AUSTRALIAN VETERINARY JOURNAL
AUSTRALIA'S PREMIER VETERINARY SCIENCE TEXT
PRODUCTION ANIMALS

ORIGINAL ARTICLE

Efficiency-corrected PCR quantification for identification of prevalence and load of respiratory disease-causing agents in feedlot cattle

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Bovine respiratory disease (BRD) is the most prevalent disease in feedlot cattle worldwide with Bovine alphaherpesvirus 1 (BoAHV1), *Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pasteurella multocida* and *Trueperella pyogenes* accepted to be common etiological agents associated with BRD. Although these agents are common in the upper and lower airways in clinical BRD cases, some also exist as normal flora suggesting their presence in the upper airways alone is not necessarily informative with respect to disease status or risk. To determine the relationship between presence, load and disease status, we investigated the relationship between load in the upper airways at induction and active BRD cases in feedlot cattle using efficiency-corrected PCR quantification. By this approach, we were able to accurately determine the prevalence and load of the key BRD agents in the upper respiratory tract showing that cattle in the hospital pen had a higher prevalence, and load, of these agents both singly and in combination compared to cattle sampled at feedlot induction. A combination of agents was the most accurate indicator of BRD risk with cattle with four or more agents detected in the upper airway more likely to be undergoing treatment for BRD than non-BRD ailments. In addition, *M. bovis* was rarely detected at feedlot induction but was identified at high prevalence in cattle in the hospital pen. These findings present a potential new technological approach for the investigation, analysis and identification of BRD-associated viral and bacterial agents for Australian feedlot systems as well as for BRD disease management and treatment.

Keywords bovine respiratory disease; BRD; efficiency-corrected quantification; *Mycoplasma bovis*; polymerase chain reaction; qPCR

Aust Vet J 2022;100:539–549 doi: 10.1111/avj.13200

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Despite significant research, bovine respiratory disease (BRD) still represents one of the greatest production costs to cattle production globally. BRD is a multifactorial syndrome with a number of bacterial and viral agents that can lead to disease.[1] It can cause severe, and sometimes fatal, respiratory disease in cattle in both intensive and pasture systems[2] with BRD being the most prevalent disease in veal calves, weaned dairy heifers and weaned/unweaned beef calves. It is also the leading cause of clinical disease and death in feedlot populations.[3–5] In Australian feedlot systems, BRD can account for more than 80% of morbidity and up to 78% of mortalities annually in a single feedlot operation.[6] Globally the economic impact of BRD is estimated to be over $3 billion/year.[7]

The incidence and prevalence of BRD in cattle can be influenced by many factors. These include pre-exposure to viral or bacterial organisms[8]; vaccination history[9]; backgrounding[10]; co-mingling of non-consanguineous cohorts[11]; time on feed[12]; antimicrobial resistance[13]; seroconversion[12]; transportation time to the feedlot[14] and the presence of other co-morbidities and other undetermined factors.[15] Environmental risk factors such as the time of year of entry to the feedlot (also known as feedlot induction), the region, prevailing weather conditions,[16] source of the cattle, cattle breed and induction weight have all been positively correlated with BRD risk.[17] Despite much research, the broad-ranging nature of these factors makes determining the underlying risk of BRD difficult which is further complicated by the absence of predictive tests for the disease.

In the Australian feedlot industry, BRD is most commonly diagnosed by pen riders by identification of cattle showing visual clinical signs, including changes in behaviour, respiratory pattern, appetite and social interactions. This method is heavily reliant on the experience of the pen rider as cattle will often mask clinical signs of disease in the presence of humans due to the predator–prey hierarchy.[18] Some other disease detection methods used in recent years include; the remote early disease identification (REDI) system,[19] disease biomarkers[20] and thoracic ultrasound (TUS).[21] However, none of these techniques' correlate the presence or absence of disease-causing agents back to clinical findings and lack the required level of accuracy to replace human intervention, therefore leaving a gap between disease status and causality.

To investigate disease mechanisms in BRD, real-time PCR has been employed as a rapid and sensitive means of identifying the microbial organisms associated with BRD in affected animals.[22, 23] However, except for some true pathogens, many BRD-associated bacteria are

part of the normal flora of the upper respiratory tract. Therefore, without quantitative analysis the use of traditional PCR is limited in determining disease causality or acting as a predictive tool. As it is hypothesised that the differentiation between normal and disease-causing agent load is likely to be one of increasing load, there is an inherent need to accurately compare agent load within and between animals to determine disease risk or status by this measure.

Emerging evidence of the importance of microbial load is now being reported for respiratory diseases in cattle. Recently, PCR threshold values were reported for *Pasteurella multocida*, *Mycoplasma bovis and Histophilus somni* that showed a significant correlation with the risk of BRD.[24] This supports a hypothesis that upper airway agent load could be a more useful predictor of BRD risk than presence or absence of an organism alone. However, PCR thresholds that are determined relative to clinical disease are often markedly different to those acquired in an analytical assay and can be affected by inherent inhibitory factors within the clinical sample. Without consideration of the amplification efficiency in association with the quantification cycle (Cq) value, the performance of the test on a biological sample is unknown[25] and can lead to misleading results.

Although quantitative PCR (qPCR) is emerging as a useful diagnostic tool, most assays currently rely on the use of standard curves to establish the concentration in a clinical sample. This largely ignores the effect of the composition of the biological sample on the inherent efficiency of the reaction. As such, many 'quantitative' assays may only be considered as 'semi-quantitative' for this reason. If commensal agents need to be considered in a disease state, this becomes highly problematic. To overcome this, 'efficiency-corrected' (EC) quantification[25] was developed to provide accurate DNA concentrations relative to agent load for both human and animal clinical samples in a reproduceable manner. This technique can accommodate the inherent differences in efficiency found between individual animals and samples, each machine run, and time of analysis allowing both inter- and intra-animal comparisons.

To determine if load could be an important indicator of disease with relation to BRD in feedlots, a study was designed to compare the presence, load, and agent combinations of common BRD-associated agents (BoAHV1, *H. somni*, *Mannheimia haemolytica*, *M. bovis*, *P. multocida* and *Trueperella pyogenes*). Efficiency-corrected quantitative PCR was applied to nasal swabs collected from asymptomatic cattle at feedlot induction and an independent cohort of animals receiving treatment in the hospital pen at two Australian feedlots. The term 'asymptomatic' was used to indicate cattle that were either subclinical or unaffected by respiratory disease at time of sampling. The findings from this study indicate there is a causal relationship between agent load and combination with the presence or absence of BRD in feedlot cattle. These findings and the use of the screening approach could be highly informative for better management of BRD disease treatment and/or prevention in Australian feedlot systems and worldwide. Longitudinal studies using qPCR are warranted to investigate the predictive value of this as a potential screening test.

Materials and methods

Animals

This study was conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes (2013) and was approved by Charles Sturt University Animal Care and Ethics Committee (Protocol A18070) and New South Wales Department of Primary Industries Elizabeth Macarthur Agricultural Institute Animal Care and Ethics Committee (Protocol M18/07). Informed consent for use of animals included in this study was obtained from the owner/manager of the cattle, in writing, at each location prior to sampling. The study was designed and reported using the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines[26] for experiments involving live animals.

Study population

The sample size was calculated based on predicted rates of BRD during the collection season (Autumn) for Australian feedlots[12] to capture animals likely to be experiencing clinical disease, compared to those apparently unaffected. A total of 461 asymptomatic animals were sampled upon presentation to two feedlot locations (Feedlot 1 and Feedlot 2) at feedlot induction (Feedlot 1: n = 220, Feedlot 2: n = 241). On the same day as cattle were sampled at induction, a convenience cohort of 152 animals were sampled at presentation to the hospital pen (Feedlot: 1 n = 54, Feedlot 2: n = 98). Induction and hospital cohort animals were independent of one another but were coincident in time. Although sampled, two induction animals from the Feedlot 1 induction cohort and two animals from Feedlot 2 hospital cohort were excluded from analysis as full production data were unavailable.

The majority of cattle were steers with only one heifer sampled which was located in the Feedlot 1 hospital pen. Four hundred and fifty-nine induction animals (Feedlot 1: n = 218, Feedlot 2: n = 241) and 150 hospital pen animals (Feedlot 1: n = 54, Feedlot 2: n = 96) were included in the analysis. Induction pens for sampling were selected randomly based on operational activities with no specific selection criteria applied. Production data including pull reason (reason reported for treatment), pull dates and treatments were supplied by the respective feedlots for all animals sampled for comparison of production outcomes.

Disease definition in hospital cohorts

At both Feedlot 1 and Feedlot 2, diseased animals were initially identified by pen riders on horseback as cattle showing overt clinical signs of disease and were removed from their home pen to the hospital pen for treatment. BRD was diagnosed by the presence of an accumulation of clinical signs that included depression; lethargy; neck extension; altered respiratory pattern; drooping ears; discharge from the eyes, nose, and mouth; cough; inappetence and altered social interactions. Pen riders also drafted animals to the hospital pen for treatment of non-BRD related ailments including lameness, bulling (dominance injuries), preputial prolapse, necrotic laryngitis, rumen acidosis and scours.

Data obtained from each feedlot defined the reason for hospitalisation at an individual animal level and each presentation to the hospital crush. At Feedlot 1, BRD was not noted as a specific pull reason, rather the term 'respiratory' was used to cover all respiratory-like ailments which could include BRD and necrotic laryngitis. Therefore, animals pulled for respiratory were assumed to have had BRD if the treatment records included either tulathromycin, florfenicol, and/or ceftiofur.

Cattle at Feedlot 2 were classified as BRD cases if they received tulathromycin and/or ceftiofur on presentation to the hospital crush. Prevaccination status differed slightly between the two locations with Feedlot 1 cattle receiving an inactivated *M. haemolytica* vaccine (Bovilis MH®, Coopers Animal Health, NSW, Aust) prior to induction. All other induction treatments were comparable.

Clinical samples

Nasal swabs were collected from entire pen cohorts presenting at feedlot induction and from a convenience sample of cattle presenting for treatment in the hospital pens on the same sampling date. Sampling was therefore opportunistic and in alignment with normal feedlot handling practices. Samples were collected from the external nares of the nasal cavity to a depth of approximately 5 cm using a dual head swab (Puritan Opti-Tranz® Plus duo HydraFlock® swabs, Sarstedt, Mawson Lakes, SA, Aust) or using single head swabs (PS, Viscose; Sarstedt, Mawson Lakes, SA, Aust). Following collection, swabs were placed into sterile phosphate buffered saline (PBS pH 7.4) and stored on ice for transportation to the laboratory. Samples either underwent immediate DNA extraction or were briefly stored at −80°C prior to DNA extraction. To facilitate DNA extraction, swabs were vortexed for 30 s in PBS, boiled at 100°C for 10 min and centrifuged for 5 min at 12,000×*g*. The resultant supernatant was transferred to a sterile Eppendorf tube for PCR analysis.

Bacterial and viral standards

Bacterial standards were cultured from frozen pure isolates sourced from the New South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute (NSW DPI EMAI). All isolates for *H. somni*, *M. haemolytica*, *P. multocida* and *T. pyogenes* were cultured on Tryptic Soy Agar EH with Sheep Blood agar (MicroMedia; Edwards Group Pty Ltd, Narellan, NSW, Aust) at 35°C in 5% CO_2 for 24 h. *M. bovis* isolates were cultured on Mycoplasma Agar containing Supplement G (Oxoid; Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Aust) at 35°C in 5% CO_2 for 7–10 days. The identities of the bacterial standards were confirmed by MicroFlex MALDI-ToF Biotyper mass spectrometry (Bruker Pty Ltd; Prestons, VIC, Aust) in addition to the standard rapid biochemical testing: catalase, gram stain, indole, and oxidase tests.

Selected bacterial colonies were washed twice in PBS (pH 7.4) before performing DNA extraction using a QuickGene DNA tissue Kit S (Kurabo; Gene Target Solutions, Dural, NSW, Aust) and Kurabo QuickGene-810 Nucleic Acid Isolation System according to the manufacturer's instructions. DNA was eluted (20 µL volume) and the purity and concentration were determined using standard spectrophotometry (Nanovue, GE Healthcare, Edwards Group Pty Ltd, Narellan, NSW, Aust) and fluorometry (Qubit™ 3.0, Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Aust) using a Qubit™ dsDNA HS Assay Kit (Q32851; Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Aust), respectively. A synthetic control for BHV-1 (Integrated DNA Technologies, Singapore Science Park II, Singapore) was used as a positive control (5′-CAA TAA CAG CGT AGA CCT GGT CTT TGC CGA CGC GCC GGC TGC GGC CTC CGG GCT TTA CGT CTT TGT GCT GCA GTA CAA CGG CCA CGT GGA AGC TTG GGA CTA CAG C-3′).

Quantitative PCR

The specificity of the primer and probe sequences used in this study (Table 1) were evaluated in silico using NCBI BLASTn Nucleotide collection (nr/nt) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the stringency set at ≤20,000 target sequences. Singleplex hydrolysis probe assays were developed for BoAHV1, *M. bovis*, *P. multocida* and *T. pyogenes* as described previously[27] with minor modification to increase specificity. Primer and probe sequences were developed de novo for *M. haemolytica* and *H. somni*. Fluorescent reporter and quencher molecules were assigned to each target, based on the thermocycler manufacturer's recommendations (Mic™ thermocycler, Bio Molecular Systems, Upper Coomera, QLD, Aust). All reaction volumes totalled 20 µL, of which 5 µL was the template.

The hydrolysis probe reactions (*M. bovis*, *P. multocida*, *T. pyogenes*. *M. haemolytica* and BoAHV1) contained PerfeCta® MultiPlex qPCR ToughMix® (Quanta Biosciences, Beverly, MA, USA), molecular grade water (Sigma-Aldrich, St Louis, MO, USA) and 200 nM of both forward and reverse primers and probes. Thermal cycling conditions were as reported by Tsuchiaka et al.[28] with minor modifications: 95°C for 1 min, 45 cycles of 95°C for 10 s, 55–60°C for 20 s and 72°C for 20 s.

SYBR Green assays (*H. somni*) contained PerfeCta® SYBR® Green FastMix® (Quanta Biosciences), molecular grade water (Sigma-Aldrich) and 200 nM of both forward and reverse primers. Reactions were performed using the following cycling conditions: 95°C for 2 min, 45 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 20 s and preconditioning of 95°C for 19 s. Product melt was completed from 72°C to 95°C at 0.3°C/s.

Establishing cycle threshold (Cq) and efficiency (E) criteria

Individual sample efficiencies and Cq values were determined using the MicPCR software (MicPCR v2.10.0, Bio Molecular Systems, Aust). Efficiency-corrected DNA concentrations were calculated using the method reported by Ruijter et al.[25] Briefly, this 'dynamic method' calculated a corrected baseline from the average baseline value prior to detection by subtracting the average of the measured values, taking the slope into account (Figure S4). The fluorescence level of 5%, which was preset by the micPCR software, was used as the cut-off for all samples. The window of linearity (W-o-L) is automatically set by the software to provide the least variation between individual efficiencies and the mean efficiency of the samples in the data set. A corrected efficiency was then used by adding 1 to the efficiency from the raw data, where 1 = no amplification and 2 = doubling every cycle.

To quantify each target agent, efficiency cut-offs were determined based on average efficiencies for each target ±0.1 using a cycle threshold of 40 cycles. By applying efficiency and Cq cut-offs, samples outside the range of accuracy were excluded from quantification and were reported qualitatively as 'detectable' or 'not detectable'. Efficiency-corrected quantification was undertaken to determine absolute DNA concentrations and thus the load of BRD agents in the upper nasal cavity of cattle, using the previously published equation.[25]

Table 1. Primer and probe sequences for PCR amplification and quantification of bovine respiratory disease associated disease-causing agents

Agent	Target gene		Sequence (5′-3′)	Amplicon size	Reference
BoAHV1	gE	F′	CAATAACAGCGTAGACCTGGTC	85	27
		P′	**FAM™–**TGCGGCCTCCGGGCTTTACGTCT **BHQ-1**		
		R′	GCTGTAGTCCCAAGCTTCCAC		
M. Haemolytica	UDP-*N*-acetylglucosamine 2-epimerase	F′	GCAAACACTTTCTACTGTAACTTCT	102	This study
		P′	**CAL Fluor® Orange 560–** CAACCTGTTATTGCCGAATACAAACCAACT-**BHQ-1**		
		R′	GTTGCTGTATCGCCATGAAC		
H. Somni	Acyl – CoA ligase	F′	CTCATACTCAAGTGCGGTACAA	100	This study
		R′	GCCTCGGTATTGGCGATTTA		
M. bovis	oppD	F′	TCAAGGAACCCCACCAGAT	71	27
		P′	**FAM™–**TGGCAAACTTACCTATCGGTGACCCT-**BHQ-1**		
		R′	AGGCAAAGTCATTTCTAGGTGCAA		
P. Multocida	Kmt-1	F′	GGGCTTGTCGGTAGTCTTT	148	27
		P′	**CAL Fluor® Orange 560–** CGGCGCAACTGATTGGACGTTATT-**BHQ-1**		
		R′	CGGCAAATAACAATAAGCTGAGTA		
T. Pyogenes	Plo-Pyolysin	F′	ATCAACAATCCCACGAAGAG	99	27
		P′	**CAL Fluor® Orange 560-**CTCGACGGTTGGATTCAGCGCAATA-**BHQ-1**		
		R′	TTGCAGCATGGTCAGGATAC		

BoAHV1, Bovine alphaherpesvirus 1; F′, forward primer; *H. somni*, *Histophilus somni*; *M. bovis*, *Mycoplasma bovis*; *M. haemolytica*, *Mannheimia haemolytica*; *P. multocida*, *Pasteurella multocida*; P′, probe; R′, reverse primer; *T. pyogenes*, *Trueperella pyogenes*.

Statistical analysis

The statistical software R,[29] was used for all statistical analyses. Fischer's exact test was used to determine the statistical significance (i.e., if $P < 0.05$) of agent prevalence between the induction and hospital cohorts, within both inter- and intra-feedlot comparison. Statistically significant differences in the agent load between cohorts, within the location and between Feedlot 1 and Feedlot 2 locations, were investigated by the pairwise comparisons of least-square means using Tukey's honest significant differences method. Interactions between the cohort (induction or hospital) and location with respect to absolute agent load were modelled using generalised linear regression. GraphPad (GraphPad Prism 9 Version 9.1.1 for Windows, La Jolla, CA, USA, www.graphpad.com) was used to transform the data by converting data into a number between 1–100 based on the highest and lowest value for each agent. GraphPad was then used to generate heatmaps for individual animals for visual comparison.

Results

A total of 150 nasal swabs were collected from animals presenting at the hospital crush across the two feedlot locations with 411 animals sampled at feedlot induction. Of these, 49 hospital animals were identified as being treated for non-BRD ailments (Non-BRD: Feedlot 1 n = 18; Feedlot 2 n = 31) and 101 hospital pen animals identified as treated for BRD (BRD: Feedlot 1 n = 36; Feedlot 2 n = 65). Of the two induction cohorts sampled across the two feedlot locations, 411 animals received no hospital treatment during their time on feed (No ailment: Feedlot 1 n = 184; Feedlot 2 n = 227), 33 were pulled and treated for other ailments (Non-BRD: Feedlot 1 n = 21; Feedlot 2 n = 12) and 15 were pulled for BRD (Feedlot 1 n = 13; Feedlot 2 n = 2). Ailment denoted as BRD or non-BRD, was as determined by the treatment documented as given by trained feedlot staff according to local treatment protocols (see 'Disease definition in hospital cohorts' section in Materials and methods).

Prevalence, combination and concentration of potential BRD agents were higher in hospital compared to induction cohorts

For induction and hospital animals sampled at Feedlot 1 (n = 272), 79.8% were positive for at least one agent with the prevalence of all agents greater in the hospital cohort compared to the induction cohort (all $P < 0.001$, Figure 1A). Similar findings were observed at Feedlot 2 with 72.4% of the induction and hospital animals (n = 337) positive by PCR analysis for at least one agent. The prevalence of all agents were significantly higher in the hospital cohort compared with the induction cohort (BoAHV1 $P < 0.001$, *H. somni* $P < 0.001$, *M. haemolytica* $P = 0.0075$, *M. bovis* $P < 0.001$, *P. multocida* $P = 0.0397$ and *T. pyogenes* $P < 0.001$, Figure 1B).

Of all the agents analysed, *M. bovis* showed the most marked difference in prevalence between induction and hospital cohorts (Figure 1). At Feedlot 1, *M. bovis* was not detected in the induction cohort whilst hospital animals showed a prevalence of 90.7%.

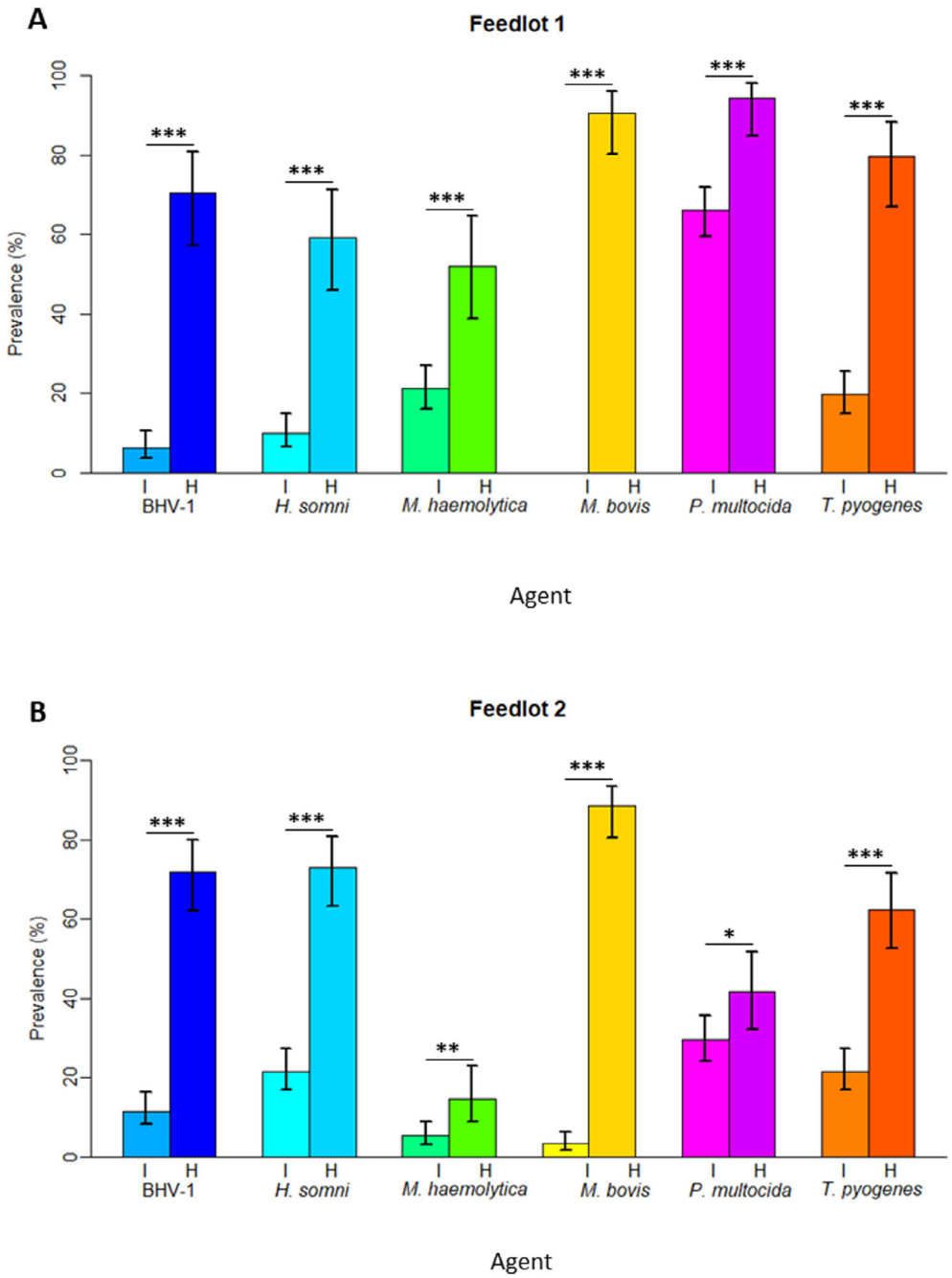

Figure 1. Prevalence (% ± CI) of the BRD-associated agents identified in nasal swabs collected at two Australian feedlots (A, Feedlot 1, B, Feedlot 2) from induction (I) and hospital (H) pen animals. Fischer's exact test: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. BHV1, Bovine alphaherpes virus 1; *H. somni, Histophilus somni; M. bovis, Mycoplasma bovis; M. haemolytica, Mannheimia haemolytica; P. multocida, Pasteurella multocida; T. pyogenes, Trueperella pyogenes.*

Similarly at Feedlot 2, 3.3% of animals were positive for *M. bovis* at induction but 88.5% were positive for this microorganism in animals from the hospital pen. The prevalence of all identified agents was significantly higher in animals sampled at hospital pen presentation compared with those sampled at induction at both feedlots.

The frequency of identified agents was evaluated both within and between feedlots comparing single agent presence with combinations of agents. The presence of a single agent was more common in induction animals than in hospital animals (Figure 2) with *P. multocida* being the most prevalent single agent detected at induction in both feedlots (Feedlot 1%–36.7%, Feedlot 2%–14.9%, Figure 2).

In contrast to those animals sampled at induction, the majority of animals presenting to the hospital pen had more than one agent present. The most common combination of agents identified in hospital animals at Feedlot 1 was BoAHV1, *H. somni*, *M. haemolytica*, *M. bovis*, *P. multocida* plus *T. pyogenes* (24.1%, Figure 2). In Feedlot 2, the most prevalent combination was BoAHV1, *H. somni*, *M. bovis* and *T. pyogenes* (18.8%, Figure 2). The primary difference between the two feedlots was the presence of *P. multocida*, which was consistently more prevalent at Feedlot 1 than at Feedlot 2. These data suggest that animals might frequently enter the feedlot without a significant microbiological burden but that this may increase significantly between induction and hospital cohorts.

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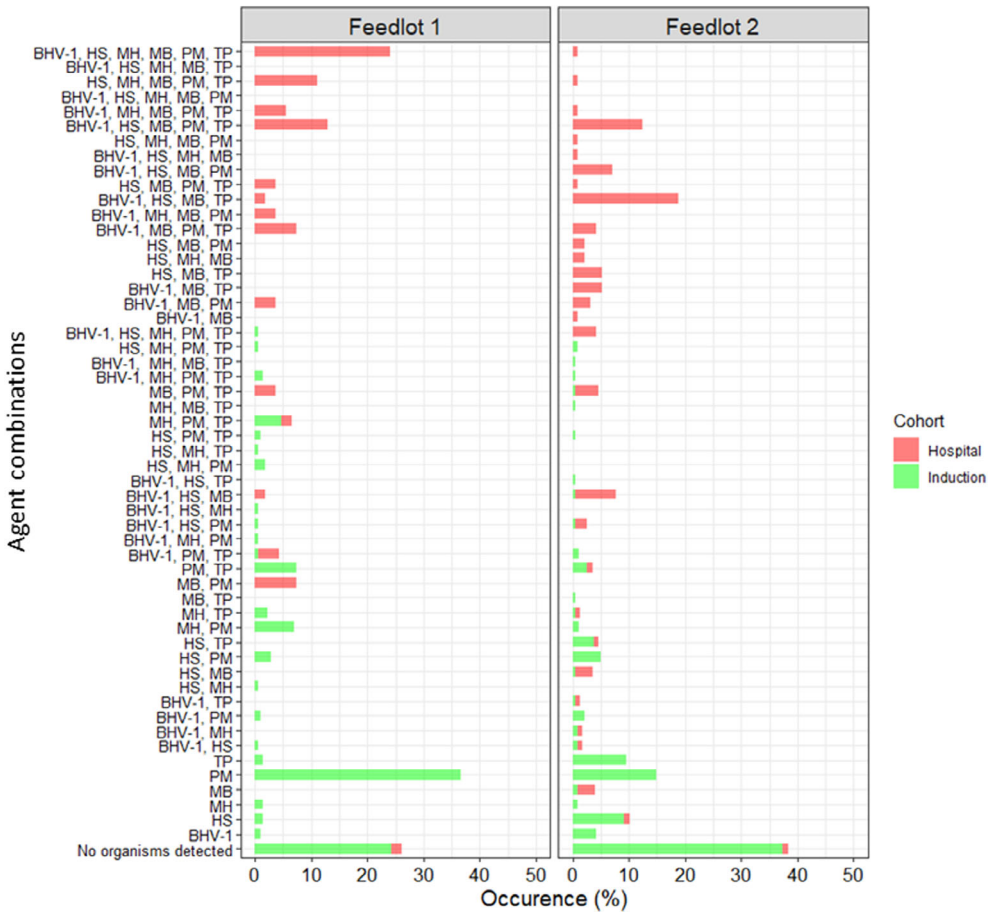

Figure 2. Combinations of PCR detectable agents associated with bovine respiratory disease in nasal swabs collected from induction and hospital pen cohorts at two Australian feedlots. Induction: Feedlot 1 n = 218, feedlot 2 n = 241; hospital: Feedlot 1 n = 54, feedlot 2 n = 96. BHV1, Bovine alphaherpes virus 1; HS, *Histophilus somni*; MB, *Mycoplasma bovis*; MH, *Mannheimia haemolytica*; PM, *Pasteurella multocida*; TP, *Trueperella pyogenes*.

The mean load of each agent was considered by location and cohort. The absolute concentrations within individual animals, as determined by efficiency-corrected PCR quantitation, were ranked as high, medium or low loads. These assays proved to be robust with respect to organism concentration (using the proxy of Cq value) and did not affect individual efficiency (E) values (Figures S1–S3). This suggests that DNA concentrations in samples from clinical BRD cases did not affect the efficiency or the dynamics of the individual PCR reactions. This verifies the stability and robustness of the assays, despite the different agent loads, and allows accurate concentrations to be determined using this methodology. The majority of cattle, both at induction and in the hospital pen, showed a low detectable load of all agents at both locations (Figure 3), however, the majority of animals exhibiting high loads were identified within hospital pens at both locations. Specifically, the highest loads of *H. somni*, BHV-1, *M. bovis* and *P. multocida* in Feedlot 1 were found in the hospital cohort with only two select cattle showing high agent loads in the induction cohort (Figure 3A). Although in Feedlot 2, the animals that displayed the highest agent loads were all in the hospital cohort and being treated for BRD (Figure 3B).

The mean concentrations of all agents across both locations and all cohorts were then considered. At both Feedlot 1 and 2, the concentration of BoAHV1 was found to be significantly greater in hospital cohorts compared to induction cohorts sampled concurrently (Figure 4). *P. multocida* concentrations were significantly greater in the hospital cohort at Feedlot 1 ($P < 0.001$, Figure 4) but were not significantly greater at Feedlot 2. *H. somni* showed the greatest variation in load between hospital pen animals between the two locations, showing a significantly lower concentration at Feedlot 1, but no significant difference between induction and hospital cohorts at Feedlot 2. Concentrations of *M. haemolytica* also did not differ between hospital and induction animals at either location (Figure 4). Concentrations of *T. pyogenes* were greater in hospital pen cattle at Feedlot 2 when compared to their induction counterparts ($P \leq 0.01$) but no significant difference was observed between cohorts at Feedlot 1 ($P = 0.999$, Figure 4). Collectively, these data show differences in the upper airway potential pathogen profile between the two locations, principally in terms of the concentrations of *P. multocida* and *H. somni*. The concentrations of *M. bovis* in hospital pen animals at both locations were similar, despite few or no animals showing

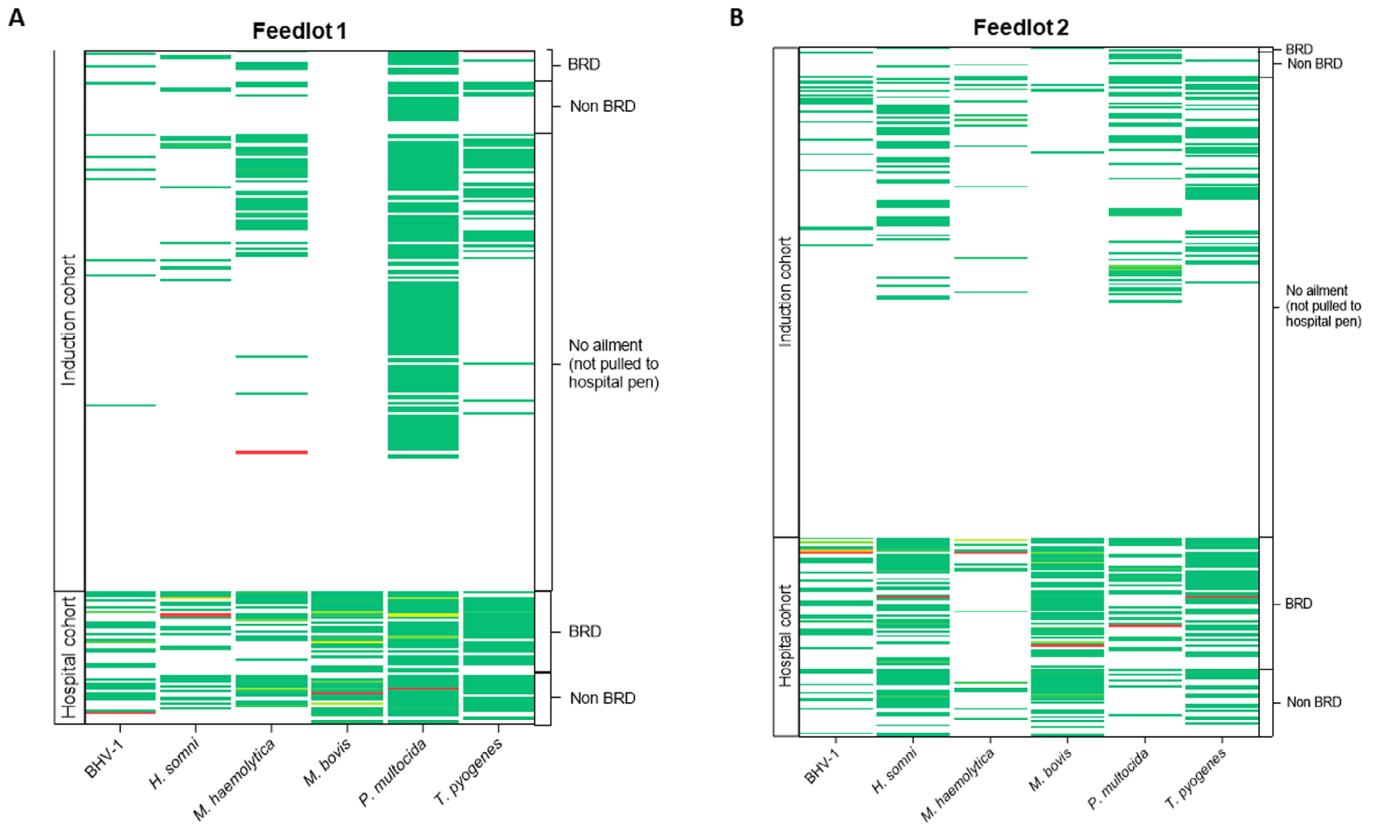

Figure 3. Microorganism load as determined by EC quantification PCR of individual animals from induction and hospital cohorts at two Australian feedlots (A and B). Heatmap is sorted on pull reason and the number of agents present per animal (most to least) and grouped by cohort. Induction: Feedlot 1 n = 218, feedlot 2 n = 241; hospital: Feedlot 1 n = 54, feedlot 2 n = 96. Normalised absolute agent load is on a colour scale for each agent; red – High agent load, yellow – Medium agent load, green – Low agent load, white – Agent not detected or not quantifiable. BHV1, Bovine alphaherpes virus 1; *H. somni, Histophilus somni*; *M. bovis, Mycoplasma bovis*; *M. haemolytica, Mannheimia haemolytica*; *P. multocida, Pasteurella multocida*; *T. pyogenes, Trueperella pyogenes*.

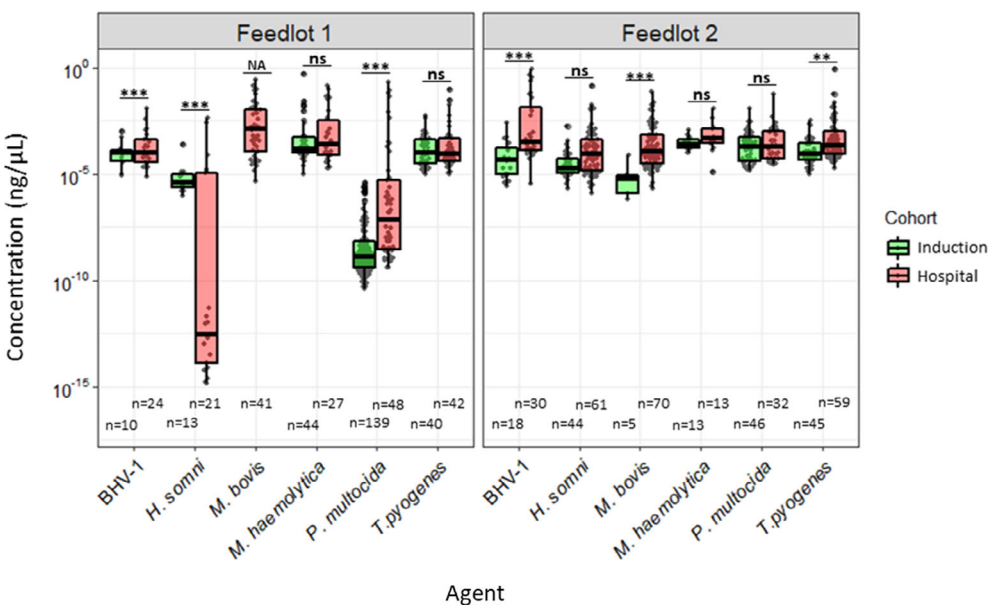

Figure 4. Absolute concentration of bovine respiratory disease agents identified in nasal swabs collected from collected from asymptomatic animals at feedlot induction and hospital presentation at two Australian feedlots (induction, Feedlot 1: n = 218; Feedlot 2 n = 241; hospital, Feedlot 1: n = 54: Feedlot 2: n = 96). Tukeys HSD test: ns P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. BHV1, Bovine alphaherpes virus 1; *H. somni, Histophilus somni*; *M. bovis, Mycoplasma bovis*; *M. haemolytica, Mannheimia haemolytica*; *P. multocida, Pasteurella multocida*; *T. pyogenes, Trueperella pyogenes*.

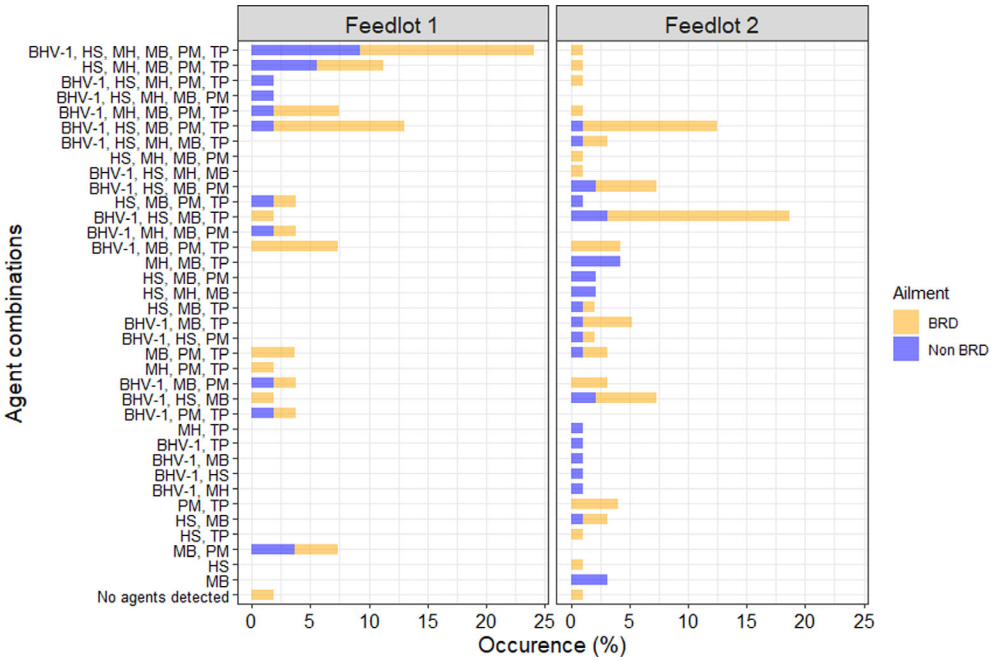

Figure 5. Combinations of bovine respiratory disease agents identified in nasal swabs collected from hospital pen animals at two Australian feedlots (Feedlot 1 n = 54, Feedlot 2 n = 96), grouped by presenting disease in feedlot hospital sessions. BHV1, Bovine alphaherpes virus 1; HS, *Histophilus somni*; MB, *Mycoplasma bovis*; MH, *Mannheimia haemolytica*; PM, *Pasteurella multocida*; TP, *Trueperella pyogenes*.

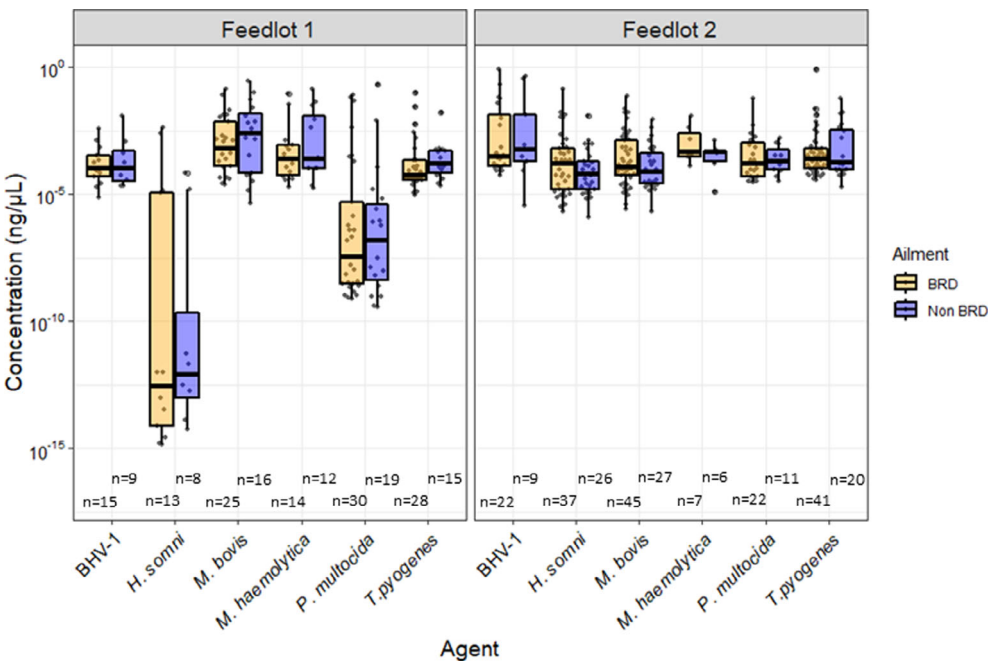

Figure 6. Absolute concentration of agents commonly associated with bovine respiratory disease from nasal swabs collected from hospital pen animals treated for bovine respiratory disease (BRD) or other ailments (non-BRD) at two Australian feedlots (Feedlot 1: n = 54; Feedlot 2: n = 96). BHV1, Bovine alphaherpes virus 1; *H. somni*, *Histophilus somni*; *M. bovis*, *Mycoplasma bovis*; *M. haemolytica*, *Mannheimia haemolytica*; *P. multocida*, *Pasteurella multocida*; *T. pyogenes*, *Trueperella pyogenes*.

presence of this agent at feedlot induction at both locations (Figures 3 and 4).

Upper airway load of BRD-causing agents was greater in hospital animals treated for BRD compared to non-BRD ailments

Potential pathogen load was considered by treatment reason for cattle sampled from the hospital pens at both feedlots. The proportions of animals treated for BRD (Feedlot 1 = 66.7%, Feedlot 2 = 67.7%) and non-BRD ailments (Feedlot 1 = 33.3%, Feedlot 2 = 32.3%) were similar between Feedlots. Cattle treated for BRD showed a greater number of agents in combination, as well as higher concentrations of agents, than their non-BRD counterparts (Figures 5 and 6). Also, BoAHV1, *H. somni*, *M. bovis* and *T. pyogenes* were found in combination in the highest percentage of cattle undergoing treatment for BRD in Feedlot 2, In contrast, the greatest percentage of cattle with BRD in Feedlot 1 had a combination of five or all six agents tested (Figure 5). When disease presentation (BRD versus non-BRD) was considered compared to agent load, no significant differences between the two treatment cohorts emerged at either location (Figure 6). Together these

data indicate that both combination of disease-causing agents in the upper airways, and the concentrations of these agents, might contribute to respiratory disease presentation in feedlot cattle, with some variation dependent on location.

Discussion

Disease initiation and progression in cases of respiratory disease in feedlot cattle is complex. The majority of the associated bacteria (*M. haemolytica*, *H. somni*, *P. multocida* and *T. pyogenes*) are normal flora and their presence alone does not necessarily indicate disease.[18] We hypothesise that the accurate determination of agent concentration and the combination of commensal and infectious agents within the nasal cavity may assist in determining subclinical to clinical thresholds that may ultimately predict the likelihood of clinical disease. Comparison of induction and hospital pen cohorts sampled in the same location and on the same collection date, showed different patterns of microorganism load, combination and prevalence of six known BRD-causal agents. Specifically, both combination and concentration of potential BRD agents in the upper airways appeared to be important in defining active repiratory disease in feedlot cattle compared to asymptomatic induction counterparts. We identified that increased load and combination of BoAHV1, *H. somni*, *M. haemolytica*, *M. bovis*, *P. multocida* and *T. pyogenes* in hospital animals correlated with a diagnosis of BRD. This study also identified a substantial increase in the prevalence of *M. bovis* after feedlot induction, with this organism potentially contributing to clinical BRD. This finding identifies a difference in the epidemiology of *M. bovis*, with the organism multiplying and being transmitted within the feedlot population, rather than arriving in detectable concentrations in a large proportion of the cattle inducted in the feedlot.

Although the use of PCR-based approaches to identify the presence of microorganisms that can contribute to clinical BRD is not new, this study is the first to utilise PCR assays in combination with efficiency-corrected quantification[25] to determine the concentration of BRD-associated agents (BoAHV1, *H. somni*, *M, haemolytica*, *M. bovis*, *P. multocida* and *T. pyogenes*) in a commercial feedlot setting. We accurately determined the intranasal concentration of agents commonly associated with BRD in asymptomatic cattle and diseased cattle in feedlots (Figures 3, 4 and 6) which could be compared directly both between cohorts and locations. EC quantification is superior to the more commonly applied standard curve quantification method which frequently over- or under-estimatesf DNA load by incorrectly assuming an amplification efficiency of 100% in clinical samples.[30] Clinical samples are dissimilar from analytical standards as they commonly contain amplification inhibitors that reduce the efficiency of the PCR reaction. By applying corrections for reaction efficiency at an individual sample level the inherent variability found in clinical samples is compensated for which allows the accurate comparison both within and between animals and cohorts.[25] Therefore, EC quantification gives the capability to compare BRD-associated microorganism presence and load over time, between cohorts and between locations in a way that has been previously impossible using standard curve methodologies.

Mycoplasma bovis *as an integrated player in BRD risk in feedlot*

This study highlighted the importance of *M. bovis* inside the feedlot operation. *M. bovis* is a slow growing, host-specific commensal that has been implicated in a number of bovine diseases including mastitis, arthritis, pneumonia, and in some reproductive losses,[31] and particularly in the development of lung lesions in cattle.[32] Whilst it is accepted that *M. bovis* can be causal for respiratory disease outbreaks in cattle, particularly when present in combination with other BRD-causing microorganisms,[33, 34] this study is the first to identify that cattle entering the Australian feedlot system at induction are not carrying detectable levels of *M. bovis* in their upper respiratory tract compared to cattle located in the hospital pen at the same location. Our findings are similar to those of a Canadian study that reported low *M. bovis* prevalence in animals at induction (1.7%) which increased over time in the feedlot (≤15 days – 72.2%, 55 days 85.7%).[35] This suggests that for the vast majoirty of cattle, infection with *M. bovis* occurs after feedlot entry, not prior to it. The emerging importance of *M. bovis* as a respiratory pathogen suggests that a new approach to the management of *M. bovis* in feedlot cattle needs to be considered, and that the epidemiological drivers of *M. bovis* spread within Australian feedlots requires further investigation.

The disease initiation threshold for *M. bovis* is still open to question. Experimental trans-tracheal inoculation with *M. bovis* failed to produce significant clinical disease in the upper respiratory tract in calves whilst others were consistently infected after ingestion of *M. bovis* contaminated milk.[36] It is therefore possible that *M. bovis* only presents as a pathogen under conditions of physiological stress or via certain routes of infection, and, similar to some other fugitive agents, may hide from immunological clearance by establishing in the tonsillar crypts or other protected niches in the respiratory tract. This hypothesis is supported by reports that *M. bovis* could be isolated from the tonsillar crypts and mainstem bronchi of clinically asymptomatic calves postslaughter[37] and may indicate one mechanism by which our nasal swabs showed no detectable levels of *M. bovis* by sensitive PCR analysis. It is possible that these refugia could facilitate colonisation of the upper airways during periods of stress or immune compromise as well as limit our ability to detect the organism by PCR from nasal swabs alone. Further studies are needed to investigate this possibility.

Presence of know BRD-causing organisms and BRD risk

Despite *M. haemolytica* commonly being reported as the most prevalent agent associated with BRD,[38] a causal relationship was not apparent from our analysis (Figure 1). The animal with the highest load of *M. haemolytica* was identified at induction (Figure 3A) and was not pulled for hospital treatment during its time on feed despite *M. haemolytica* being a known respiratory agent in cattle. Conversely, the steer showing the highest load of *T. pyogenes* in Feedlot 1 was identified in the induction cohort and was pulled for BRD after 13 days on feed (Figure 3A). Given the relatively low incidence of BRD in this study cohort, the utility of PCR quantification as a predictive tool for BRD at feedlot induction requires further investigation using larger cohorts for detailed correlation of induction pathogen load to disease outcome during time on feed.

Our study showed *P. multocida* to be present in the nasal secretions of hospital cattle at both feedlot locations but that this did not correlate directly with BRD risk. Timsit, Hallewell, Booker, Tison, Amat and Alexander[39] reported *P. multocida* was the most common bacterium isolated from the lower respiratory tract via trans-tracheal aspiration in both BRD affected (54.8%) and asymptomatic cattle (25.2%). We report similar findings in that *P. multocida*, alone or in combination, was commonly found in nasal secretions of both induction (46.8%) and hospital cattle in both locations (60.7%, Figures 1 and 2). This suggests that *P. multocida* is likely to be contributing, in an additive sense, to disease onset and severity.

Agent number and combination as an indicator of BRD in feedlot steers

Our data showed that animals treated for BRD did not necessarily show higher loads of BRD-associated agent in their nasal secretions than those treated for non-BRD ailments (Figure 6), however, the number of agents in combination was increased in hospital pen animals being treated for BRD. More specifically, hospital animals with four or more agents present were more likely to be treated for BRD than for other ailments (Figure 5). These findings suggest that the number of different BRD-associated agents, as well as their load, may contribute to the risk of an animal developing BRD in the feedlot, and possibly its subsequent disease progression. As the hospital animals sampled in this study were a separate and independent population to the induction animals sampled, a longitudinal study would be required to further investigate the possible contribution of load and combination of agents on BRD risk in feedlot cattle.

Conclusions

In conclusion, this study has identified that efficiency-corrected quantitative PCR analysis is a powerful tool for examination of upper airway microorganism presence and load at an individual animal and whole cohort level, and that both combination and concentration might play role in the pathogenesis of respiratory disease in feedlot cattle in Australia. Our findings also identify that *M. bovis* might contribute to BRD risk by increasing both pathogen load and their combinations in cattle after feedlot entry and this could represent an emerging challenge for feedlot cattle health management.

Acknowledgments

The authors acknowledge Leah Stroud, Karen Williams, and Veronika Vicic for their expert technical assistance. We also thank Joe McMeniman, Matthew Van der Saag, Mark Westman and Mark Hazelton for helpful comments on the manuscript. Thank you also to Gang Xie for his assistance with the statistics, as well as helpful comments on the manuscript. We extend our gratitude to the managers and staff of the two feedlots who contributed to the study – without their assistance, this work would not have been possible. Open access publishing facilitated by Charles Sturt University, as part of the Wiley - Charles Sturt University agreement via the Council of Australian University Librarians.

Conflicts of interest and sources of funding

The authors declare no conflicts of interest. This research was supported by Meat and Livestock Australia Donor Company, Charles Sturt University, and the New South Wales Department of Primary Industries (Project P.PSH.0873). Rebecca Barnewall was supported by the Australian Government Research Training Program Scholarship and scholarship awards from The Graham Centre for Agricultural Innovation and Meat and Livestock Australia (B. STU.2001).

Data availability statement

The data that support the findings of this study are restricted by a funding arrangement; the corresponding author can relay requests for data sharing upon reasonable request.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.13200/suppinfo.

Appendix S1 Supporting Information

(Accepted for publication 27 July 2022)