res.* 2017;78(3):350–358. doi:10.2460/ajvr.78.3.350
15. McGuirk SM, Peek SF. Timely diagnosis of dairy calf respiratory disease using a standardized scoring system. *Anim Health Res Rev.* 2014;15(2):145–147. doi:10.1017/S1466252314000267
16. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics.* 1977;33(1):159–174. doi:10.2307/2529310
17. Pass DA, Thomson RG. Wide distribution of *Pasteurella haemolytica* type 1 over the nasal mucosa of cattle. *Can J Comp Med.* 1971;35(3):181–186. [4254890]
18. Allen JW, Viel L, Bateman KG, Rosendal S. Changes in the bacterial flora of the upper and lower respiratory tracts and bronchoalveolar lavage differential cell counts in feedlot calves treated for respiratory disease. *Can J Vet Res.* 1992;56(3):177–183.
19. Godinho KS, Sarasola P, Renoult E, et al. Use of deep nasopharyngeal swabs as a predictive diagnostic method for natural respiratory infections in calves. *Vet Rec.* 2007;160(1):22–25. doi:10.1136/vr.160.1.22
20. Magwood SE, Barnum DA, Thomson RG. Nasal bacterial flora of calves in healthy and in pneumonia-prone herds. *Can J Comp Med.* 1969;33(4):237–243.
21. DeRosa DC, Mechor GD, Staats JJ, Chengappa MM, Shryock TR. Comparison of *Pasteurella* spp. simultaneously isolated from nasal and transtracheal swabs from cattle with clinical signs of bovine respiratory disease. *J Clin Microbiol.* 2000;38(1):327–332. doi:10.1128/JCM.38.1.327-332.2000
22. Capik SF, Richeson J, Lubbers BV, Loy JD, Cernicchiaro N, Beyene TJ. Between-location heterogeneity of bilateral nasopharyngeal swabs and bronchoalveolar lavages based on M ALDI-TOF identification of bovine respiratory disease pathogens. In: *Bovine Respiratory Disease Symposium 2019, Denver, CO*. BRDS; 2019:122.
23. Capik SF, White BJ, Lubbers BV, et al. Characterization of *Mannheimia haemolytica* in beef calves via nasopharyngeal culture and pulsed-field gel electrophoresis. *J Vet Diagn Invest.* 2015;27(5):568–575. doi:10.1177/1040638715597724
24. Clawson ML, Murray RW, Sweeney MT, et al. Genomic signatures of *Mannheimia haemolytica* that associate with the lungs of cattle with respiratory disease, an integrative conjugative element, and antibiotic resistance genes. *BMC genomics* 2016;17(1):982. doi:10.1186/s12864-016-3316-8
25. Cook RJ. Kappa and its dependence on marginal rates. In: *Encyclopedia of Biostatistics.* John Wiley & Sons, Ltd; 2005.
26. Chai J, Capik SF, Kegley B, Richeson JT, Powell JG, Zhao J. Bovine respiratory microbiota of feedlot cattle and its association with disease. *Vet Res.* 2022;53(1):4. doi:10.1186/s13567-021-01020-x
27. Credille B. High-risk cattle management and stocker calf health: modulation of the bovine respiratory microbiome from a systems perspective. *Vet Clin North Am Food Anim Pract.* 2022;38(2):229–243. doi:10.1016/j.cvfa.2022.03.001

Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org

Unauthenticated | Downloaded 07/21/24 10:28 PM UTC