Antimicrobial activity *in vitro* against *Mannheimia haemolytica*, *Pasteurella multocida*, and/or *Histophilus somni* from cattle with naturally occurring bovine respiratory disease and its association to clinical outcome

Running title: What results of AST for three bacterial pathogens associated with BRD mean to clinical outcome

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Antimicrobial activity *in vitro* against *Mannheimia haemolytica*, *Pasteurella multocida*, and/or *Histophilus somni* from cattle with naturally occurring bovine respiratory disease and its association to clinical outcome

Abstract

Antimicrobial resistance is frequently blamed for clinical failures of treatment or control of bovine respiratory disease (BRD). *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* are generally recognized as the four main bacterial pathogens associated with BRD. This study used data from randomized, controlled, clinical trials that recorded qualitative classification of clinical outcomes (success, or failure) and of results of antimicrobial susceptibility testing (AST; classified as resistant, or not resistant to florfenicol, gamithromycin, tildipirosin, or tulathromycin). Association of results *in vitro* to clinical outcome for treatment or control of naturally occurring BRD (1 319 calves) was quantitatively evaluated. Clinical outcome was not significantly (P = 0.4643) associated (Fisher's exact test) with qualitative results of AST *in vitro* for pathogens that were not exposed to antimicrobial medication *in vivo* (971 head of cattle). Clinical outcome was significantly (P < 0.0001) associated with qualitative results of AST *in vitro* for pathogens that were exposed to antimicrobial medication *in vivo* (348 head of cattle). For pathogens not exposed to antimicrobial medication *in vivo*, 1.85 % (95 % confidence interval [CI] = 0.38 to 5.32) of clinical failures were attributable to antimicrobial resistance. For pathogens exposed to antimicrobial medication *in vivo*, 51.72 % (95 % CI = 32.53 to 70.55) of clinical failures were attributable to antimicrobial resistance. In conclusion, antimicrobial resistance of bacterial pathogens associated with

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BRD and not exposed to antimicrobial medication *in vivo* is a quantitatively minor cause of clinical failure for treatment or control of naturally occurring BRD.

***Keywords*:** Bovine respiratory disease; clinical outcome; antimicrobial resistance; quantitative assessment; evidence-based medicine; antimicrobial stewardship.

Study contribution

Quantitative results from this study are evidence (evidence-based medicine) that antimicrobial resistance is less of a predictor of clinical failure than antimicrobial susceptibility is a predictor of clinical success. The quantitative contribution of antimicrobial resistance to clinical failure is relatively small and is different when bovine respiratory disease involves pathogens exposed to antimicrobial medication *in vivo*. Quantitative measurements of the clinical contribution of antimicrobial resistance support antimicrobial stewardship, informed decisions by attending veterinarians, Managers of feedlots, and/or owners of cattle regarding the selection, purchase, and/or use of antimicrobials for treatment or control of bovine respiratory disease in feedlot cattle.

Introduction

Bovine respiratory disease (BRD) is a multifactorial and complex disease, resulting in fibrinous pneumonia and/or bronchopneumonia , and has been extensively reviewed.[1–6]. The proportional contribution of each factor is not known. Management practices are beneficial but losses associated with BRD continue.[2, 7, 8] *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* are generally recognized as the four main bacterial pathogens associated with BRD.[4, 5] Antimicrobial

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resistance is frequently blamed for clinical failures of treatment or control of BRD.[9] To explain clinical failures for cattle with BRD, attempts are made to isolate bacterial pathogens associated with BRD, and determine their antimicrobial activity *in vitro*, (antimicrobial susceptibility tests, AST).[10, 11]

Antimicrobial activity *in vitro* is considered an important guide for selecting the most appropriate medication for administration.[12–15] and predicting clinical efficacy; however, the relationship between assessment *in vitro* and treatment outcome *in vivo* is "imperfect".[15–18] McClary et al.[16] reported that the proportion of clinical failures attributable to recovery of *M. haemolytica* resistant to tilmicosin (population attributable fraction) was 0.2 %. Sarchet, et al.[17] evaluated the association between clinical outcome and AST results with tulathromycin against two pathogens (*M. haemolytica*, and *P. multocida*). These studies did not calculate the accuracy of the test, did not include the influence of the prevalence of clinical failure by chance alone on quantitative values for outcome variables positive predictive value or negative predictive value, and did not separate pathogens not exposed to antimicrobials *in vivo* from those exposed to antimicrobials *in vivo*.

Veterinary Diagnostic Laboratories (VDL) perform AST according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[19] or the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[20] Clinical breakpoints (susceptible, intermediate, or resistant) are ordinal, qualitative, interpretive criteria used to correlated AST to clinical outcome.[15] These are established by members of the CLSI or EUCAST after reviewing AST results determined by broth dilution and agar gel diffusion methods against wild-type bacterial isolates.[19, 20] Wild-type pathogens are defined as the

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subpopulation of bacteria that do not express acquired resistance.[21, 22] Non-wild type pathogens are defined as the bacterial subpopulation that express acquired resistance after exposure to antimicrobial agents. Larger proportions of non-wild type bacteria exhibit resistance to one or more antimicrobials.[21–25] Clinical breakpoints are specific to the bacterial pathogen, the bodily system from which they were isolated (or the disease), and the antimicrobial agent when used according to approved labeling.[19] Standard procedures *in vitro* for the assessment of *Mycoplasma* species associated with BRD have not been developed.

Agreement between AST results and clinical efficacy has been suggested to be at least 80 % for cattle with mild to moderate BRD and resistant pathogens are expected to substantially reduce clinical efficacy.[15] Data were not published to support that expectation. Approximately 21.3 % of cattle entering feedlots were medicated to control BRD; 13.4 % of cattle placed in those feedlots were treated because of BRD, and 81.7 % of those responded to treatment.[26] Data for tulathromycin, tildipirosin, gamithromycin, or florfenicol, regarding substantial evidence of efficacy for treatment or control of BRD, obtained from Freedom of Information summaries, also support that a similar proportion (ranged from 76 % to 89.4 %) of clinical success was observed.[27–30] Results of AST were not included in those reports.

Results of quantitative assessment of test accuracy can be used to answer the critically needed question about the influence of antimicrobial resistance on clinical outcome and to develop realistic expectations for the clinical application of AST results.[31] Minimum requirements for the quantitative assessment of the association of results of AST with clinical outcome are: 1) reliable, standardized *in vitro* procedures (known as the

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index test), and 2) a reference standard comparator which is the clinical response to medication *in vivo*.[31] Accuracy of the test is a quantitative assessment (cardinal data) of the ability of the index test to properly classify patients by the probability of clinical outcome.[31]

The purpose of this retrospective study was to 1) analyze the association between of results of AST with clinical outcome for treatment or control of naturally occurring BRD, and 2) quantify the accuracy of results of AST for florfenicol, gamithromycin, tulathromycin, or tildipirosin ("accuracy of the test") against three bacterial pathogens (*M. haemolytica, P. multocida*, and/or *H. somni*) associated with BRD.

Materials and methods

Ethical statement

The ethical statement is not required for reasons stated in the following paragraph.

Design and Sources of data

This is a retrospective study of the relationship between qualitative results of AST *in vitro* and qualitative clinical outcomes for treatment or control of naturally occurring BRD. Data for this study were from published studies, and from unpublished studies that are proprietary property of Merck Animal Health. Experimental units (individual animals) in those studies were representative of beef cattle that enter commercial feedlots in North America. Among those source studies, the breeds, sex, castration status, age, size, and origin of the cattle may have differed, but within a study, those factors were consistent. *A priori* for each respective study and independent of results of AST, protocols established criteria for inclusion or exclusion of cattle from the study, the establishment

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of a diagnosis of clinical BRD (case definition), the observation period or duration of the study, for samples (deep nasopharyngeal or bronchoalveolar) to be obtained, the techniques used for sampling, handling, and submission of samples, the administration of medication, and the classification of clinical response. Samples were obtained before antimicrobial medication was administered per protocol and were submitted to VDL for the isolation of bacterial pathogens (*M. haemolytica*, *P. multocida*, and/or *H. somni*) and for AST. These VDL followed guidelines by the CLSI and were not aware of the experimental treatment or the clinical outcome of the animals.

Within a given source, the same case definition, variation, and incidence of misdiagnosis were applied randomly and impartially to all experimental units. Clinical success was declared when a calf survived to the end of the study after only one initial administration of antimicrobial medication because of BRD. In other words, no other antimicrobial medication was administered during the study. A clinical failure was declared when antimicrobial medication was subsequently administered, according to protocol, after the initial experimental treatment. In other words, an antimicrobial was administered more than once during the study. These definitions are consistent with those of pivotal studies leading to regulatory approval of the respective antimicrobial products. At intervals stated in the respective protocols, animals enrolled in those studies were observed by trained personnel who were blinded to the experimental treatment administered and the results of AST.

Data were included in this study if bacterial isolation and results of AST *in vitro* were traceable to clinical outcomes for the individual animal. Duplicated data and those not traceable were excluded. Cattle were only counted once, even if more than one isolate

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(any combination of *M. haemolytica*, *P. multocida*, and/or *H. somni*) was obtained from that individual. Results of AST for pathogens isolated from an individual animal were combined. If multiple isolates (any combination of *M. haemolytica*, *P. multocida*, and/or *H. somni*) were obtained from an individual animal, and any of those isolates was resistant to the respective experimental treatment, the clinical response for that animal was attributed (100 %) to that resistant organism, as if it was the only contributing pathogen. If all isolates were not resistant, results of AST were recorded as "not resistant."

Cattle in two sources[32, 33] were not previously medicated, and data from those sources were provided to the CLSI for establishment of clinical breakpoints for the respective antimicrobial products against wild type pathogens (*M. haemolytica*, *P. multocida*, and *H. somni*). One source[34] was a dissertation describing research submitted as part of the requirements for an academic graduate degree. Two sources[35, 36] were proprietary data from clinical field trials conducted in commercial feedlots in Canada, utilizing cattle obtained through auction markets, with unknown histories of previous antimicrobial administration. These data provided a unique opportunity to study the longitudinal effects of resistance to tulathromycin.

Organization of the data

To avoid confusion with definitions used by CLSI, and for purposes of this study, pathogens associated with BRD and isolated from cattle with no known administration of antimicrobial medication were classified as "not exposed to antimicrobial medication *in vivo*" (NEVO). Pathogens associated with BRD and isolated from cattle that had been medicated with antimicrobials were classified as “exposed to antimicrobial medication

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in vivo" (EVO). An *a priori* assumption was that pathogens NEVO would have different patterns of results of AST than would pathogens EVO, and that any phenotypically expressed, qualitative resistance was due to exposure to antimicrobial medication *in vivo*.[21–25] Therefore, data for pathogens NEVO were presented and analyzed separately from data for pathogens EVO. It should be emphasized that pathogens, not cattle, were classified as NEVO or EVO.

Not exposed to antimicrobial medication in vivo

In this study, at least one of the three bacterial species associated with BRD was isolated from 971 head of cattle with unknown or no previous exposure to antimicrobial medication, and these pathogens were classified as NEVO. Antimicrobial medications used in sources with NEVO pathogens included gamithromycin, tildipirosin, or tulathromycin. Each medication was administered at the site, route, and dose per the respective labeled instructions.

Exposed to antimicrobial medication in vivo

At least one of the three bacterial species associated with BRD was isolated from 348 head of cattle that had been medicated with antimicrobials, resulted in *in vivo* exposure of those bacteria to antimicrobial medication. These pathogens were classified as EVO. Antimicrobial medication used in sources with pathogens EVO included florfenicol, gamithromycin, tildipirosin, or tulathromycin. Each medication was administered at the site, route, and dose per respective labeled instructions.

Data for the pathogens NEVO were combined for quantitative evaluation of the accuracy of results of AST and are presented separately from combined data for

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pathogens EVO. Data were summarized for 1 319 animals and were apportioned as described by Bossuyt[37] for quantitative assessment of the accuracy (specificity, sensitivity, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio, and accuracy) of results of AST of four medications *in vitro* against three bacterial pathogens associated with BRD (**Figure 1**, **Tables 1** & **2**).[32–38]

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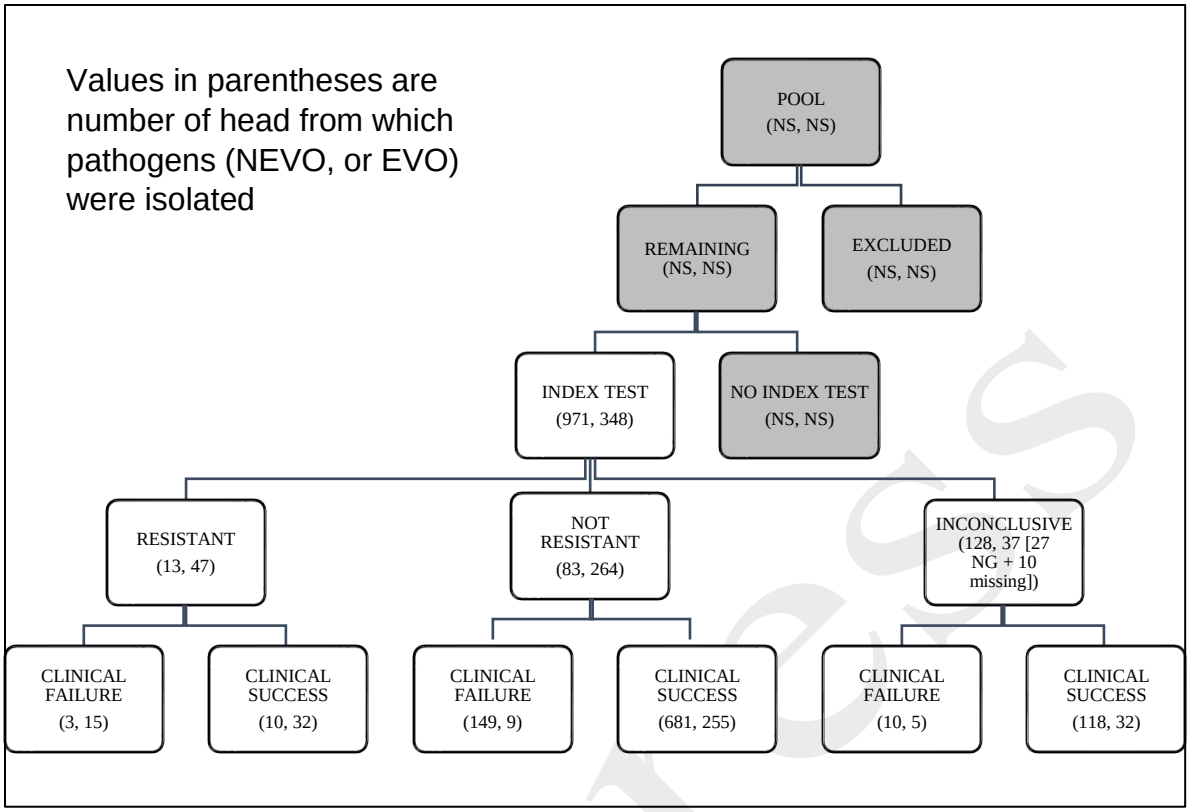

Figure 1. Flow chart derived from Bossuyt et al.[37] of numbers of animals for six studies (4 treatment, and 2 control) of naturally occurring BRD from which isolates of *M. haemolytica*, *P. multocida*, and/or *H. somni* NEVO related to *in vitro* AST of gamithromycin, tildipirosin, or tulathromycin[32, 33, 36]; and for three studies of the treatment of naturally occurring BRD from which isolates of *M. haemolytica*, *P. multocida*, and/or *H. somni* EVO related to *in vitro* AST of gamithromycin, or florfenicol[35, 36]. Only those animals for which clinical outcome was traced to results of the index AST were included (indicated by squares with no fill). Not stated = NS; Not resistant = Susceptible plus Intermediate results; Inconclusive = results of no growth (NG) plus missing or lost reports of AST.

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Table 1. Data recorded in 2 × 2 table and calculations[31, 38] used to quantitatively assess the accuracy of antimicrobial activity *in vitro* against three bacterial pathogens (*M. haemolytica*, *P. multocida*, *H. somni*) associated with naturally occurring BRD

Result of AST (See **Table 3)**	Clinical Outcome		Total
	Failure	Success	
Resistant (≥ XX µg/mL)	a	c	a + c
Not Resistant (< XX µg/mL)	b	d	b + d
Total	a + b	c + d	a + b + c + d

Prevalence (Prev) = [(a + b)/(a + b + c + d)]; probability of a clinical failure by chance alone; also called the pre-test probability of clinical failure; proportion of isolates from calves with clinical failure in the entire population of calves at the time of sampling (pre-treatment).

***Positive Predictive value (PPV)** = {(Se × Prev) / [Se × Prev] + [(1 − Sp) × (1 – Prev)]}; probability of clinical failure when isolate was R; also called the post-test probability of clinical failure; proportion of all isolates from calves with clinical failure that were R.

***Negative Predictive value (NPV)** = {[Sp × (1 − Prev)] / [(1 − Sp) × Prev] + [Sp × (1 − Prev)]}; probability of clinical success when isolate was NR; proportion of isolates NR that resulted in clinical success.

Sensitivity (Se) = [a/(a + b)] True positive; probability of isolate that was R from clinical failure; proportion of clinical failures with isolates that were R; proportion of clinical failures attributable to R.

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Specificity (Sp) = [d／(c + d)] True negative; probability of isolate that was NR from clinical success; proportion of clinical successes with isolates that were NR; proportion of clinical successes attributable to NR.

PPV − Prev = Change in predictability of clinical failures by knowing that an isolate was R *in vitro*.

Positive likelihood ratio (PLR) = [Se／(1 − Sp)]; Ratio of the probability of an isolate that was R was associated with clinical failure (Se), related to the probability of an isolate that was R was associated with clinical success (1 − Sp). (R = Failure : R = Success)

Negative likelihood ratio (NLR) = [(1 − Se)／Sp]; Ratio of the probability of an isolate that was NR was associated with clinical failure (1 − Se), related to the probability of an isolate that was NR was associated with clinical success (Sp). (NR = Failure : NR = Success)

***Accuracy** = {(Se × Prev) + [Sp × (1 − Prev)]}／100 = probability that a patient was correctly classified.

*** PPV, NPV,** and **Accuracy** are dependent on **Prevalence** of clinical failure (see formulae).

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Table 2. Summary of data for isolates of *M. haemolytica*, *P. multocida*, and/or *H. somni* from 1 319 head of cattle. Not exposed *in vivo* = NEVO; Exposed *in vivo* = EVO

NEVO								
			Respective interpretive criteria†					
Source (reference)	Med*	Head	Resistant		Not Resistant		Inconclusive	
			Clinical response					
			Failure	Success	Failure	Success	Failure	Success
32	gam	127	3	0	47	77	NR	NR
33	tildip	524	0	6	74	444	NR	NR
34	gam	17	0	2	2	9	0	4
35	tulath	303	0	2	26	151	10	114
Total		971	3	10	149	681	10	118
EVO								
34	gam	8	2	5	0	1	0	0
35	florfen	299	0	5	7	252	5	30 (10 lost)
36	florfen	41	13	22	2	2	0	2
Total		348	15	32	9	255	5	32

*Med = medication; gam = gamithromycin; tildip = tildipirosin; tulath = tulathromycin; florfen = florfenicol

† Not resistant = (susceptible plus intermediate)

Index Test and Reference Standard

For this study, the index test was provided by CLSI standards for performing AST *in vitro*.[19] Interpretive criteria of qualitative results for the index test (AST) against three bacterial pathogens (*M. haemolytica*, *P. multocida*, *H. somni*) were clinical breakpoints established by the CLSI (**Table 3)** for the respective antimicrobial (florfenicol, gamithromycin, tulathromycin, or tildipirosin) that was administered in the respective source studies. For this study, those interpretive criteria were condensed into two ordinal categories (resistant; not resistant = susceptible + intermediate). In Van Donkersgoed and Berg,[35, 36] results reported as “no growth” indicated that no bacterial pathogens

associated with BRD were identified. Reports for some AST were lost. Lost reports and results of “no growth” were collectively defined as inconclusive results.

Table 3. Minimum inhibitory concentrations (MIC) and interpretive criteria (susceptible; intermediate; resistant) against *M. haemolytica*, *P. multocida*, or *H. somni* for the antimicrobial medications that were the subject of this retrospective study.[19]

	Interpretive criteria (MIC, µg/mL)		
Antimicrobial	Susceptible	Intermediate	Resistant
florfenicol	≤ 2	4	≥ 8
gamithromycin	≤ 4	8	≥ 16
tildipirosin	≤ 8	16	≥ 32
tulathromycin	≤ 16	32	64

The binomial ordinal classification of clinical outcome (clinical failure or clinical success) for control or treatment of cattle with naturally occurring BRD provided a finite clinical endpoint and was the reference standard. Using the two binomial ordinal classifications of clinical outcome and the result of AST, a 2 × 2 contingency table (**Table 2**) was constructed. Data for pathogens NEVO were separated from data for pathogens EVO. This helped to minimize the potential exaggeration of subtle effects when small numbers of cattle comprised the studies used as sources.

A 2 × 2 contingency table with resistant vs. not resistant and another table with resistant vs. (not resistant + inconclusive) were generated to compare association of results of AST with clinical outcome (**Table 4).** This study focused on comparisons of

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resistant vs. (not resistant + inconclusive) but both sets of analyses are presented to demonstrate the importance of including inconclusive results.

Analyses

Association of clinical outcome with results of AST was analyzed using Fisher's exact test (**Table 4**).[38] Quantitative assessment of the accuracy of the index test was performed using the formulae, definitions, and abbreviations presented in **Table 1**.[38]

Results

There was no significant association (Fisher's exact test, P = 0.4643, **Table 4**) between clinical outcome and AST results for pathogens NEVO. A significant association (Fisher's exact test, P < 0.0001, **Table 4**) between clinical outcome and AST results was found for pathogens EVO.

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Table 4. Contingency tables (2 × 2) for NEVO or EVO pathogens associated with BRD. For each subpopulation of pathogen, the association of clinical outcome (clinical failure or clinical success) with results of AST (Resistant or Not Resistant) as well as Resistant or (Not Resistant + Inconclusive) was evaluated.[38] Not exposed *in vivo* = NEVO; Exposed *in vivo* = EVO.

Clinical Outcome	NEVO			EVO	
	Resistant	Not Resistant		Resistant	Not Resistant
Failure	3	149		15	9
Success	10	681		32	255
Fisher's exact test (*P*-value)	0.7136			< 0.0001	
Clinical Outcome	NEVO			EVO	
	Resistant	(Not Resistant + Inconclusive)		Resistant	(Not Resistant + Inconclusive)
Failure	3	159		15	14
Success	10	799		32	287
Fisher's exact test (*P*-value)	0.4643			< 0.0001	

Data and results expose the importance of inconclusive results of AST. Although different summary values were generated (**Table 5**), the interpretations were the same. In the subpopulation of cattle from which pathogens NEVO were isolated, the probability of clinical failure by chance alone (prevalence, Prev) was 16.68 % (95 % CI = 14.39 to 19.18) with 1.85 % (95 % CI = 0.38 to 5.32) of those clinical failures attributed to resistance of those pathogens NEVO to the antimicrobial agent used in the respective source (**Table 5).** The probability that a resistant pathogen NEVO was isolated from an animal classified as a clinical failure (positive predictive value, PPV) was 23.08 % (95 % CI = 7.70 to 51.88). The ability to predict a clinical failure increased 6.4 % by knowing that

an associated pathogen NEVO was resistant *in vitro* (PPV − Prev). The probability that a non-resistant pathogen NEVO was isolated from an animal classified as a clinical success (negative predictive value, NPV) was 83.40 % (95 % CI = 83.09 to 83.71), with 98.76 % (95 % CI = 97.74 to 99.41) of the clinical successes associated with a non-resistant pathogen NEVO + inconclusive results (specificity, Sp). An animal, with a pathogen NEVO that was resistant, was 1.50 (95 % CI = 0.42 to 5.38) times more likely to experience clinical failure than clinical success (positive likelihood ratio, PLR). An animal with a non-resistant NEVO pathogen was 0.99 (95 % CI = 0.97 to 1.02) times more likely to experience clinical failure than clinical success (negative likelihood ratio, NLR).

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Table 5. Summary of quantitative statistics of accuracy of AST for *M. haemolytica*, *P. multocida*, and/or *H. somni* associated with BRD. Data presented contrast Resistant v Not resistant as well as Resistant v (Not resistant + Inconclusive). Not exposed *in vivo* = NEVO; Exposed *in vivo* = EVO

	NEVO				EVO			
	Resistant v Not Resistant		Resistant v (Not Resistant + Inconclusive)		Resistant v Not Resistant		Resistant v (Not Resistant + Inconclusive)	
Variable	Point Estimate	95 % CI	Point Estimate	95 % CI	Point Estimate	95 % CI	Point Estimate	95 % CI
Prev (%)	18.03	15.49 to 20.80	16.68	14.39 to 19.18	7.72	5.01 to 11.26	8.33	5.65 to 11.75
Se (%)	1.97	0.41 to 5.66	1.85	0.38 to 5.32	62.50	40.59 to 81.20	51.72	32.53 to 70.55
Sp (%)	98.55	97.35 to 99.30	98.76	97.74 to 99.41	88.85	84.63 to 92.25	89.97	86.13 to 93.04
PLR	1.36	0.38 to 4.90	1.50	0.42 to 5.38	5.61	3.57 to 8.79	5.16	3.19 to 8.34
NLR	0.99	0.97 to 1.02	0.99	0.97 to 1.02	0.42	0.25 to 0.71	0.54	0.37 to 0.78
PPV (%)	23.08	7.71 to 51.86	23.08	7.70 to 51.88	31.91	23.01 to 42.37	31.91	22.46 to 43.13
NPV (%)	82.05	81.69 to 82.40	83.40	83.09 to 83.71	96.59	94.41 to 97.94	95.35	93.35 to 96.77
PPV – Prev (%)	5.05	---	6.4	---	24.19	---	23.58	---
Accuracy (%)	81.14	78.33 to 83.73	82.60	80.06 to 84.93	86.82	82.54 to 90.37	86.78	82.76 to 90.16

CI = Confidence Interval; Prev = Prevalence of clinical failure: Se = Sensitivity; Sp = Specificity; PLR = Positive Likelihood Ratio; NLR = Negative Likelihood Ratio; PPV = Positive Predictive Value; NPV = Negative Predictive Value.

In the subpopulation of cattle from which pathogens EVO were isolated, the probability of clinical failure by chance alone (Prev) was 8.33 % (95 % CI = 5.65 to 11.75). The sensitivity (Se) of the test was 51.72 % (95 % CI = 32.53 to 70.55) of those clinical failures attributed to resistance of EVO pathogens to the antimicrobial agent used in the respective source. The probability that a resistant pathogen EVO was isolated from an animal with a clinical failure (PPV) was 31.91 % (95 % CI = 24.46 to 43.13). The ability to predict a clinical failure increased 23.58 % by knowing that a pathogen EVO was resistant *in vitro* (PPV−Prev). The probability that a non-resistant pathogen EVO was isolated from an animal classified as a clinical success (NPV) was 95.35 % (95 % CI = 93.35 to 96.77), with 89.97 % (95 % CI = 86.13 to 93.04) of the clinical successes associated with non-resistant (Sp) pathogens EVO. An animal with a pathogen EVO that was resistant, was 5.16 (95 % CI = 3.19 to 8.34) times more likely to be classified as a clinical failure than a clinical success (PLR). An animal with a pathogen EVO that was not resistant was 0.54 (95 % CI = 0.37 to 0.78) times more likely to be classified as a clinical failure than a clinical success (NLR).

Regardless of whether the pathogens were NEVO or EVO, quantitative assessment of the test's accuracy indicated that clinical response of 80 % to 90 % of the animals was correctly classified by results of AST *in vitro*. Values for NPV, and for Sp indicated that the value for accuracy was influenced more by (not resistant + inconclusive) results to predict clinical success, than by the ability of resistance to predict clinical failure (PPV and Se). As indicated by the value and associated 95 % confidence intervals for PPV, resistance was far less predictive of clinical failure than a coin flip (50:50 or 50 %).

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The prevalence of clinical failure by chance alone (Prev, also called the pre-test probability of clinical failure) was approximately twice as high for cattle that had not been medicated previously compared to cattle that had been medicated. Because Prev influences quantitative values for PPV, NPV, and accuracy, in this study, those values were calculated with adjustments for Prev in each subpopulation (see formulas **Table 1**).[31, 38−41]

The difference between PPV (also called the post-test probability of clinical failure) and Prev (PPV–Prev) represented the change in the ability to predict clinical failures by knowing that an isolate was resistant, beyond the probability of a clinical failure by chance alone.[31] For NEVO pathogens, that value was 5 % to 6 %. For EVO pathogens it was approximately 24 % (**Table 5**). That quantitative value provides information to change or confirm opinions about performing the test and helps remove biases that impair judgement regarding clinical expectations for treating an animal infected with a pathogen that is resistant *in vitro*.

Discussion

To the best of the coauthors' knowledge, this is the first study to thoroughly evaluate the quantitative association between results of AST and clinical outcomes for cattle medicated to control or treat naturally occurring BRD. Likewise, the coauthors are not aware of references for an acceptable or adequate quantitative threshold for the accuracy of qualitative results of AST applicable to bacterial pathogens associated with BRD.

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Medical diagnostic procedures should add value to daily management strategies[42] and should be evaluated quantitatively to assess their accuracy using simple calculations.[31, 38] Veterinary medical literature is replete with articles about antimicrobial resistance and bacterial pathogens associated with BRD but there is a paucity of investigations that evaluate the association of AST results *in vitro* with clinical outcome for cattle with BRD.[16, 18]

Sources of data

All sources of data used in this study provided most of the information described by Bossyut[37] (**Figure 1).** Clinical response and the predictive value of the results of the AST for the clinical outcome, were free of bias associated with knowing the results of AST. Separating data representing pathogens NEVO from data representing pathogens EVO helped to minimize potential exaggeration of subtle effects when small numbers of cattle were studied.

The names used to categorize the two subpopulations of pathogens (EVO or NEVO) were inconsequential. Any name, number, or letter could have been used for qualitative, ordinal, binomial classification and would not have changed the data, calculations, results, discussion, or interpretation of this study.

Sampling technique and subpopulation of cattle

The Clinical and Laboratory Standards Institute does not stipulate sampling procedures for BRD. Regardless of the sampling technique used, pathogens obtained from cattle that had not been medicated are used for the development of qualitative clinical

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breakpoints.[19] These clinical breakpoints are not validated for non-wild-type pathogens or for pathogens EVO.

In routine clinical practice, samples from the respiratory tracts of cattle submitted to VDL for bacterial isolation and AST *in vitro* are usually from animals classified as clinical failures that had been treated multiple times and/or with multiple antimicrobials, were unresponsive to treatment, and/or were moribund or dead.[9, 43] By submitting samples only from moribund or dead animals, and omitting samples from clinically healthy or recovered cohorts, the attending veterinarian limits their ability to interpret and apply results of AST. Moribund or dead cattle do not represent the general population of cattle and are not the same as the subpopulation of cattle used by CLSI to establish interpretive criteria with wild-type pathogens. Pathogens isolated from such samples should be considered non-wild-type pathogens, as described by CLSI.

Variability inherent in sampling procedures, probability of isolating pathogens, and handling and shipping of samples should be minimized and randomized among all animals being evaluated.

Pathogens isolated

In an individual animal with BRD, the proportional contribution of multiple bacterial isolates is not known.[6, 44–46] Likewise, the proportional contributions by organisms with phenotypic, qualitative antimicrobial resistance are not known. For this study, 100 % of the clinical response was intentionally attributed to any organism isolated from that animal that was resistant to the antimicrobial medication used in the respective source. This was done for two reasons: first, due to abundant concerns about resistance factors that are

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readily transferred among bacteria,[5, 47–49] and second, because it is not possible to quantify the relative contribution of individual bacterial pathogens or of antimicrobial resistance of those pathogens. The coauthors recognized that procedure may have overweighted the influence of resistant pathogens.

Index test – Antimicrobial Susceptibility Testing

Guidelines from CLSI[19] apply to any laboratory; however, comparing results among laboratories and surveys is challenging.[24] In this study, the same laboratory was used for all samples within a given source, removing the laboratory as a source of variation. Any modifications to CLSI standards published after the studies were conducted must not be applied to the data presented here. Applying updated interpretive criteria would inappropriately distort the results and render them invalid.

Interpretive criteria apply to results from CLSI-described procedures *in vitro* and samples obtained from specific bodily systems and species of patient regardless of sampling technique.[19] To assess the accuracy of AST adequately and appropriately, it is obligatory that pathogens be obtained from animals classified as clinical successes as well as those classified as clinical failures.[31] Incomplete data (samples only from clinical failures) are biased and invalid for use in the quantitative assessment described in this study. Results of AST for pathogens isolated from samples obtained during necropsy or only pathogens EVO isolated from cattle that were previously medicated, were clinical failures, and died or were euthanized, do not provide sufficient information to select medication against subpopulations of pathogens NEVO in cattle that will be medicated in the future. Evidence does not support that rationale.

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Anholt et al.,[43] stated that susceptible strains of targeted bacterial pathogens obtained from animals or samples typically submitted to VDL would have been removed, resulting in higher proportions of resistant isolates. If that conclusion was correct, there would be no value to sample any animal that had been medicated with antimicrobials because isolates obtained from those animals should all be resistant. Data from sources used in this study do not support that conclusion.

Anholt et al.[43] reported that 66% of *H. somni* and all (100 %) *M. haemolytica*, *P. multocida*, *Trueperella pyogenes*, and *Mycoplasma bovis* (note: CLSI clinical breakpoints do not exist for *Mycoplasma bovis*) were resistant to at least one of the antimicrobials evaluated. They also stated that 90.2 % of all isolates were resistant to at least one of the macrolides evaluated. Those data show that a relatively substantial portion of antimicrobial treatments failed in animals with resistant pathogens, but they do not confirm that treatment failed because of resistant pathogens.

Among laboratories, definitions differ for “no growth” when results of AST are recorded. For some laboratories, no growth means no bacteria grew from the sample (sample was sterile), while for others it means no bacterial pathogens associated with BRD were identified from the bacterial growth. Some people intentionally disregard results of no growth mistakenly rationalizing that decision by thinking such information is non-contributory and further justify that irrational assessment as “only logical”.[12, 13] For epidemiologic purposes, and as used in this study, results of AST that were reported as no growth or results that were lost, were classified as inconclusive results. Inconclusive results of AST are not defined by CLSI, and yet they are reported by trained laboratory personnel following the same critical guidelines. Inconclusive results of AST are as

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valuable as other interpretive criteria and are crucial to thoroughly quantitatively evaluate the accuracy of the index test.[31, 37] In this study, inconclusive results of AST were evaluated (when included) to demonstrate the void created by not recording these critical data. Omission of inconclusive results of AST propagates misleading interpretations.

A more pertinent topic for discussion might be whether it is appropriate to continue applying clinical breakpoints established with wild-type pathogens to pathogens EVO. In the future, other methods may better describe results of AST *in vitro* as the index test for both subpopulations of pathogens,[22, 50–52] but epidemiologic data about clinical outcome as the reference standard must be incorporated for the accuracy of the test to be quantitatively evaluated.

Unfortunately, results reported from VDL are insufficient to accurately calculate quantitative values needed to develop medication protocols and expectations for response to them. For those quantitative calculations, standardized procedures *in vitro* as described by CLSI or EUCAST are an absolute requirement, and inconclusive results should be included.

Calculations and interpretations of results

After appropriately organizing the qualitative data regarding clinical outcome (success or failure) and results of AST (resistant or not-resistant), quantitative values for Se, Sp, Prev, and accuracy of the test were simple mathematic operations.[31, 38–40] Prevalence (by chance alone) of the condition of interest does not necessarily cause differences in Se and Sp; however, Se and Sp were significantly associated with prevalence in about 1 in 3 studies of human conditions. [39, 41]

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The intrinsic accuracy of a diagnostic test with binomial results does not depend on prevalence and is expressed by values for Se and Sp.[40, 41] However, Se and Sp do not provide information about the diagnostic accuracy and predictability for a particular patient or subpopulation. For that, PPV and NPV are needed; however, PPV and NPV are functions of accuracy of the test and the prevalence of the condition (clinical failure by chance alone). As clearly seen in the respective formulae (see **Table 1),** precise values for PPV, NPV, and accuracy are dependent on Prev.[31, 39–41] Precise as well as accurate measures or estimates of prevalence are important to access accuracy of the diagnostic test.[41]

Values for specificity (Sp) with pathogens NEVO or pathogens EVO were comparable to the value of 80 % clinical success predicted by non-resistant isolates in other studies.[15, 17] In this study, values for PPV and Se indicated that antimicrobial resistance was a better predictor of clinical failures when pathogens EVO were isolated rather than pathogens NEVO. A very low proportion of clinical failures was attributable to resistance of pathogens NEVO. These findings suggest that non-resistant pathogens associated with BRD may be far more relevant to clinical decisions than are findings of pathogens that are resistant.

The quantitative value of PPV−Prev provides information to change or confirm opinions about performing the index test; and helps remove biases that impair judgement regarding clinical expectations for treating an animal infected with a pathogen that is resistant *in vitro*. Knowing that a pathogen NEVO was resistant increased the predictability of clinical failure very little compared to knowing the probability of a clinical failure by chance alone (Prev). This contrasts with the statement that “bacterial resistance

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translates as a substantial reduction in clinical efficacy".[15] In this study, the quantitative increase in predictability was less than that of flipping a coin (50 % probability). Anyone wanting to quantify the role of phenotypic antimicrobial resistance as a contributing factor to clinical failure should include Prev in those calculations, because Prev is not constant (**Table 5**).[32–36]

Application

In clinical practice, clinical outcome is always retrospectively related to results of AST because cattle are responding to medication or may have been medicated with another antimicrobial(s) before results of AST are received by the attending veterinarian. Data needed to evaluate the quantitative accuracy of a test (see **Table 1**) can be recorded by trained personnel at many feedlots. Mathematic operations and calculations with those data are simple, and statistical evaluation is available from public domain websites. Utilizing and applying these calculations for making decisions represents a change in thinking. The simplicity of this approach is overshadowed by the complexity of BRD and the managerial dogma that has surrounded it for decades.

It is important for attending veterinarians, researchers, and diagnostic laboratory technicians to recognize that pathogens isolated after patients have been medicated will likely yield different results of AST.[21–25] Critical to clinical patient care are data regarding realistic expectations for response to treatment of patients that have different subpopulations of pathogens. There is no scientific logic to assume that genotypic or phenotypic antimicrobial resistance alone can definitively determine clinical expectations. Without epidemiologic data regarding clinical outcome of patients from which pathogens

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were isolated, the contributions of *in vitro* results ends when laboratory personnel record them.

Results of AST are intended to help the attending veterinarian select medication that will increase the likelihood of clinical success and decrease the likelihood of clinical failure.[12, 13, 15] However, “selection” is not the subject or objective of studies about efficacy or about accuracy of the test. Selection imposes human bias into experimental design, which is unacceptable in randomized, controlled studies evaluating clinical efficacy, establishing clinical breakpoints, or calculating accuracy of the test.

In this study, the accuracy of AST and knowing that any of three (3) bacterial pathogens associated with BRD was resistant, contributed little to change the probability of clinical failures above that of chance alone. Different results may be expected if mono-pathogenic bacterial diseases were the condition of interest.[53] Results of this study suggest that the probability of clinical success would be approximately 82 % for cattle infected with pathogens that are susceptible; and approximately 77 % for cattle infected with pathogens that are resistant to the medication administered. If proportions of clinical success outside of this range occur, antimicrobial resistance may be a contributing factor, but other factors must be investigated. [1–6]

Since the 1950’s antimicrobial resistance has influenced decisions about administration of antimicrobials.[54, 55] This question remains: *How will antimicrobial resistance of pathogens associated with BRD impact future management of BRD?*[56]

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Conclusions

Antimicrobial resistance of bacterial pathogens associated with BRD and NEVO is a quantitatively minor cause of clinical failure for control or treatment of BRD. Current clinical breakpoints for pathogens associated with BRD are more useful for predicting clinical success with pathogens NEVO or EVO, and of clinical failure with pathogens EVO. Attending veterinarians can use the approach presented in this study to quantitatively evaluate the accuracy of AST for their respective clients and to optimize the diagnostic service medically and financially.

Quantitative components of accuracy of the test provide better information to couple qualitative results of AST *in vitro* with clinical outcome *in vivo.* They are more prudent than relying solely on AST to predict clinical outcomes. Inconclusive results of AST must not be disregarded as “logically non-contributory” or “irrelevant”. Submitting only samples from clinically ill or deceased patients and using those results of AST to tailor health protocols, ignores other factors that contribute to clinical success or failure. Those data alone are not adequate to determine the quantitative influence of phenotypic resistance of pathogens on clinical outcome. Future efforts should shift from inordinate focusing on antimicrobial resistance to addressing other major causes of clinical failure for cattle with BRD.

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Data availability

All relevant data are within the manuscript.

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Conflict of interest

The coauthors of this study are a current employee (LKB), a retired employee (JB), two (2) independent, contracted, non-employee investigators (JVD, GWB) of Merck Animal Health, USA.

Authors' contributions

For this study, and according to the CRediT taxonomy, the respective coauthor's contributions are as follows:
Conceptualization: LK Bryant, J Van Donkersgoed, J Berg, GW Brumbaugh
Data curation: LK Bryant, J Van Donkersgoed, J Berg, GW Brumbaugh
Formal analysis: LK Bryant, GW Brumbaugh
Funding acquisition: LK Bryant
Investigation: LK Bryant, J Van Donkersgoed, J Berg, GW Brumbaugh
Methodology: LK Bryant, J Van Donkersgoed, J Berg, GW Brumbaugh
Project administration: LK Bryant
Resources: LK Bryant
Supervision: LK Bryant
Validation: LK Bryant, J Van Donkersgoed, J Berg, GW Brumbaugh
Writing: LK Bryant, J Van Donkersgoed, J Berg, GW Brumbaugh

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