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Respiratory pathogens in veal calves: Inventory of circulating pathogens

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ABSTRACT

In the veal industry in The Netherlands, each year around 1.2 million "white" veal calves are produced on around 1100 farms. Bovine respiratory disease (BRD) causes serious health issues in these calves, also resulting in high usage of antimicrobials. To reduce antimicrobial usage, a more targeted treatment regime is needed, for which it is necessary to identify the causative agent. This study aimed at determining associations between pathogens and clinical disease, between prevalence of pathogens and BRD outbreaks, and BRD and performance. A cohort study was conducted involving ten veal farms, in which calf respiratory health was evaluated for the first 12 weeks. Whenever there was an outbreak of BRD, as determined by the farm veterinary surgeon, samples were taken from diseased and control calves through broncho-alveolar lavage. From these samples a broad spectrum of micro-organisms were isolated. Performance data were also collected. A total of 23 outbreaks happened during the 12 week study period, mostly in the first six weeks. BRD associated pathogens found were: BHV1, BPI3V, BRSV, BVDV, *Pasteurella multocida*, *Mannheimia haemolytica*, *Trueperella pyogenes*, *Histophilus somni*, *Mycoplasma bovis*, *Mycoplasma bovirhinis* and *Mycoplasma dispar*. For most BRD associated pathogens, there was no clear association between presence or prevalence of the micro-organisms and clinical issues. Only *T. pyogenes (*7.4% in healthy, 14.6% in diseased calves, p 0.013*)*, *M. bovis* (37.6% and 63.2% respectively, p 0.001) and BVDV (9.9% and 16.9% respectively, p 0.03) were found more often in diseased animals. BPI3V was found in a few early outbreaks, which might suggest involvement in early outbreaks. It appears to be difficult to associate specific pathogens to outbreaks at the species level. BRD is the major reason for treatment with antimicrobials. More specific knowledge about the association between pathogens and health/disease could help to reduce antimicrobial use.

1. Introduction

In The Netherlands, each year around 1.2 million "white" veal calves are produced on around 1100 farms. In accordance with relevant European legislation white veal calves are generally reared on milk replacers supplemented with solid feeds (Bus et al., 2019), and slaughtered at an age of approximately 6 months. The husbandry of veal calves in The Netherlands consists initially of individual housing, followed after several weeks by group housing until they are slaughtered. Bovine respiratory disease (BRD) is a serious problem in veal calves (Pardon et al., 2012, Pardon et al., 2013, Bergevoet et al., 2010). This, not only because of the welfare problems associated with the disease, but also because BRD leads to a high use of antimicrobials. Although the use of antimicrobials has been significantly reduced in the Dutch veal sector, it is still high compared to the use in some of the other livestock sectors (Bos et al., 2013, Autoriteit Diergeneesmiddelen (Dutch Animal Medicines Authority), 2020). The majority of antimicrobials, either for individual treatment or for group treatment, are prescribed for BRD: Pardon et al. (2012) report that respiratory disease was the reason for 53% of antimicrobial group treatments in Belgium. Schnyder et al. (2019) report that the main indication for antimicrobial use in Switzerland was BRD (81.1%), and Fertner et al. (2016) found that in Denmark respiratory disease was the primary indication for almost 79% of antimicrobial use (in rose veal calves). Pardon et al. (2013) found that BRD has a severe effect on performance: with each episode of BRD and the subsequent treatment with antimicrobials, the hot carcass weight, fat cover and carcass colour were more negatively affected.

In the Netherlands, as in other countries Europe-wide, there is a general consensus that the use of antimicrobials in the livestock production sectors should be reduced as much as possible in order to slow

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down the development of antimicrobial resistance (Bos et al., 2013, Bokma et al., 2019). In the veal sector in The Netherlands, young calves from a diverse background are brought together on farms and fattened together until slaughter age. This practice leads to a high disease pressure and results subsequently in a relatively high use of antimicrobials. BRD is one of the most important disease problems during fattening in veal calves. For that reason, efforts are needed to control BRD in veal calves.

BRD can be associated with a variety of pathogens. Examples are bovine respiratory syncytial virus (BRSV), bovine parainfluenzavirus-3 (BPI3V), bovine coronavirus (BCV), bovine herpesvirus-1 (BHV-1), bovine viral diarrhoea virus (BVDV), *Mannheimia haemolytica*, *Pasteurella multocida* (Pardon et al., 2011), *Histophilus somni* (Angen et al. 2009), *Trueperella pyogenes* (Wisselink et al., 2017), *Mycoplasma bovis*, *Mycoplasma dispar* and *Mycoplasma bovirhinis* (Nicholas, 2011). Several yeasts, fungi and parasites may also play a role (Aslan et al., 2002). For reduction of antimicrobial use, a more targeted treatment regime should be applied (Pardon et al., 2012), however for this we need to identify the causative agent. In case of a bacterial infection, an antimicrobial treatment might be considered. Ideally, the causative micro-organism should be isolated and / or its antibiotic susceptibility profile should be evaluated.

The premise of this study was, that identification of potential BRD associated pathogens might assist in the decision making on whether to treat of not treat with antimicrobials. The study aimed at determining associations (1) between presence of pathogens and clinical disease in individual animals and (2) between prevalence of pathogens and BRD outbreaks. In more detail, the questions to be addressed were: 1) During outbreaks of BRD, do we find associations between the presence of BRD-associated pathogens and clinical manifestation of BRD in individual animals, where clinical manifestation was defined in terms of 'diseased' vs. 'healthy' on the basis of clinical scoring, in terms of rectal temperature, and in terms of breathing frequency? 2) Which BRD-associated pathogens were detected more frequently during outbreaks than at sampling times without outbreak?; How often where different BRD-associated pathogens present during BRD outbreaks? Could the presence of micro-organisms in an outbreak predict the agents detected in the next outbreak?

2. Materials and methods

A cohort study was set up on 10 commercial white veal farms in the Netherlands. The number of ten farms was the maximum deemed practically possible, and was chosen to best capture likely variation between farms. On the farms, calves were sampled by bronchoalveolar lavage when outbreaks of bovine respiratory disease were diagnosed and the samples were examined for pathogens. Presence of pathogens potentially associated with BRD was analysed against signs of disease.

2.1. Selection of farms

Farms were selected on the basis of the following criteria:

- Except for the veal calves, there were no other bovines or other ruminants on the farms.
- The farms used an All-In/All-Out policy (all calves are delivered to the farm at the same time, i.e. within a period of a week, in a cleaned and disinfected stable; no calves were added during the rearing period; calves were transported to the abattoir in batches of the same weight; the stable must be completely empty and cleaned before arrival of a new batch of calves)
- Arrival date of the calves on the farms should be planned such that the first months of their lives were within the "BRD season"; arrival was from October to December 2013
- Calves on the farms should either originate from the Benelux (Belgium, The Netherlands and Luxemburg) and Germany (BG group, six farms), or from Poland, Latvia or Estonia only (Eastern European (EE) group, four farms).
- Willingness of the farmer to participate in the study and a farm setup that consisted of at least one rearing unit with a capacity of 300 veal calves or more. If more rearing units were present, only calves within one and the same selected rearing unit were included in the study.
- To prevent carry-over of micro-organisms, calves were not moved from one rearing unit into another or vice versa during the study period.

Veal production in The Netherlands is organised in highly integrated value chains with a key role for a small number of integrators that connect the different parts of the value chain. Such integrations are companies within which all production stages are combined from the fattening farms and feed mills to the abattoir, and in which calves are placed in farms under contract agreements. Feed is provided through the integration, and the integration also organises the processing of the calves. The 10 herds participating in the study all belonged to one of the two largest white veal 'integrations' in the Netherlands.

The calves arrived on the farms in this study between early October 2013 (Farm 1) and early December 2013 (Farm 10) in an empty and cleaned and disinfected rearing unit. The calves were followed over 12 weeks (84 days) until January 2014 on Farm 1 and March 2014 on Farm 10. Calves on each farm originated from multiple herds and were transported from the herd of origin to the veal herds, at the minimum age of 14 days old, after a short stay at a collection centre. The calves were fed with veal calf milk replacer and were kept on wooden slatted floors.

All farms have a veterinarian that is specialized in veal calves. During the first weeks of the rearing period, the veterinarian visits the farms regularly.

The project team has at no point intervened in the daily running of the farm, and has at all times taken an observer role.

2.2. Selection of calves for sampling

On the day of arrival (D0) and the last day of the experiment (D84), and every time there was an outbreak of lower respiratory tract disease for which a metaphylactic treatment was planned, calves were examined clinically using the criteria described in Table 1 (Cortjens et al., 2019), to assess whether they were clinically healthy or diseased. An animal was deemed diseased if it scored at least a 1 on the respiration rate, irrespective of any other signs. All calves scoring 0 on respiratory rate were not included in the controls if they had other respiratory signs, and calves scoring 0 on all aspects were considered healthy.

The study was conducted in one unit (barn) per farm. The number of calves per unit varied between 400 and 710 calves. On D0, for a random selection of 100 calves girth measurements, measured right behind the front legs, were taken as a proxy of weight (a random list of 100 rank numbers was produced, and researchers would select calves in order of this list). Calves to be sampled on D0 and at later occasions were, as much as possible, randomly selected out of these 100 calves. On D0, bronchoalveolar lavages were taken from 20 healthy calves from the group of 100 (calves were sampled in order of the ranking list if they fitted the criteria, if not, the next calf in the list was sampled, until a total of 20). The number of 20 healthy calves was chosen based on a sample-size calculation requiring a probability of at least 80% to select at least one animal carrying a given pathogen if the prevalence of the pathogen was 8% or higher. Every time the farmer suspected an outbreak of lower respiratory tract disease, the veterinary surgeon of the farm was consulted. The definition used to declare an outbreak was as follows: an outbreak (or a new outbreak) was declared, if the veterinary surgeon confirmed the diagnosis and decided a metaphylactic treatment had to be started, and a previous, if any, metaphylactic treatment had not been given less than 7 days ago. If an outbreak was declared, clinically diseased calves and calves that appeared healthy (controls) (Table 1)

Table 1
Clinical score to assess the health of a calf.

Parameter	Score 0 (not present)	Score 1 (mild)	Score 2 (moderate)	Score 3 (serious)
General impression	Clear, alert, normal appetite, normal behaviour	Reduced response, reduced appetite, no other abnormalities	Listlessness, clearly	Soporous, hardly responding to stimuli, no appetite and not able to stand without help
Ocular and/or nasal discharge	No discharge	Ocular or nasal discharge is intermittently fluid or mucous	Ocular and nasal discharge has increased. Persistent mucous or clear mucus with flakes of (white/yellow) pus	Serious ocular or nasal discharge, persistently purulent or bloody
Coughing	No coughing	Every now and then spontaneous or induced* dry (non-productive) cough	Frequent spontaneous or induced* dry or productive cough	Frequent spontaneous productive cough, induced* cough turns into coughing fit
Respiratory rate	Normal breathing (RR <50 breaths/minute)	Increased breathing rate (RR 51–70 breaths/minute)	Increased and/or abdominal breathing (RR 71–100 breaths/minute)	Seriously increased breathing. Calf is clearly tight (breathing with extended neck, open mouth, foaming at the mouth)(RR >100breaths/minute

A calf with score 0 for all parameters was classified as healthy; all calves with a score of at least 1 on respiratory rate as diseased.
* In upper respiratory tract infection coughing can be induced by applying pressure by hand to the larynx or trachea (mid-trachea, or just before the chest entrance)

were selected to be sampled: bronchoalveolar lavages were done of 16 control calves and 10 diseased (case) calves, if present, before any treatment was administered. The 16 control calves were assessed again three days later; those found diseased then were excluded as control calf from the analysis as they would probably already had been subclinically affected at the time of sampling. The number of 10 diseased calves was chosen based on a sample-size calculation requiring a probability of at least 80% to select at least one animal carrying a given pathogen if the prevalence of the pathogen was 15% or higher. The number of 16 control calves was chosen with the aim that at least 10 calves would still qualify as control calf after the re-assessment three days later. At the second and subsequent sampling moments, the control calves were selected from the group of 20 healthy calves sampled at D0, excluding calves that had been sampled as clinically diseased at any earlier point in time; if this would not allow the selection of up to 16 controls, remaining controls would be selected from the wider group with girth measurements on D0 following the list of rank numbers. Finally, on D84 a girth measurement was done of all calves that had been sampled during the experimental period, (both 100 of D0 and all additional calves), and just as on D0, bronchoalveolar lavages were taken from 20 healthy calves.

For every individual animal the following records were collected:

- Identification number and sex
- Age at arrival or birth date
- Start date and date of slaughter
- If lost: date of mortality
- If lost: cause of mortality
- If antimicrobials used individually or generally: date, substance (active ingredient and brand name), dosage, application method, indication, duration and frequency of treatment

At group level the average weight per calf was recorded, and per unit group (metaphylactic) treatments with antimicrobials and other use of drugs (date, substance (active ingredient and brand name), dosage, application method, indication, duration and frequency of treatment. Supplementary file Suppl_info_antibiotics.docx in the Supplementary information shows the antimicrobials used as a treatment in the case of an outbreak. During outbreak treatment, all calves were treated by adding the antimicrobials to the milk replacer.

2.3. Bacteriological and virological examinations

Bronchoalveolar lavage fluid (BALF) samples were collected via the ventral nasal meatus as described by Fogarty et al. (1983). Between 35 and 75 mL BALF was obtained from each calf after introduction of 100 mL PBS using a silicon tube with a diameter of either 6 millimetre (internal) and 10 millimetre (external), or 3 millimetre (internal) and 7 millimetre (external) depending on the size of the calf. Directly after sampling 5% fetal calf serum (FCS) was added to the BALF and the BALF samples were transported within 24 h under cooled conditions (ice packs) to the laboratory. At the laboratory, clots of mucus were aspirated under aseptic conditions using a pipet and 10 mL BALF was spun down for 10 min at 4600g at 4 °C. The BALF pellet was resuspended in 250 µl PBS, and two vials with 80 µl each were prepared. To one of the vials 80 µl glycerol was added (pepton glycerol medium with 30% glycerol) for bacteriological examination, and both vials were stored at − 80 °C. The remaining BALF was centrifuged (200g, 10 min, 4 °C), the supernatant was aliquoted and stored at − 80 °C for virus isolation.

In order to isolate bacterial lung pathogens *P. multocida*, *M. haemolytica* and *T. pyogenes* BALF samples were plated on heart infusion agar (HIS, ACU 7269 C, Acumedia Manufacturers Inc. Lansing, MI) supplemented with 5% defibrinated sheep blood. For *H. somni*, the BALF samples were plated on chocolate blood agar, using 7% defibrinated horse blood and Columbia blood agar (CM 331, Oxoid, Badhoevedorp, The Netherlands). Both the HIS plates and the chocolate blood agar plates were incubated in air enriched with 5% CO_2. The plates were examined after culturing for 24 and 48 h at 37 °C. Colonies with a morphology consistent with *P. multocida*, *M. haemolytica*, *T. pyogenes* and/or *H. somni* were subjected to MALDI-TOF mass spectrometry (Bruker MALDI Biotyper Microflex, version 3.1 with reference database V4.0 (5627 MSPs) Bruker Daltonics GmbH, Germany). Single colonies were subcultured twice and stored at − 80 °C in the presence of 15% glycerol.

For the simultaneous detection of *M. bovis, M. bovirhinis* and *M. dispar*, BALF samples were analysed with a 16 S rRNA PCR combined with denaturing-gradient gel electrophoresis (DGGE) by the Animal and Plant Health Agency (APHA, Mycoplasma Team, Addlestone Surrey, UK, McAuliffe et al., 2003).

The BALF samples were also tested for the presence of BHV1, BPI3V, BRSV and BVDV. The samples were incubated on 70–80% confluent monolayers of embryonic bovine tracheal (EBTr) cells (WBVR origin) for three (BHV-1) or six (BRSV, BVDV and PI3) days at 37 °C (5% CO2). Presence of virus was determined by an immunoperoxidase monolayer assay (IPMA). BRSV was detected by using mouse monoclonal antibodies: MAB3 directed against the F-protein of BRSV (Van der Poel et al., 1996, Langedijk et al., 1998) followed by incubation with rabbit anti-mouse peroxidase (Dako P0260) as conjugate. BHV1, BVDV and BPI3V were detected by using a polyclonal / hyperimmune serum: an antiserum against BHV1 (WBVR origin: cow 628 pr. 91 pr. 89–3) followed by incubation with rabbit anti-cow peroxidase (Dako P159), an anti-BVDV swine serum (WBVR origin: swine 8353 09–11–2004) followed by incubation with rabbit anti-swine peroxidase (Nordic MUbio RASw/IgG (H+L)/PO) to detect BVDV and an anti-BPI3V bovine serum (WBVR origin, 23jan01) followed by incubation with rabbit anti-cow peroxidase (Dako P0159) to detect BPI3V. For all viruses, staining was performed with 3-amino-9-ethyl carbazole (AEC) (Sigma A-6926) as

substrate. Plates were read microscopically for stained cells.

2.4. Statistics

Differences in the prevalence of specific BRD associated lung pathogens detected in BALF samples from diseased and control calves were tested in a stratified analysis (with outbreak, numbered across all farms, as stratum) using the Mantel-Haenszel test (Kuritz et al., 1988). The same method was used to study the association of the rectal temperature with the presence of specific micro-organisms and to study associations between the presence of micro-organisms and a breathing frequency higher, respectively lower than 70 per minute.

The prevalence of specific micro-organisms through subsequent outbreaks was evaluated using the Chi-squared test (Plackett, 1983), a test to study differences between expected and observed frequencies. This test was also used to assess trends over time in the prevalence of micro-organisms.

Predictability of prevalence of specific micro-organisms on the basis of prevalence on D0 or on the basis of prevalence in preceding outbreaks was assessed using the Fisher Exact test (Agresti, 1992), a test to examine significance of association (contingency) between two kinds of classification. It was also used to assess prevalence at D0, during outbreaks and at D84.

The Independent T Test (Levin, 2011), a test to compare sets of normally distributed data, was used to compare growth performance between control and diseased calves.

The Mann- Whitney test (Mann and Whitney, 1947), a test to evaluate differences in means from two populations, was used to compare time of onset of BRD between EE and BG farms.

IBM SPSS was used as Statistical software tool.

3. Results

Epidemiological aspects: (1) associations between presence of BRD-pathogens and clinical manifestation of BRD, 2) association between prevalence of pathogens and BRD outbreaks.).

In the ten units on the ten farms there were 23 BRD outbreaks (as defined in materials and methods) in the first 12 weeks (Fig. 1); two of these outbreaks occurred on D0. Outbreaks were seen in all ten units, with at least 2 outbreaks per unit. In the EE groups, the first outbreak happened significantly earlier than in the BG group (Mann-Whitney test): in the EE group on average three days after D0, in the BG group on average 14 days after D0. The majority of outbreaks, 19 out of 23, happened in the first six weeks of the study period (Fig. 1.).

The sampling regime should have led to 230 diseased animals being sampled. However, during the 23 outbreaks in total 219 samples from diseased calves and 376 samples from control calves were taken. The reason that less than 230 (23 outbreaks times 10) diseased calves were sampled was, that during three of the outbreaks only less than 10 calves could be found that were diseased according to the clinical scoring based on breathing frequency. From the control animals, 17 in total were found to be diseased three days later and thus discarded for the statistical analyses.

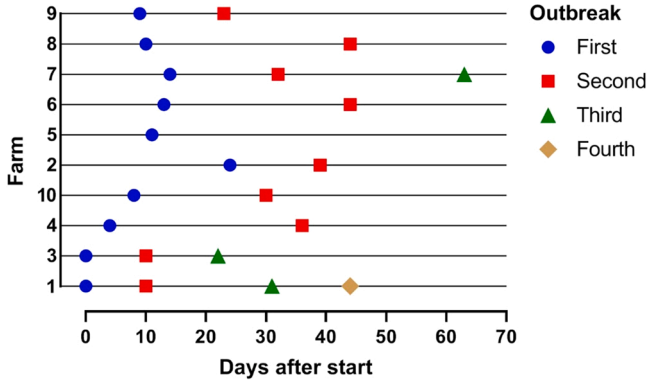

Fig. 1. Timing of outbreaks on ten veal calf farms. Veal calves on farms 1, 3, 4 and 10 originated from countries in Eastern Europe (EE), the veal calves on the other farms originated from Western European countries (BG).

In Table 2, the results of the microbiological analysis are displayed. The detected prevalence of all different pathogens considered generally differed considerably between sampling times, ranges being 0.0%-max for all pathogens except *M. dispar* (4.5%-max), with max ranging between 15% (*H. somni*) and 95% (*M. bovirhinis, M. dispar*); for details we refer to the Supplementary file (OverviewOfDetectedMO.xlsx).

Six different BRD associated pathogens were identified in more than half of the outbreaks: *P. multocida*, *M. haemolytica*, *T. pyogenes*, *M. bovis*, BVDV and BPI3V. The other three BRD-associated pathogens investigated (BRSV, BHV1 en *H. somni*) were detected in less than half of the outbreaks: respectively in ten, seven and two of the 23 outbreaks. On average more than five bacteria species and around three virus species were identified per outbreak. When analysed per individual outbreak, there are only six instances where micro-organisms were identified significantly more often in diseased compared to control calves (Fischer exact test, $p < 0.05$): *M. bovis* in three outbreaks (second outbreaks on farm 1 and 10, first on farm 2); and BPI3V, *T. pyogenes* and *M. bovirhinis* each in one outbreak (second outbreak on farm 2, first on farm 8, second on farm 8, respectively). In contrast, there were three instances where micro-organisms were identified significantly more often in control compared to diseased calves (Fischer exact test, $p < 0.05$): *P. multocida* in two outbreaks (second outbreak on farm 4 and first on farm 8) and *M. bovirhinis* in one outbreak (second outbreak on farm 9). When analysing the data across all outbreaks on all farms, three micro-organisms were identified significantly more often in diseased compared to control calves: *T. pyogenes*, *M. bovis* and BVDV (Mantel-Haenszel test).

We also investigated if there are combinations of two different micro-organisms that co-occur in the same animal more frequently than expected from random co-occurrence. I.e. we investigated the potential association between the detection of two different micro-organisms in the same animal, during outbreaks but irrespective of disease (i.e. combining diseased and controls in one analysis). A total of 17 combinations of MOs were investigated: all 15 possible combinations of one of the 5 viruses and one of the three bacteria: *P. multocida*, *M. haemolytica* and *M. bovis*, and the combinations *P. multocida* & *M. haemolytica* and *P. multocida* & *M. bovis*. The only combination for which a significant (and positive) association was found is BPI3V and *P. multocida* ($p = 0.023$, Mantel-Haenszel test); we note that this result should be interpreted with caution as it is part of a multiple comparison (17 combinations of tests). For the combinations BRSV & *P. multocida*, and BVDV & *M. haemolytica*, the patterns are so different between individual outbreaks that the model underlying the Mantel-Haenszel test is not valid (no common odds ratio between outbreaks); for all other combinations studied no association was found ($p > 0.05$).

In Fig. 2 the pattern of prevalence across sampling times is given for a selection of two viral and two bacterial pathogens; for the detailed patterns of the other pathogens studied we refer to the Supplementary file OverviewOfDetectedMO.xlsx. It is observed that in eight out of ten farms the prevalence detected for *P. multocida* at D84 was higher than at D0. This increase in detected prevalence corresponded to a significant temporal trend (Chi-squared trend test, $p < 0.05$) in five out of the eight farms. Generally, subsequent outbreaks seemed to have different micro-organisms involved; the presence of micro-organisms in an outbreak could not predict the agents detected at the next outbreak (conditional independence hypothesis tested using Fisher Exact test). The same applied to the presence of micro-organisms on D0.

BPI3V was mainly detected during outbreaks; on D0 and D84 it was rarely found. BVDV was only detected in three of the farms on D84. BVDV and BRSV were more often detected during outbreaks ($p < 0.05$, Fischer Exact). BRSV, though relatively rare, was significantly more

Table 2
The prevalence of BRD-associated pathogens in diseased and control calves.

Agent	Control calves			Diseased Calves			All calves			*P*-value	Common Odds Ratio (95% CI)	
	N tested	N (%) positive		N tested	N (%) positive		N tested	N (%) positive				
BHV1	355	9	(2.5)	219	2	(0.9)	574	11	(1.9)	0.578	0.5	(0.1–2.4)
BPI3V	355	37	(10.4)	219	33	(15.1)	574	70	(12.2)	0.18	1.5	(0.9–2.7)
BRSV	355	13	(3.7)	219	12	(5.5)	574	25	(4.4)	0.7	1.3	(0.5–3.4)
BVDV	355	35	(9.9)	219	37	(16.9)	574	72	(12.5)	**0.037**	**1.8**	**(1.1–3.0)**
P. multocida	338	118	(34.9)	211	62	(29.4)	549	180	(32.8)	0.183	0.8	(0.5–1.1)
M. haemolytica	339	24	(7.1)	212	22	(10.4)	551	46	(8.3)	0.22	1.6	(0.8–3.2)
T. pyogenes	339	25	(7.4)	212	31	(14.6)	551	56	(10.2)	**0.013**	**2.1**	**(1.2–3.8)**
H. somni	338	0	(0.0)	212	1	(0.5)	550	1	(0.2)	0.813		
M. bovis	338	127	(37.6)	209	132	(63.2)	547	259	(47.3)	**< 0.001**	**3.5**	**(2.3–5.2)**
M. bovirhinis	338	84	(24.9)	210	58	(27.6)	548	142	(25.9)	NA		
M. dispar	338	127	(37.6)	210	90	(42.9)	548	217	(39.6)	0.471	1.2	(0.8–1.7)

Significant differences between diseased and control calves are displayed in bold (Mantel-Haenszel test).
NA: not applicable

often detected during first outbreaks.

In Table 3 an overview is given of the clinical scores found in the 219 diseased calves. By definition, diseased calves had a respiratory rate over 50 per minute. In 42 calves that was the only sign (score 0 on General Impression). 133 calves had mild signs (score 1 on General Impression), 33 had moderate signs (score 2) and one had serious signs (score 3). There were ten missing scores on General impression. The most common sign was a mild cough.

In Fig. 3, the rectal temperatures measured in all calves, both diseased and controls, are given. The average rectal temperature of the control calves was 38.9 °C, in diseased calves 39.5 °C. The Mantel-Haenszel test was used to study if the rectal temperature was associated with the presence of specific micro-organisms. No associations could be demonstrated in diseased calves nor in control calves. There were also no associations (Mantel-Haenszel test) between the presence of specific micro-organisms and a breathing frequency higher, respectively lower than 70 per minute, except for *M. bovis*. For *M. bovis* there was a negative association ($p = 0.003$) in diseased calves, meaning that in diseased calves, *M. bovis* was detected more often if the breathing frequency was below 70 per minute.

Table 4 shows the results of the mortality, rejected lungs and antibiotic use recording. Mortality ranged between two and six percent. Lung rejections varied between zero and six percent. The number of treatments with antimicrobials varied greatly between farms: between 12 and 162. The majority of treatments, if the records allowed sufficient analysis, was given for respiratory issues. A majority of farms applied second choice antimicrobials. According to the Dutch Formularium for veal calves and beef cattle of the Royal Dutch Veterinary Association, in principle, second choice antimicrobials should only be used in exceptional cases where first choice antimicrobials do not provide the desired effect, to ensure availability of go-to treatment options. On some farms for a third to half the treatments a second choice antimicrobial was used.

4. Discussion

In this study, clinical signs of bovine respiratory disease were used as the basis of declaring an animal diseased and declaration of an outbreak. This is how, in practice, most veterinary surgeons would decide if treatment is required at the time this study was conducted. More modern diagnostic tools, such as thoracic ultrasound (TUS) can be used to detect pneumonia (Leenen et al., 2020). Leenen et al. (2020) found that out of 110 healthy looking calves, 31 could be found with pneumonia on the basis of TUS and were therefore classified as having subclinical pneumonia. In this study however, the emphasis was not on distinguishing between the presence of pneumonia or upper respiratory tract symptoms. In both cases, it is likely that a treatment could be indicated. *T. pyogenes*, *M. bovis* and BVDV were the only pathogens found significantly more often in diseased than in healthy calves (Table 2). This may mean that they have a primary association with respiratory disease. Biesheuvel et al. (2021) found that *M. haemolytica* had a primary association with respiratory disease in veal calves, however in our study we could not confirm this.

P. multocida was the micro-organism most commonly detected at D0 and D84. In five of the farms the prevalence of *P. multocida* increased significantly between D0 and D84 (Fig. 2). *P. multocida* was not detected significantly more often in diseased than in control calves. This may mean that *P. multocida* generally behaves like an opportunistic rather than a causative agent in respiratory disease, possibly taking advantage of respiratory disease caused by other micro-organisms. Pardon et al. (2011) also detected a variety of pathogens without finding a specific one that could be linked to individual outbreaks. In our study the pathogens were identified at species level. Subtyping of strains, for example the serotyping of *M. haemolytica* (Mason et al., 2022), and identification of virulence factors, might shed more light on the role of specific microorganisms.

H. somni is known as an organism commonly associated with BRD, however in this study *H. somni* seemed not to be very prevalent. In an evaluation of a real-time PCR for detection of *H. somni* it was found that real-time PCR was more sensitive than culture (Wisselink et al., 2017). In other studies on *H. somni*, this difference was also found (Angen et al., 1998; Bell et al., 2014). The explanation for this difference is that *H. somni* is a slow growing organism with small colonies that can be easily overgrown by other organisms (Angen et al., 1998). It can be concluded that the number of *H. somni* positive samples might be underestimated in this study.

M. bovis was the most frequently detected pathogen in this study, confirming the importance of *M. bovis* infections in veal calves in The Netherlands (Ter Laak et al.1992). *M. bovis* is distributed worldwide, with variable prevalence, and considered to be one of the major emerging pathogens of cattle (Nicholas, 2011). Controlling the infection is difficult because of the absence of a safe, effective commercial vaccine in Europe. Moreover, a reduced susceptibility to antimicrobials for this organism has been reported and this contributed to difficulties in controlling the infection (Gautier-Bouchardon, 2018). This highlights the importance of continuing research to control *M. bovis* infections in veal calves.

M. dispar and *M. bovirhinis* were also frequently detected in this study. These two Mycoplasmas are regularly isolated from the nasal cavity of cattle with respiratory disease and can act as non-pathogenic commensals in the respiratory tract (Nicholas, 2011).

Generally, all BRD-associated viral and bacterial pathogens that could be expected to be associated with the clinical signs of BRD were detected in BALF samples of the calves during the outbreaks of respiratory disease (Table 1, Fig. 2). This did however not imply that these agents were identified as the causative agents of the disease. Most outbreaks occurred in the first six weeks of the study (between moment of

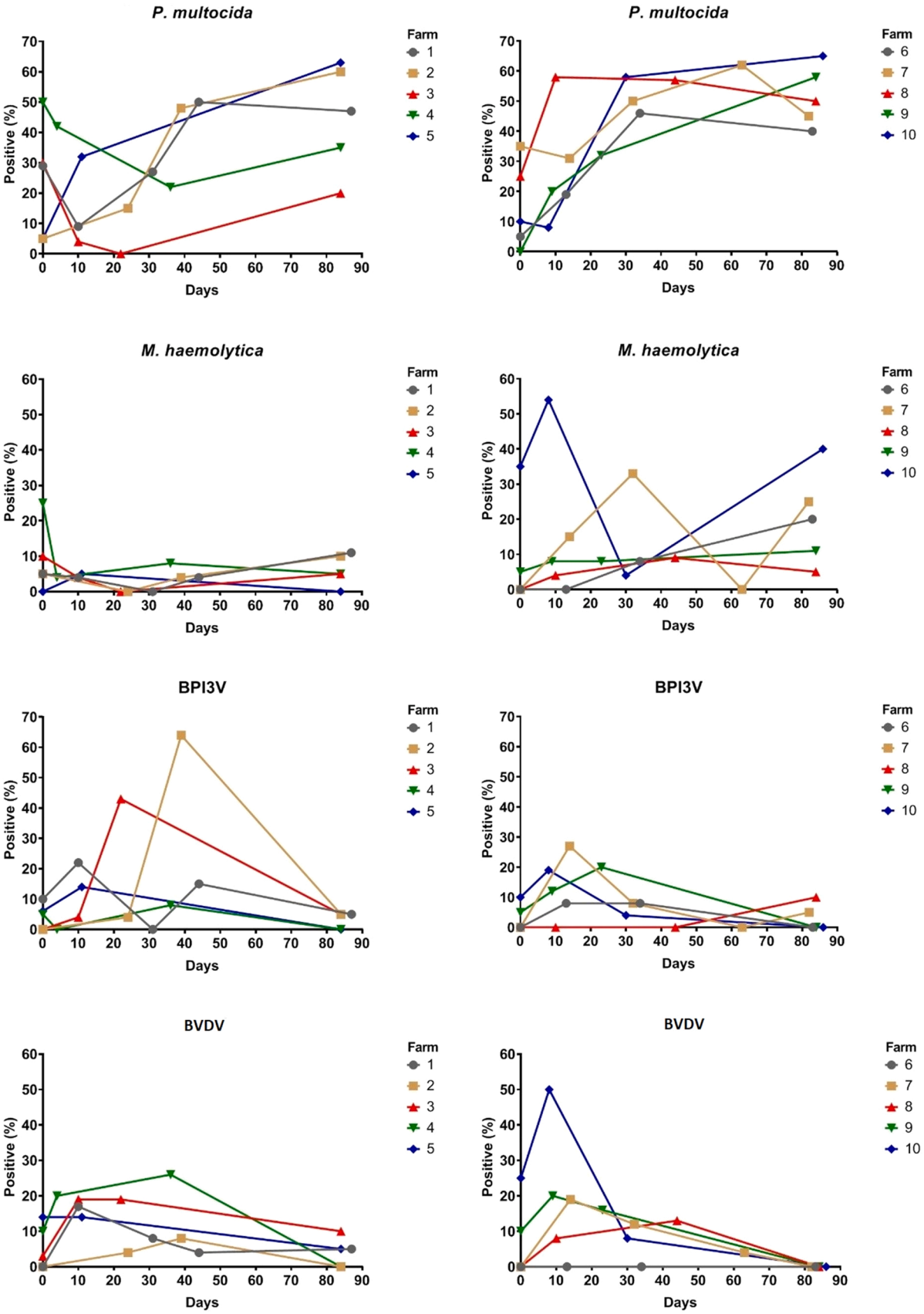

Fig. 2. Percentage BALF samples positive for *P. multocida, M. haemolytica*, PI3V and BVDV on ten veal farms, on D0 and D84 and on time points in between where there was an outbreak of BRD. For outbreaks the percentage positive is across both healthy and diseased animals, whereas at D0 and D84 only healthy animals were sampled unless there was an outbreak (which happened on D0 for two farms). For readability of these graphs we have omitted the error bars on the percentage values. For a representative example with error bars we refer to the Supplementary Figure in the supplementary materials.

Table 3
Overview of clinical scores of diseased veal calves.

Criterium	Respiratory rate (%)			Coughing (%)				Ocular and/or nasal discharge (%)				General impression (%)			
Score	50–70	71–100	> 100	0	1	2	3	0	1	2	3	0	1	2	3
Number of calves	145 (66.5)	66 (30.3)	7 (3.2)	97 (46.4)	99 (47.4)	13 (6.2)	0 (0)	104 (48.8)	85 (39.9)	24 (11.3)	0 (0)	42 (20.2)	133 (63.9)	32 (15.4)	1 (0.5)
Total	218			209				213				208			

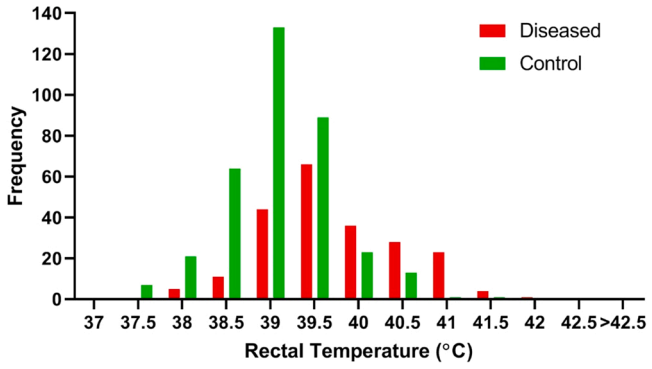

Fig. 3. Frequency of rectal temperatures of diseased and control calves.

arrival and 59–70 days of age). Outbreaks differed between EE and BG calves, but an age component cannot be ruled out. In subsequent outbreaks on the same farm the BRD-associated pathogens found were considerably different from earlier outbreaks on the same farm. Pardon et al. (2011) also found differences in the pathogens detected in subsequent outbreaks.

The results overall do not give a clear indication of primary association of a specific pathogen with respiratory disease. Some BRD-associated pathogens were only seen during outbreaks or with higher prevalence during outbreaks than either on D0 and D84; particularly BPI3V and BRSV. BRSV was only found rarely, and mostly in first outbreaks. This may suggest a primary association, but it cannot be concluded on the basis of the results of this study, also because of the fact that the differences were not significant. This study does therefore not provide support for the premise that finding BRD associated pathogens and identifying them at species level might aid in directing treatment regimes. The fact that both diseased and control animals carried the micro-organisms may be explained by the fact that both control and diseased calves were usually within the same groups and therefore in close proximity. The way a distinction was made in this study between control and diseased calves may have relevance here as well. There is no absolute certainty that control calves were indeed healthy, or were affected without showing overt signs. Leenen et al. (2020) showed, that clinically healthy calves could harbour pneumonia that could be identified through ultrasonography in calves. Buczinski et al. (2014) concluded that clinical examination did not identify all individuals with lung lesions.

The fact that the number of metaphylactic treatments of groups of calves is variable between farms, as well as the large differences in the number of individual treatments, and the use of second choice antibiotics on several farms, is notable (Bokma et al., 2019, Bos et al., 2013). Second and further choice antimicrobials should be used as little as possible, in order to make sure that there are sufficient go-to treatments available in case of antimicrobial resistance and to protect availability of essential antimicrobials as much as possible.

The significant difference in the moment of onset of BRD between calves on farms with calves from EE and from BG may be associated with the difference in average age on D0, with BG herds having an average age of 18 days, and EE herds of 25 days. Pardon et al. (2015) found an association with the onset of BRD in calves and the level of immunoglobulins (Ig). Calves with Ig levels under 7.5 g/L had an increased risk of developing BRD. Whether that means that there is a causal relationship is not clear. They also found that if calves were serologically negative for BCV or BRSV, they were at a higher risk of developing BRD. As the EE calves in our study were older, it is possible that they had further reduced Ig levels than the BG calves. As Pardon et al. (2015) state, at this age, Ig levels are determined by the amount of colostrum the calves have been provided with. Having originated from Eastern Europe, transport duration may also have affected the immune status negatively (Hudson et al., 2020). It is also possible that age-dependent immunocompetence, causing differences in the pathogenesis of infections depending on the age of the calves, plays a role (Antonis et al., 2010).

Knowing what causes a disease, is a prerequisite for an effective treatment. BRD is the major reason for treating calves in the veal sector. If the causative agent is a bacterium, an antimicrobial treatment might

Table 4
Overview of mortality, rejected lungs and antibiotic use.

Farm	Number[1]	Number dead / emergency slaughter	Percentage loss	Percentage condemned lungs	Number of individual treatments with antibiotics	Average number of Individual treatments per animal	Number of herd treatments	Percentage of animals individually treated for BRD	Percentage of individual treatments with 2nd choice antibiotic per animal
1	54	1	2%	4%	36	0.67	5		33
2	92	3	3%	0%	95	1.03	2		1
3	100	5	5%	3%	NA		3		NA
4	84	5	6%	4%	80	0.95	6	71	11
5	84	3	4%	0%	12	0.14	6		0
6*	100	5	5%	2%		0.60	4	34	17
7	100	0	0%	3%	95	0.95	4		38
8	99	6	6%	6%	70	0.71	3		0
9*	99	7	7%	1%		1.27	3	80	13
10	87	1	1%	1%	162	1.86	3		33
Totaal	899	36	4%	2%		0.93		62	

NA: no data on antibiotic use available at detailed level.
[1] Calves of which the chest circumference has been determined on D0.
* Antibiotic use information is present, but not (or not yet) in an analysable format.

be considered. If the causative agent is a virus, an antimicrobial treatment might not be necessary or even unwanted. Understanding BRD and the role of potential pathogens in BRD is pivotal for the development of different control strategies, which might be an effective immunisation strategy, improved biosecurity but also system adjustments. These control strategies will contribute in the transition towards a more sustainable veal industry.

5. Conclusions

While a variety of potential pathogens could be detected during outbreaks of BRD in veal calves, it seems difficult to associate specific pathogens to outbreaks at the species level. This covers the first two aims of the study. BRD is the major reason for treatment with antimicrobials; reduction of antimicrobial use in the veal sector could be advanced with more specific knowledge about the association with (specific strains of) pathogens and health/disease.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2022.109571.

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