Bacterial and viral pathogens of bovine respiratory disease in veal calves during the first 12 weeks of the fattening period.

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Abstract

Pathogens causing bovine respiratory disease (BRD) were investigated during the first 12 weeks of the fattening period at 10 veal farms. Broncho-alveolar lavage samples were taken from 20 calves at arrival, 84 days after arrival and before an antibiotic therapy was used in case of an outbreak of BRD. All samples were tested for viruses: BHV1, PI3, BRSV, BVDV and BCV, and bacteria: *M. haemolytica, P. multocida, H. somni, A. pyogenes.* At arrival at the farm, 15,7% of the calves had bacteria in their lungs and 26,7% had viruses in their lungs. This percentage increased during outbreaks of BRD. The percentage calves with bacteria in their lungs increased to 60,5% at 12 weeks. *P. multocida,* PI3 and BCV were most commonly found during the course of the study.

Introduction

The white veal industry is specialized in raising calves at a lower iron diet to obtain pale meat. Calves housed in the Dutch veal industry are mostly males and originate from dairy cattle farms located in many different European countries. Despite good care, some diseases are common among veal calves. Apart from diarrhoea, arthritis and otitis, bovine respiratory disease (BRD) is very significant because it is the most common disease and the second cause of mortality in veal (Pardon *et al.,* 2012).

BRD is one of the major contributors to reduced animal welfare because of their severe clinical signs. The most frequent symptoms seen in calves with respiratory disorders are rapid breathing, abdominal breathing, serous to purulent nasal and ocular discharge, coughing and fever. Calves can also show a loss of appetite, dehydration and reduced growth(Leruste *et al.,* 2012; Pardon *et al.,* 2012; Snowder *et al.,* 2006).

BRD is responsible for a lower carcass weight, and a poor meat quality and colour. and increase the use of antibiotics (Pardon *et al.,* 2013) Respiratory diseases causes great economic losses in the veal calf sector worldwide (Snowder *et al.,* 2006).

Many risk factors play an important role in the pathogenesis of BRD in the veal industry. Risk factors for BRD mentioned in the literature are a low body weight on arrival at the veal fattening farm, long and poor transport conditions, farmer experience to anticipate on problems, and the season of arrival at the farm. Arrival in autumn is associated with more respiratory disorders (Bähler *et al.,* 2012; Briscic *et al.,* 2012; Moore *et al.,* 2002). Other risk factors are the housing conditions at the farm. The risk of infection is higher in the absence of individual baby-boxes at the start, and at average group size of >15 calves/pen (Briscic *et al.,* 2012)

Treatment of calves with bovine respiratory disease usually consists of antimicrobial treatment with antibiotics. The use of antibiotics is restricted to certain conditions to minimize selection for antibiotic resistance development of bacteria and the consequences for human health. The WHO has given some advices and measures to prevent further resistance development and to restrict this impacts on human health(D'Hoe *et al.,* 2012; Gezondheidsraad, 2011) Their advices for the agricultural sector have resulted in an increased attention for the guidelines of the use of antibiotics(D'Hoe *et al.,* 2012). Thereby the use of antibiotics is more restricted.

Presence of antimicrobial resistance has been reported of several BRD pathogens (Hendriksen *et al.,* 2008; Kehrenberg *et al.,* 2001; Lubbers *et al.,* 2013) Considerable resistance of *Pasteurella* spp. and *Mannheimia* spp. exist against ampicillin, oxytetracycline, trimethoprim/sulfamethoxale, tylosin and tilmicosin (Catry *et al.,* 2002; Catry *et al.,* 2005; Rérat *et al.,* 2012; Vogel *et al.,* 2001). Resistance to various antibiotics has been shown by Mycoplasma *bovis.* This pathogen is less susceptible to gentamicin, tylocin, spectinomycin, lincomycin, tetracycline and oxytetracycline(Thomas *et al.,* 2003).

Incorrect use of antibiotics cause a selection of antibiotic resistant bacteria Lees *et al*.,(2009) It is important to know which pathogens are present in order to make a correct choice of therapy.

This study is part of a larger study into the causes of BRD during the first 12 weeks of the fattening period. The aim of this study is to examine both the prevalence of bacterial and viral pathogens in the lower respiratory tract of veal calves when they arrive at the veal farms and the development of their prevalence during the first 12 weeks of the fattening period. The second aim is to get an understanding of the prevalence of respiratory pathogens in the lower respiratory tract of veal calves when an antibiotic therapy is used to treat the herd against BRD. This information can be used in the future to support the decision to use an antibacterial therapy in case of BRD.

Material and methods

animal and sampling

The two main veal integrations in the Netherlands (Van Drie Group and Denkavit) selected 10 white veal farms with at least 300 animals for this study. Al these farms work with the group housing system. The farms were divided in 2 groups: 5 were populated with calves from the Benelux or Germany and 5 with calves from Eastern European countries.

As soon as the calves arrived at the veal farm (Day 1), bronchial alveolar lavage samples of 20 healthy calves with score 0 according to table 1 were collected. These 20 calves were selected based on their individual cage number using a random number generator. The same calves were sampled at the end of the study on the 84th day, unless they were found ill.

In addition to the sampling at day 1 and day 84, bronchial alveolar lavage samples (BAL) were also collected when the local veterinarian diagnosed a BRD outbreak. An outbreak was defined as the situation in which antibiotic intervention was found necessary by the local veterinarian. When an outbreak occurred, 10 healthy (controls) and 16 diseased (case) calves were sampled immediately before the start of the antibiotic intervention. Selection of case and control calves was based on table 1. Again, a calf with score 0 was classified healthy, all other calves as diseased. This calves were randomly chosen from the healthy and diseased calves throughout the barn. Control calves were clinical observed again 3 days later to confirm that they were correctly classified as controls and excluded from the study when they were found sick.

Parameter	Score 0 (absent)	Score 1 (mild)	Score 2 (moderate)	Score 3 (severe)
Overall impression	alert normal appetite normal behaviour	reduced response reduced appetite	listlessness, clearly slow, clearly reduced appetite. calf separates himself from the group	soporeus, hardly react to stimuli, none appetite, can't stand without help
Eye and/or nasal discharge	none	alternately serous and mucus	increased. persistent mucus or serous with purulent (with/yellow) discharge	severe eye and/or nasal discharge, persistent purulent or haemorrhagic
coughing	none	occasionally spontaneous (or induced) dry (none productive) chough	frequently spontaneous (or induced) dry or chough	frequent spontaneous productive cough, induced cough results in coughing
stuffiness	Normal breathing (RR < 50 breaths/minute)	increased breathing (RR 51-70 breaths/ minute)	increased and/or abdominal breathing (RR 71-100 breaths/minute)	severe increased breathing. calf is clearly stuffiness. (breathing with stretched neck, foam at the mouth.) (RR > 100 breaths/ minute)

Table 1. Clinical score sheet used to assess the health of a calf. A calf with score 0 was classified healthy, all other calves as diseased.

BAL samples were collected by introducing a probe through the nose into the trachea. When the probe could not be introduced further, 100mL lavage fluid (Phosphate Buffered Saline, PBS, Gibco®PBS) was introduced , followed by an attempt to aspirate at least 40mL back with the same syringe. The obtained fluid was divided over two Falcon tubes with a growth medium, cooled and

send to Central Veterinary Institute in Lelystad for laboratory diagnostic. When less than 40 ml was obtained after aspiration, another 100mL PBS was introduced and again followed by an attempt to aspirate at least 40mL.

Bacteriology

For bacteriology, 10mL of lung lavage was pipetted into a 15 mL Falcontube. This Falcontube was centrifuged for 10 minutes at 4500 rpm and pellet was divided over 2 tubes. (80µL per each micronictube) Glycerol was added to 1 tube. This tube was stored at -80°C. Material of the other tube was used for bacterial culture at HIS and chocolate plates. This plates were incubated at 36°C during 48 hour. The plates were examined at 24 and 48 hour incubation on the bacteria *Mannheimia haemolytica, Pasteurella multocida, Trueperella pyogenes* and *Histophilus somni*. The results were conformed with the malditof.

Virology

The remainder of the lung lavage was centrifuged for 10 minutes at 1100 rpm and 4°C. This material is used for virology. The material was divided over cups with bacteriostatic medium and heated to 37°C. The medium was aspirated and diluted. Then 200 microliters of the same medium was added. Then, 1 ml of medium was added to a cell culture that had been incubated for 2 hours at 37°C in order to obtain a good attachment of the virus to the cells. After 3 days BHV and BCV were visible and after one week the other viruses are visible. The samples were tested to the viruses Bovine Herpes Virus type 1, Bovine Para Influenza Virus type 3, Bovine Respiratory Syncytial Virus Bovine Viral Diarrhea Virus and Bovine Corona Virus. The results were conformed with the malditof.

Results

In total 946 samples were collected at the 10 participating vealfarms, At day 1 and 84 were 400 samples collected and 546 samples were collected during the first 12 weeks of the fattening period when BRD occurred. 4 farms had calves derived from Eastern Europe countries. The other 6 farms had calves from the Benelux and Germany. Calves were on average 14 days old when they arrived on the vealfarms.

day 1 (See table 2.)

In 27.9% of the BAL samples collected from the calves on arrival (day 1) at least one bacterial species was detected. The most common bacterial species at the sampling on day 1 was *P. multocida.* This bacteria was detected in 64,2% of the BAL samples with bacteria and was present in calves on 7 farms. *H. somni* was the less common bacteria species, it was detected in 0,9% of the BAL samples and on one farm only. 4,7% of the BAL samples contained 2 different bacteria species. 67% of this results are derived from 1 farm. In this BAL samples was the combination of *P. multocida* with *M. haemolytica* or with *T. pyogenes* equally present. There were no calves with more than 2 different bacterial species in their BAL samples at the day of arrival.

In 15,6% of the BAL samples at least one virus species was detected, while in 0,9% of the BAL samples 2 different virus species and in 0,5% of the BAL samples 3 different virus species were found.

BVDV was the most detected virus with 6,6%. It was present in the BAL samples of 6 farms. BRSV was detected in 0,5% of the BAL and only at 1 farm, BHV1 at 3 farms, PI3V at 6 farms and BCV at 4 farms. There were on average 3 different viruses present at each farm. The amount of different viruses at each farm differ from 0 to 4. In 5,2% of the BAL samples was at least both 1 bacterial and 1 viral species detected.

sampling	BRSV	BVDV	BHV1	PI3V	BCV	*M. haemolytica*	*P. multocida*	*T. pyogenes*	*H. somni*
day 1	0,5% (1)	6,6% (6)	4,7% (3)	4,3% (6)	1,9% (4)	8,3% (6)	19,3% (9)	5,7% (5)	1,0% (1)
day 84	0% (0)	2,0% (3)	0% (0)	3,5% (6)	6,0% (8)	13,0% (9)	48,2% (10)	8,5% (8)	1,5% (1)

table 2. results of the samplings of all calves at day 1 and 84 on the vealfarm. In parenthesis the number of farms where the pathogens are found.

day 84 (see table 2)

The calves were on average 84 (84-87) days present on the vealfarm. In 60.5% of the samples collected at day 84 from the calves, that maintained healthy during the first 12 weeks of the fattening period, we found at least one bacterial species. *P. multocida* was the most common bacterial pathogen. It was present at all farms and detected in 48,2% of the BAL samples. *H. somni* was the less frequently detected bacteria. It was present at 2 farms and in 2% of the BAL samples. *M. haemolytica* was present in the BAL samplings at 9 farms and *T. pyogenes* at 7 farms.
There were on average 3 different bacterial pathogens found at each farm but distributed over different calves. 7% of the calves had 2 different bacterial species in there BAL samples, 2% had 3 bacterial species and 1% had 4 different bacterial species in there BAL sample.

In 10% of the BAL samples at least one virus species was detected. BRSV and BHV1 were no longer present at any farm. BCV which was detected in 6,0% of the BAL samples was the most common virus at this sampling at 8 farms the most common virus at this sampling. BVDV and PI3V were present on respectively 4 and 6 farms. On 6 farms were 2 different viruses found in the BAL samples, but only 1,5% of the calves had 2 different viral species in their BAL samples. There were no calves with more than 2 different virus species in their BAL samples. At 40% of the farms there was a combination of both 3 different bacterial and 2 different viral species found in the BAL samplings.

	days on farm	BRSV	BVDV	BHV1	PI3V	BCV	M. haemolytica	P. multocida	T. pyogenes	H. somni
Benelux	1	0,9%	6,5%	4,2%	2,5%	1,7%	6,7%	14,2%	6,7%	1,7%
E. Europe	1	0,0%	6,8%	5,4%	6,5%	2,2%	11,1%	27,8%	4,2%	0,0%
Benelux	84-87	0,0%	0,8%	0,0%	3,3%	7,5%	16,7%	53,3%	9,2%	0,0%
E. Europe	84-87	0,0%	3,8%	0,0%	3,8%	3,8%	7,5%	40,0%	7,5%	3,8%

Table 3. The results of the samplings on day 1 and 84 of the fattening period. The results are divided bij origin of the calves. Calves are deriven from Belgium, The Netherlands, Luxembourg and Germany (Benelux) or from Eastern Europe countries. (E. Europe)

Calf origin

Calves originating from Eastern European countries had both more bacterial and viral pathogens in their BAL samples when they arrived at the vealfarms. The difference is only significant in case of *P. multocida* (P = 0,02). BRSV and *H. Somni* were present in the BAL of calves from the Benelux and Germany but not in in the BAL of the calves from Eastern European on the day of arrival. See table 3. In more BAL samples of calves from Germany and the Benelux were bacteria found than in the samples of calves from Eastern Europe when the calves were 84 days on the vealfarms. The differences are not significant (P> 0,05).

sampling	BRSV	BVDV	BHV1	PI3V	BCV	M. haemolytica	P. multocida	T. pyogenes	H. somni	age in days
1	7,8% (7)	17,1% (9)	2,3% (3)	11,7% (8)	11,3% (10)	10,2% (8)	24,3% (10)	13,3% (9)	0,4% (1)	11 (4-24)
2	3,0% (2)	12,0% (8)	0,9% (2)	17,1% (7)	12,0% (5)	7,8% (7)	38,3% (8)	10,8% (7)	0,0% (0)	32 (22-44)
3	0% (0)	3,8% (2)	1,9% (1)	7,7% (1)	1,9% (1)	1,9% (1)	55,8% (2)	1,9% (1)	0,0% (0)	54 (44-63)

Table 4. Results of the samplings per outbreaks of BRD. In parenthesis the number of farms where the pathogens are found. In parenthesis the number of farms were the pathogens are found. Age in days is the average age with in parenthesis the distribution in days.

BRD outbreaks. (See Table 4)

Most farms had 2 outbreaks of BRD in the first 12 weeks of the fattening period. Farm 5 had only 1 outbreak and farm 3 and 7 had 3 outbreaks. BAL samples were taken when at least 10% of the calves had clinical signs of respiratory disease. The bacterial samplings of one sampling were contaminated, these samples were not included in the results.

Calves were on average 11 days present at the vealfarm when the first outbreak of BRD occurred. In 41,2% of the BAL samples of diseased calves from the first outbreak, at least one viral species and in 42% of samples one bacterial species was detected. BVDV was the most detected (17.1%) and BHV1 the less detected (2,3%) virus during this sampling. BHV1 was only present at 3 farms. BCV was present at all farms. The bacteria *P. multocida* was detected in 24,3% of the BAL samples and present at all farms. *M. haemolytica* and *T. pyogenes* were respectively present in 10,2% and 13,3% of the samples and were present at most farms. *H. somni* is the least common bacteria and was only found in one BAL sample at one farm. 10,9% of the calves had both a bacteria and a virus in the BAL sample. Except BHV1 and *H. somni*, all the pathogens were more present in the BAL samples of the first outbreak compared with the sampling on day 1.

A second outbreak of BRD occurred at 9 farms. Calves were on average 32 days present at the vealfarm. 37,6% of the BAL samples contained at least one virus species and 45,7% of the BAL samples contained at least one bacterial species. BVDV was present in 12% of the BAL samples and at 8 farms. BCV was also present in 12% of the BAL samples but at 5 farms. BHV1 was the least detected virus, it was only detected in 0,9% of the BAL samples and at 2 farms. *P. multocida* was the most common bacteria. It was present in 38,3% of the BAL samples and on 8 farms. *H. somni* was not detected during the second outbreak. 12% of the calves had both a bacterial and a virus species in the BAL sample.

A third outbreak of BRD occurred at 2 farms only. The calves were on average 54 day present at the farms. In 15,4% of the BAL samples could a virus be detected and in 59,6% of the BAL samples a bacteria. There were no samples with more than 1 different bacterial or viral species. 9,6% of the samples contained both a bacteria and a virus species. The most common virus was PI3V(7,7%). The most common bacteria was *P. multocida*(55,8%). BRSV and *H. somni* were not found in the BAL samples of the third BRD outbreak.

outbreak		BRSV	BVDV	BHV1	PI3V	BCV	M. haemolytica	P. multocida	T. pyogenes	H. somni
1	control	6,9%	13,8%	3,1%	11,3%	8,8%	9,4%	24,4%	6,9%	0,0%
1	diseased	9,3%	21,6%	1,0%	12,4%	15,5%	11,3%	23,7%	23,7%	1,0%
2	control	2,8%	9,9%	0,7%	13,4%	11,3%	5,6%	39,4%	10,6%	0,0%
2	diseased	3,3%	15,2%	1,1%	22,8%	13,0%	10,9%	34,8%	10,9%	0,0%
3	control	0,0%	3,2%	3,2%	6,5%	3,2%	3,2%	61,3%	0,0%	0,0%
3	diseased	0,0%	4,8%	0,0%	9,5%	0,0%	0,0%	47,6%	4,8%	0,0%

table 5. Results of the samplings of the three BRD outbreaks devided by control and BRD calves.

In Table 5 the results of control calves and calves with BRD are separately displayed. The average percentages of bacteria and viruses in the BAL samples of calves with BRD and the percentages of viruses in the BAL samples of control calves decreased during the period from the first to the third outbreak. The percentages bacteria in the BAL samples of control calves increased during the period from the first to the third outbreak.
Calves with BRD had in all samplings the highest percentages BVDV, BPI3V and *T. pyogenes* in their BAL samples compared with control calves. *P. multocida* was in each sampling less present in the BRD calves than the control calves. *M. haemolytica* was in the first two outbreaks more present in the BRD calves, but absent in BRD calves in the third outbreak. The percentage *M. haemolytica* was increased in the third outbreak.

virus	BRSV			BVDV			BHV1			BPI3V			BCV		
outbreak	OR	95BI	P	OR	95BI	P	OR	95BI	P	OR	95BI	P	OR	95BI	P
1	1,4	0,55-3,47	0,497	1,7	0,90-3,35	0,102	0,3	0,04-2,81	0,310	1,1	0,51-2,43	0,798	1,9	0,88-4,15	0,103
2	1,2	0,25-5,32	0,375	1,6	0,74-3,62	0,025	1,5	0,10-25,08	0,820	1,9	0,96-3,80	0,008	1,2	0,53-2,63	0,027
3	-	-	-	1,5	0,09-1,50	< 0,000	0,0	-	-	0,7	0,08-0,66	< 0,000	0,0	-	-

bacteria	*M. haemolytica*			*P. multocida*			*T. pyogenes*			*H. somni*		
outbreak	OR	95BI	P	OR	95BI	P	OR	95BI	P	OR	95BI	P
1	1,2	0,54-2,81	0,626	1,0	0,53-1,74	0,912	4,2	1,95-9,10	< 0,000	-	-	-
2	2,0	0,77-5,39	0,082	0,8	0,47-1,41	0,001	1,0	0,44-2,41	0,039	-	-	-
3	0,0	-	-	0,57	0,19-0,57	< 0,000	-	-	-	-	-	-

Table 6. Odds ratios and 95% confidence interval for the presence of respiratory pathogens in calves with clinical signs of BRD compared with healthy calves 95BI = 95% confidence interval, OR = Odds Ratio.

The Odds Ratios with 95 % confidence intervals are calculated for the presence of respiratory pathogens in calves with clinical signs of BRD compared with healthy calves based on Altema *et al.,* (2011). Only *T. pyogenes* was during the first outbreak significant more found in case than control calves (P< 0,05). Al other difference in prevalence of pathogens between diseased and healthy calves are not significant. During the second outbreak BVDV, PI3V and BCV were significant more often detected in the BAL samples of calves suffering from BRD than healthy calves(P<0,05).
The difference of *M. haemolytica* between healthy and diseased calves during the first and second outbreak were not significant. *P. multocida* was equally present in cases and controls during the first outbreak. During the second and the third outbreak, PM was significantly less frequently isolated from cases compared to control calves (table 5 and 6).

Discussion

This study analysed the occurrence of bacterial and viral pathogens in the lower respiratory tract of healthy and diseased veal calves up to the age of 12 weeks. Bronchoalveolar lavages (BAL) were chosen to collect the pathogens from the lungs. BAL is a quick, simple and minimal invasive technique (Nieto *et al.,* 1995). It should be noted that the results are representative of only one area of the lung so the results do not necessarily provide information about the entire anatomical region. However, BAL is regarded as a good technique to get an understanding of the microbial flora of the lower respiratory tract (Allen *et al.,* 1991).

The first objective of the present study was to determine the prevalence of respiratory pathogens in the lower respiratory tract of veal calves when they arrive at the veal farms and to determine the development of their prevalence during the first 12 weeks of the fattening period.
In the present study, respectively 27,9% and 15,6% of clinical healthy calves had at least 1 potential pathogenic bacteria or virus in the lower respiratory tract. We found that 95% of the calves had only 1 bacterial or viral species in their lower respiratory tract. It is not known if this is a normal finding

because in Scopus and Pubmed are no comparable reports available about the microbial flora of the lungs of clinical healthy calves of this age.

Because of the occurrence of respiratory disease shortly after arrival at the vealfarms high percentages of pathogens were expected in the respiratory tract. It could be that the percentages are low in the lower respiratory tract and higher in the upper respiratory tract, but this is not likely because Allen *et al.,* (1991) and Godinho *et al.,* (2007) show that the result of BAL samples are comparable with the results of nasal swabs (excepted in case of *Mycoplasma* spp). The incubation period of the most pathogens is a few days Smith *et al.,* (2015). It could be that only a few calves in the herd have pathogens in their respiratory tract and spread these pathogens to other calves after arrival. This may explain the low percentages of respiratory pathogens in the BAL samples at day 1 and that after an average of 11 days respiratory signs are present to such an extent that a herd treatment is necessary.

At the age of 12 weeks the prevalence of BAL samples with bacteria is increased to 60,5%. The prevalence of viruses is decreased from 15,6% to 10,0%. A limited amount of studies are published about the microbial lung flora of clinical healthy calves. The study of Angen *et al.* (2009) describe in their discussion some articles who found prevalences of *P. multocida* and *Mycoplasma spp.* varying from 17% to 55%. Autio *et al.* (2007)investigated a group of 144 healthy calves and found pathogenic bacteria, *Mycoplasma spp.* and pathogenic bacteria including *Mycoplasma spp.* in respectively 27%, 75% and 83% of the samples. Angen *et al.* (2009) demonstrated a prevalence of 32% of pathogenic bacteria in the samples of calves without showing clinical signs of respiratory disease. This studies confirm the results in this study about the high prevalence of pathogens in the lower respiratory tract of clinical healthy calves.

The results on the association between disease and the detection of respiratory pathogens agree with most other literature. *P. multocida* was the most detected pathogen in both healthy and diseased calves and significantly more present in control calves than in calves with BRD. In other studies *P. multocida* was the most detected bacterial pathogen in calves with BRD. (except *Mycoplasma* spp.) (Allen *et al.,* 1991; Pardon *et al.,* 2011; Autio *et al.,* 2007). However these prevalences are not as high as found in our study . In literature is *P. multocida* also a common pathogen in the lower respiratory tract of healthy control calves. The presenceof *P. multocida* was not associated with clinical signs of respiratory disease. This finding support the opinion that *P. multocida* can be classified as an opportunistic bacteria. Although commonly isolated in the BAL samples, it is not considered to be a primary causative agent of respiratory disease in veal calves (Autio *et al.,* 2007; Griffin, 2010; Catry *et al.,* 2005).

Many immune moderating stress factors as new environmental conditions, transport, abrupt feed changes and contact with other cattle are presumably necessary before *P. multocida* can colonize the lower respiratory tract (griffin , 2010; Woolums, 2015). All this factors are present before the first outbreak. Therefore *P. multocida* had probably a contribution during the first and maybe the second outbreak.

The prevalence of *M. haemolytica* during the outbreaks is within the range reported in literature. Autio *et al.,* (2007), Catry *et al.,* (2002) and Pardon *et al.,* (2010) describe respectively a prevalence of 2%, 11% and 21,5% in the samples of calves with BRD. *M. haemolytica* is particularly present during the first outbreak and is more often found in the BAL samples of diseased calves. But the difference is not significant. *M. haemolytica* is a commensal bacterium of the upper respiratory tract. This can be the explanation of the presence in healthy calves. *M. haemolytica* is able to colonise the lower respiratory tract following a period of stress but virally caused damage to the respiratory tract is necessary for infection (Allen *et al.,* 1991; Griffin, 2010).

During the first outbreak more viruses were detected in the BAL samples of diseased calves compared with the second and third outbreak. Influence of stress of the transport and collection of different calves at one farm can be present during this outbreak. This could be an explanation for the higher percentages demonstrated in de BAL samples of the first sampling because this samples on average have been taken 11 days after arrival on the veal farms.

The percentages of *H. somni* detected in the BAL samples are very low. In literature are comparable percentages described (Autio *et al.,* 2007; Pardon *et al.,* 2011; Woolums*,* 2015). It could be due to the fastidiousness of the organism in culture (Cusack *et al.,* 2003). Only Griffin (2010) described much higher percentages of *H. somni* in both healthy and sick animals but this is about adult cattle. Therefor the contribution of *H. somni* in respiratory disease in veal calves seems to be negligible.

T. pyogenes was significant more present in the BAL samples of diseased calves during the first outbreak. There was no significant difference in the prevalence during the second outbreak. *T. pyogenes* is a very common opportunistic bacteria on the mucous membranes of the upper respiratory tract. It can isolated from up to 100% of upper respiratory tracts and other body sites of healthy animals. *T. pyogenes is* capable of acting as a primary and secondary pathogen causing pneumonia, but a secondary infection following other microbial infection of immune moderating stress is more common(Jost *et al.,* 2005; Ribeiro *et al.,* 2015). This these characteristics of *T. pyogenes* can declare the difference of prevalence between the first and the second outbreak.

Comparable prevalence of BRSV, PI3V and BCV are described in Autio *et al.,* (2007) and Härtel *et al.,*(2004). During the second outbreak BVDV, BPI3V and BCV were significant more often detected in the BAL samples of calves with BRD. During the first outbreak were the prevalences of detected viruses higher, but there was no significant difference between healthy and diseased calves. During the third outbreak BVDV and BPI3V were the only viruses detected in the BAL samples of diseased calves. In general, there seems to be little association between the presence of viruses and clinical disease.

Härtel *et al.,* (2004) suspect that BRSV and BCV are causal pathogens in BRD outbreaks and PI3V not. It does not seem in this results because BRSV was not significant more present in the BAL samples of diseased calves as PI3V. The study of Härtel *et al.,* (2004) have used not only veal calves but also dairy calves. In addition, the calves in this study are older than the calves used in the present study. This can be the explanation of the difference. In the article of Härtel *et al.,* (2004) 80% of the calves have antibodies against BPI3V. Thereby it is possible that BPI3V have caused infection at younger age comparable with the age of the calves in the present study.

The percentages of calves with BRSV in their BAL samples were low in comparison with the other viruses and in comparison with other literature which described the importance of BRSV in the occurrence of BRD (Klem *et al.,* 2013; Valarcher *et al.,* 2007). An explanation can not be found in literature. There could also be an influence of maternal immunity because the general occurrence of BRSV at dairy farms (Smith *et al.,* 2015; Chase *et al.,* 2008).

BHV1 is also demonstrated in a few BAL samples. The prevalence declined during the 12 weeks of the study. The feature of BHV1 to cause a latent infection can be the explanation (Muylkens *et al.,* 2007). BHV1 was not found in samples of the second and third outbreaks. There was possible not enough immunosuppression during this outbreaks to reactivate the BHV1 virus.

BVDV was the most detected virus in the BAL samples of the first outbreak and one of the most detected during the second outbreak. No comparable studies in literature were found because other studies did not test for BVDV, they sample only from dead animals or they use serum. Pardon *et al.,* (2010) found comparable results in serum of calves as we found in this study. BVDV is during the second outbreak significant more often detected in the BAL samples of diseased calves. However the role of BVDV in the pathogenesis of BRD is not clear. It is assumed that BVDV is not capable to cause respiratory disease, but it may facilitate the colonisation of other pathogens in the respiratory tract and has an immunosuppressive effect (Pardon *et al.,* 2010; Richer *et al.,* 1988; Potgieter *et al.,* 1984).

The prevalence of BCV increased during the study period and was during the second outbreak significant more present in the BAL samples of diseased calves. BCV is a virus which is able to cause respiratory disease without secondary bacterial infection (Decaro *et al.,* 2008). However this article describes the presence of maternal immunity up to 2-3 months. Many articles describe the influence of *Mycoplasma* spp. in the etiology of BRD (Angen *et al.,* 2010; Autio *et al.,* 2007; Pardon *et al.,* 2011). There is no information about *Mycoplasma spp.* used in this study because the results

were not available at the moment of writing. *Mycoplasma spp.* can be one of the causative agens in BRD outbreak in which no significant difference were found in the precence of pathogens between healthy and diseased calves. Beside the different results of the presence of bacterial and viral pathogens there is also the influence of non-infectious causes of respiratory disease as decribed in Brscic *et al.,* (2012). This factor can also be a part of the explanation of the different results of this study.

In general, viral infections alone are not sufficient to cause BRD but they are a risk factor for BRD (Cusack *et al.,* 2003). Viruses are believed to predispose to bacterial infection in 2 ways. First, viral agents can cause direct damage to respiratory mucociliary clearance mechanisms, facilitating translocation of bacteria from the upper respiratory tract and establishment of infection in compromised lung. Secondly, viral infection can interfere with the host immune system's ability to respond to bacterial infection (Taylor *et al.,* 2010). This is demonstrated with the viruses BHV1 and BVDV and the secondary bacteria *P. multocida* and *M. haemolytica* (Jericho *et al.,* 1985) whereby a severe fibrinopurulent bronchopneumonia developed in a large part of the lungs.

This observations underline the concept of the multifactorial etiology of BRD (Autio *et al., 2007*) and the required interactions between viral and bacterial pathogens (Bosch *et al.,* 2013). This can be an explanation of the results of the outbreaks in which no significant difference were found between healthy and diseased calves. Probably the different pathogens are not capable to cause the clinical signs of BRD, but is BRD an infectious disease resulting from the interaction of bacterial and viral agents and multiple predisposing stress factors (Autio *et al.,* 2007).

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