Bovine Respiratory Disease: Is there a difference in found pathogens between calves with clinical disease and calves without clinical disease at the same farm?

In 2012 there were 1.990 veal calves farms with a total of 908.370 veal calves in the Netherlands (*PVE, 2013*).
One of the major problems in the feedlot industry is Bovine Respiratory Disease (BRD). BRD is a collective name for diseases of the lower respiratory tract of bovines. The financial damage of BRD is huge because it delays the growth speed, it causes more health problems and it decreases the quality of the carcass (*Edwards, 2010, Apley, 2006*).

BRD can cause different symptoms, the most common symptoms are fever, abnormal breathing and coughing. Symptoms as eye and nasal discharge, growth retardation and anorexia are also frequently seen (*Leruste et al., 2012, Allen et al., 1991, Brscic et al., 2012*).

A combination of multiple factors give rise to BRD. Viruses and bacteria play an important role, but also management strategies, environmental factors and genetic predisposition are important factors. (*Edwards, 2010, DeRosa et al., 2000, Brscic et al., 2012*)

When samples of calves with BRD are tested for pathogens, multiple viruses and/or bacteria can be found in their lungs. The samples taken in this experiment are being tested for 12 specific airway pathogens: *Pasteurella multocida, Mannheimia haemolytica, Trueperella pyogenes, Histophilus somni, Mycoplasma (M.) bovis, M. bovirhinis, M. dispar,* Bovine Respiratory Syncytial Virus (BRSV), Bovine Parainfluenza 3 virus (PI3V), Bovine Viral Diarrhoea Virus (BVDV), Bovine Herpes Virus 1 (BHV1) and the Bovine Coronavirus (*CVI Lelystad, 2013*).

Sometimes these pathogens are also found in the respiratory tract of healthy calves (Edwards, 2010). This means that these pathogens not always cause clinical disease. Some pathogens are commensal for the respiratory tract and need an infection with a primary pathogen before they can contribute to the occurrence of BRD. Usually BRD starts with a viral infection of the upper respiratory tract. This viral infection gives other pathogens a change to catch on in the respiratory tract and to aggrevate the clinical disease or to persist the clinical disease and in this way causes a secondary bacterial infection. If the immune system fails to respond in a way that eliminates the infection this can lead to an infection in the lower respiratory system (*Edwards, 2010*).

The supposed primary pathogens for BRD are the respiratory viruses BRSV, PI3V and BHV1. They can all cause severe respiratory problems *(Thonur et al., 2012*). BHV1 is also immunosuppressive, it inhibits the cell-meditated immunity (*Jones et al., 2011*).
Bovine Coronavirus is a pneumoenteric virus that can cause mild respiratory symptoms or pneumonia (*Saif, L.J., 2010*).

BVDV is not a virus specific for the respiratory tract but it does play an important role in BRD because it is an immunosuppressive virus, the virus infects the macrophages and lymphocytes. Because of the immunosuppressive effect of BVDV the calves infected with BVDV are more at risk for infection with other pathogens. The immuunsystem is less able to defend the calves against pathogens (*Thonur et al., 2012, Edwards, 2010, Al-Haddawi, 2007*).

Pasteurella multocida, Mannheimia haemolytica, Trueperella pyogenes and *Mycoplasma (M.) bovirhinis* are all opportunistic pathogens. They are all found both in healthy calves and in calves with BRD and they need a primary pathogen to cause BRD (*Taylor et al., 2010, Rice et al., 2007, Jost & Billington, 2005, Miles et al., 2004*).
Histophilus somni can be an opportunistic pathogen, but depending on the strain it can also be a primary pathogen or a commensal (*Elswaifi et al., 2012, Sandal & Inzana, 2010*).
M. dispar is also found in both healthy calves and in calves with BRD but an infection with only *M. dispar* can cause the calve to get BRD, although the symptoms observed in these calves are not severe (*Miles et al., 2004*).
M. bovis can also be a primary pathogen but is also found in calves without BRD and it is often found as a co-infection with other pathogens in calves with BRD (*Castillo-Alcala et al., 2012*).

Because a part of the most common pathogens are also found in calves without clinical signs of BRD, the question arises if we can find a specific pathogen in the lungs of the calves with BRD that we don't find in the lungs of healthy calves. This is important to know for the prevention and treatment for calves with BRD, especially in the framework of less antibiotic use in the feedlot industry.
The purpose of this research is to see if there is a difference between the pathogens found in the lungs of veal calves with and without clinical BRD within a farm, to see if the supposed classification of primary and secondary pathogens can be confirmed and to see if the lungs of the sick calves contain more different or other pathogens than the lungs of the healthy calves.

Material and Methods

For this study samples of 10 different veal farms are being taken. All the farms have a barn with at least three hundred veal calves in the barn used for the experiment, they don't have other bovines at their farm (milk producing cattle or beef cattle) and they work with the all-in/all-out method at barn level. Four of these farmers only have calves originating from dairy farms in the Benelux or Germany, while the other six farms only have calves from Eastern European in the barn that is included in the experiment.

As soon as the barn of the farmer is filled with calves, 100 calves are tagged based on a random list made on the computer with Excel. N random numbers are drawn between 0 and 1 with the "rand()" function, where N is the number of animals present in the barn. Each of these N numbers are ranked, using the "rank.eq(…)" function. The first 100 numbers on the ranking list were the pens with individual calves selected and tagged for this study. Twenty

calves got a white tag and served as preferred control calves during an outbreak of BRD. Eighty out of the 100 calves got a blue tag.

When there is a disease outbreak of BRD, just before all the calves in the barn are treated with antibiotics, broncho alveolar lavage is being performed on 16 healthy (control) calves and on 10 sick calves. These calves are chosen from the 100 calves that are tagged before. The clinical observation to see if the calves are healthy or not, was based on a standard form (Table 1).

A calf is marked as a control calf when it scores a zero at all the parameters shown in table 1. If a calf scores at least an one at at least one parameter, it was excluded from being a control calve. This means that if a calf was ill because of a completely different cause then respiratory diseases and his general impression had a score of at least an one it could not be a control calf. The control calves were chosen out of the twenty white tagged calves, when this group of 20 calves did not contain 16 healthy calves, the rest of the calves were chosen out of the 80 blue tagged calves.
In that case the blue tag was replaced for a white tag. If a control calf (white tagged calf) was defined as a calf with BRD the white tag was replaced for a red tag and the calf served as a sick animal during this outbreak.

A calf is sick, was determined as a calve with BRD, when he scores a minimum of one on the scale of oppression, this means that he has a respiration rate of at least 50 breathings per minute. Depending on how many sick calves of the 10 that needed to be sampled were found in the group of calves with white tags, the rest of the sick calves were picked out of the group calves with the blue tags. If the calf was sick and being sampled the blue tag was replaced for a red tag. Once a calf was tagged with a red tag, it could never be a control calf again.

This is done with every outbreak of BRD and the outbreak is defined as outbreak X.

Parameter	Score 0	Score 1	Score 2	Score 3
General impression	Clear, alert, normal appetite, normal behaviour	Decreased response, decreased appetite, no further abnormality's	Lifeless, clearly lethargic, clearly decreased appetite, calf separates itself from the group	Sopor, calf hardly reacts to stimuli, calf doesn't eat and isn't able to stand without help
Eye/nasal discharge	No eye/nasal discharge	Eye/nasal discharge varying aqueous-mucous	Eye- or nasal discharge is increased. Persisted mucous or clear mucus blend with puss like (white/yellow) discharge	Badly eye- or nasal discharge. Persistently puss like or blood-stained
Coughing	No coughing	Occasionally spontaneous or induced dry cough	Frequent spontaneous or induced dry or productive cough	Frequent spontaneous productive cough, induced coughing advances in a cough attack
Oppression	Normal breathing (frequency < 50 respirations/minute)	Accelerated breathing (frequency 51-70 respirations/minute)	Accelerated and/or abdominal breathing (frequency 71-100 respirations/minute)	Accelerated breathing, calf is clearly oppressed, breaths with stretched neck, open mouth and foam in the mouth

Table 1: Clinical observation form to determine if the calf is healthy or sick. Calves with at least a one on the score of impression are marked as calves with BRD and were sampled as sick calves. Calves with a score of 0 on all parameters were sampled as healthy calves (source CVI)

The broncho alveolar lavage was performed by two people. Both people wore gloves, for each calf new gloves were used. One person held the calf, the other inserted a sterile flexible silicon tube of 0.7 mm thick and 103 cm long with one rounded edge in the ventral nostril, the rounded edge first, across the trachea into the lungs. Then a syringe of 100 ml Phosphate Buffered Saline pH 7.2 was sprayed empty into the lungs, immediately followed by the withdrawal of the liquid. For every calf a new (sterile) tube and syringe was used. The sample from the lungs was put in a tube with foetal bovine serum/ foetal calf serum and cooled with ice packs and was sent to the Central Veterinary Institute in Lelystad where the samples were analysed on the following pathogens*: Pasteurella multocida, Mannheimia haemolytica, Trueperella pyogenes*, *Histophilus somni*, BRSV, PI3V, BVDV, BHV1 and the Bovine Coronavirus. Virus isolation was carried out by a standard tissue culture and also a multiplex PCR was performed. To test for present bacteria standard bacteriological research was performed. To test for *M. bovis*, *M. bovirhinis* and *M. dispar* the Central Veterinary Institute sent samples to England to the Animal Health and Veterinary Laboratories Agency (AHVLA).

Three days after the broncho alveolar lavage a clinical observation was done on the control calves to check if they were still healthy and they were not in the incubation period at the sampling day. If control calves were sick during this observation they were regarded as sick calves for the outbreak. The clinical observation to determine whether the control calves were still healthy was done on basis of the score form as shown in table1. The calves needed to score a zero at every parameter to be regarded as healthy and their samples were defined as samples of healthy calves. If they scored at least an one at the parameter oppression their samples were defined as samples of sick calves with BRD. If they scored at least an one at

one of the other parameters and a zero for the parameter oppression their sample was excluded from the experiment.
The results of the found pathogens of every separate outbreak within a farm are compared between the sick and the healthy calves and a 2-sided Fisher's exact test, with $\alpha < 0.05$, is performed to see if the results are significantly different. The Fisher's exact test is used for every pathogen individually and only performed when the pathogen was found as well in the healthy as in the clinical sick calves.

Ik vind het zelf altijd wel handig om de M+M's in de verleden tijd te zetten. Jij hebt tegenwoordige en verleden tijd door elkaar gebruikt. Probeer het wel consequent te houden.

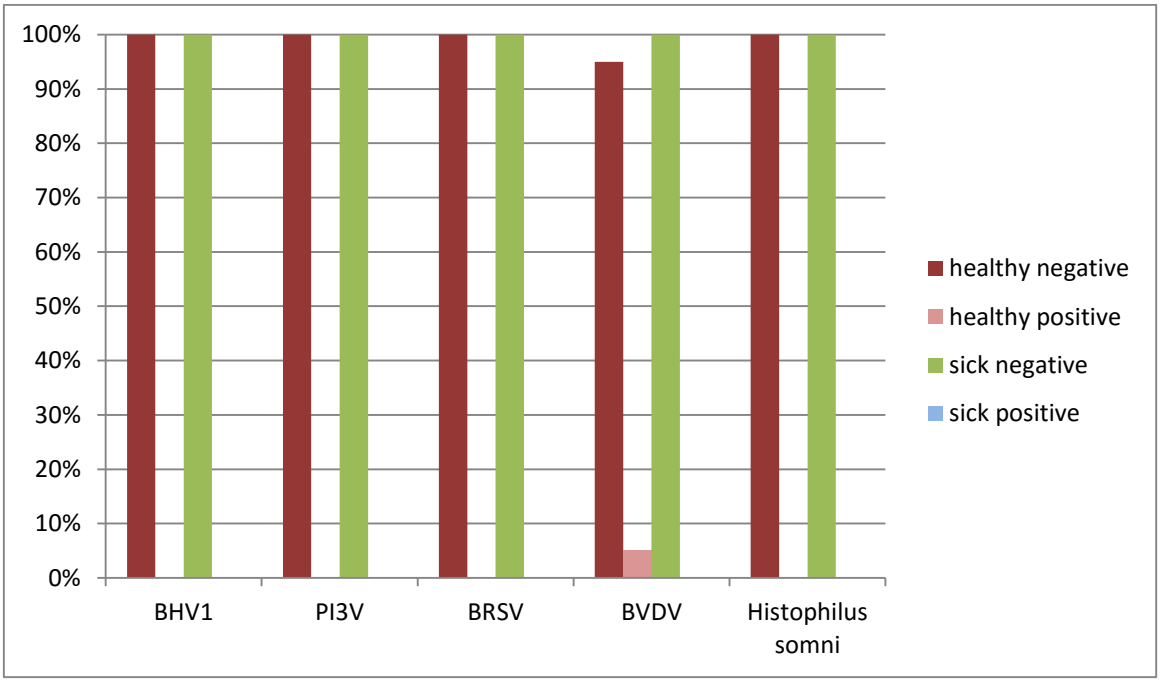
Results

From the outbreaks of BRD the data of 3 outbreaks where available (outbreak 1, 2 and 3). These outbreaks were all from the same farm. The available data of these outbreaks contained 4 or 5 pathogens of the 12 pathogens mentioned earlier. This means that only the data from these three outbreaks and these 5 pathogens, namely BHV1, PI3V, BRSV, BVDV and *Histophilus somi*, are worked with. The other data will not be discussed in this report. A negative result found for a pathogen means that this pathogen was not found in the obtained sample, with a positive result the pathogen was found in the obtained sample.

Outbreak 1
For outbreak 1 30 animals were tested, 20 healthy and 10 sick calves. The data available for analyses included BHV1, PI3V, BRSV, BVDV and *Histophilus somni*. Table 2 gives an overview of the results in number of animals. Graph 1 gives in overview in percentage of the healthy and sick calves and the found pathogens.

	BHV1		PI3V		BRSV		BVDV		*Histophilus somni*	
Result	negative	positive	negative	positive	negative	positive	negative	positive	negative	positive
Healthy	17	0	17	0	17	0	18	1	20	0
Sick	10	0	9	0	9	0	8	0	10	0
Total	27	0	26	0	26	0	26	1	30	0
	27		26		26		27		30	

Table 2: Overview of the found pathogens, the amount, healthy and clinical sick calves that were tested positive/negative for the pathogens. A total of 30 samples were taken, 20 samples of healthy calves, 10 samples of sick calves.
Note: Some values are missing, not for every sample all the results were available.



Graph 1: Percentage of the found pathogens in the samples of outbreak 1.
Healthy negative → the samples of the healthy calves were negative for the pathogen
Healthy positive → the samples of the healthy calves were positive for the pathogen
Sick negative → the samples of the sick calves were negative for the pathogen
Sick positive →the samples of the sick calves were positive for the pathogen
Note: No positive results for any pathogen in the samples of the sick calves (sick positive) were found.

As shown in table 2 and graph 1 only 1 difference in found pathogens was found between the control and sick calves. There was one control calf positive for BVDV.

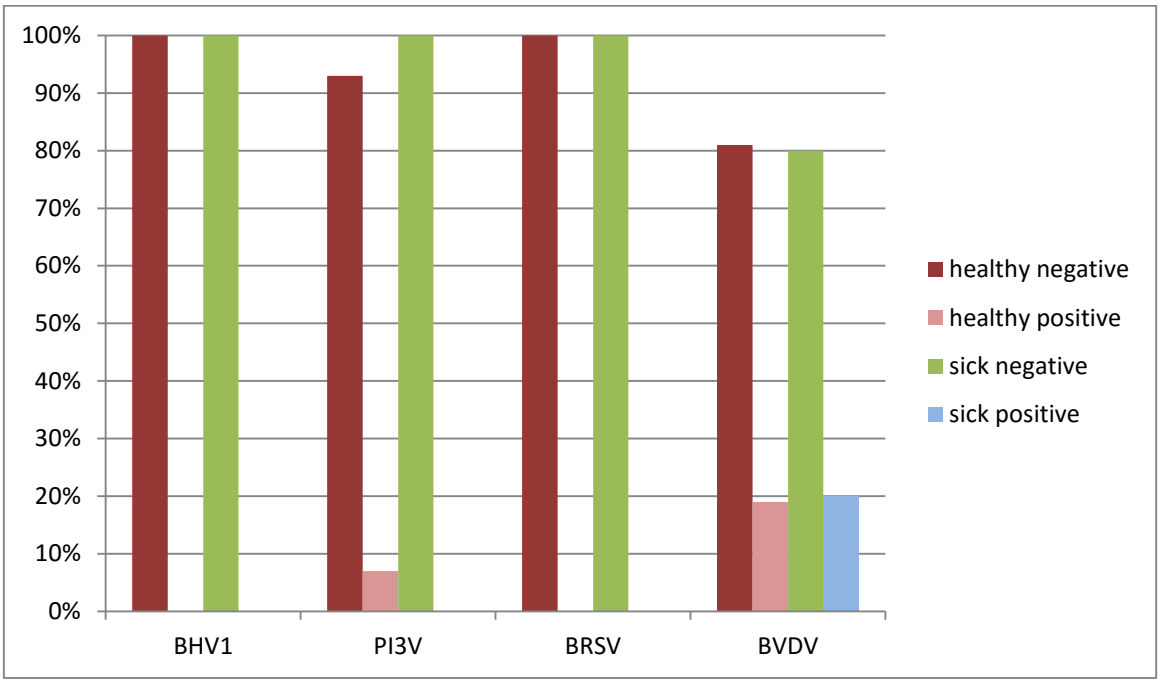
A 2-sided Fisher's exact test was performed for BVDV with a result of $\alpha > 0.05$ ($\alpha = 1.000$) meaning no significant difference was found between the found pathogens in healthy and sick calves for BVDV. This means that there was no significant difference found between found pathogens between the healthy calves and the sick calves for outbreak one. There was not a pathogen that was more present in the sick calves then in the healthy calves or contrariwise.

Outbreak 2

For outbreak 2 26 calves were tested, 16 healthy and 10 sick calves. The available data included the pathogens BHV1, PI3V, BRSV and BVDV. Table 3 gives an overview of the results in numbers and graph 2 gives an overview in percentage of the healthy and sick calves and the found pathogens.

	BHV1		PI3V		BRSV		BVDV	
	negative	positive	negative	positive	negative	positive	negative	positive
Healthy	16	0	14	1	15	0	13	3
Sick	10	0	9	0	9	0	8	2
Total	26	0	23	1	24	0	21	5
	26		24		24		26	

Overview of the found pathogens, the amount, healthy and clinical sick calves that were tested positive/negative for the pathogens. A total of 30 samples were taken, 20 samples of healthy calves, 10 samples of sick calves.
Note: Some values are missing, not for every sample all the results were available.



Graph 2: Percentage of the found pathogens in the samples of outbreak 1.
Healthy negative → the samples of the healthy calves were negative for the pathogen
Healthy positive → the samples of the healthy calves were positive for the pathogen
Sick negative → the samples of the sick calves were negative for the pathogen
Sick positive →the samples of the sick calves were positive for the pathogen

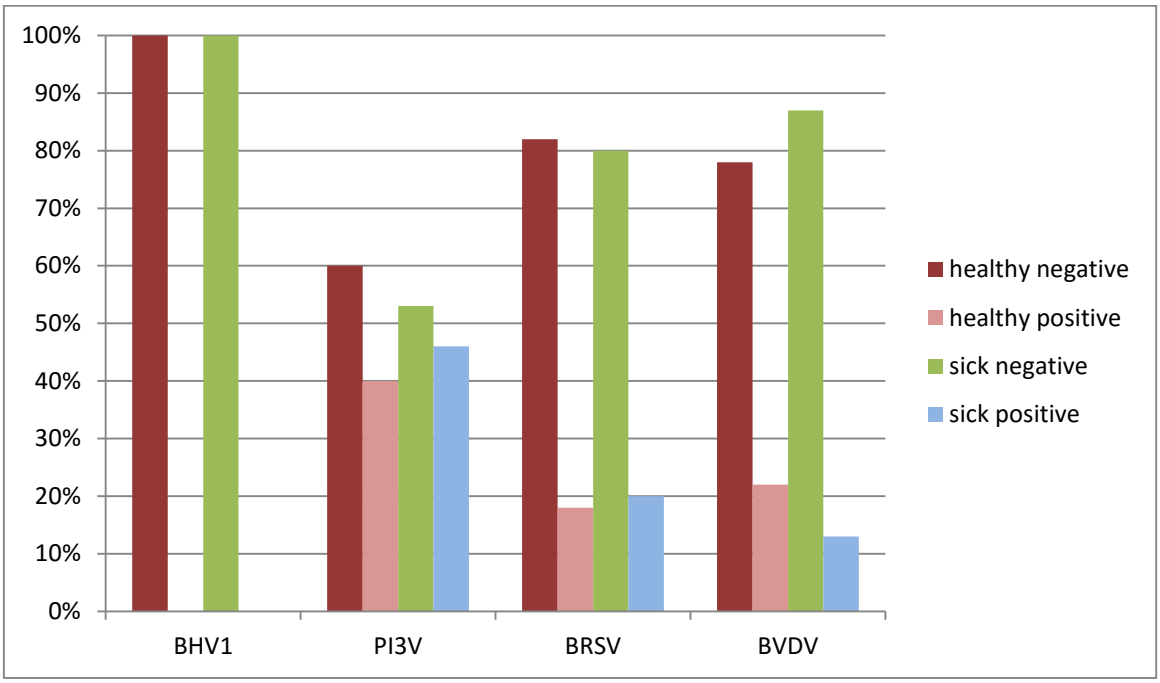
As shown in table 3 and graph 2 there were positive and negative results for PI3V and BVDV. A 2-sided Fisher's exact test was performed for both pathogens. For both pathogens the result was $\alpha > 0.05$ (PI3V $\alpha = 1.000$ and BVDV $\alpha = 1.000$) which means no significant difference was found between the found pathogens in healthy and sick calves, meaning there was no significant difference found in the found pathogens during outbreak 2. This means that there was not a pathogen that was more present in the sick calves then in the healthy calves or contrariwise.

Outbreak 3

For outbreak 3 26 animals were tested, 11 calves were control calves, 15 calves were sick calves. The samples were tested for BHV1, PI3V, BRSV and BVDV. Table 4 gives an overview of the results in numbers and graph 3 gives an overview in percentage of the healthy and sick calves and the found pathogens.

	BHV1		PI3V		BRSV		BVDV	
	negative	positive	negative	positive	negative	positive	negative	Positive
Healthy	11	0	6	4	9	2	7	2
Sick	15	0	8	7	12	3	13	2
Total	26	0	14	11	21	5	20	4
	26		25		26		24	

Overview of the found pathogens, the amount, healthy and clinical sick calves that were tested positive/negative for the pathogens. A total of 30 samples were taken, 20 samples of healthy calves, 10 samples of sick calves.
Note: Some values are missing, not for every sample all the results were available.



Graph 3: Percentage of the found pathogens in the samples of outbreak 1.
Healthy negative → the samples of the healthy calves were negative for the pathogen
Healthy positive → the samples of the healthy calves were positive for the pathogen
Sick negative → the samples of the sick calves were negative for the pathogen
Sick positive →the samples of the sick calves were positive for the pathogen

As shown in table 4 and graph 3 there was no difference found between healthy and sick calves for BHV1, all the samples had a negative result. For the other three pathogens positive and negative results were found for both healthy and sick calves. A 2-sided Fisher's exact test was performed for these three pathogens. For all three pathogens the outcome of the test was $\alpha > 0.05$ (PI3V $\alpha = 1.000$, BRSV $\alpha = 1.000$ and BVDV $\alpha = 0.615$), which means that no difference was found between the found pathogens in healthy and sick calves, meaning there was no significant difference found between the found pathogens during outbreak 3. This means that there was not a pathogen that was more present in the sick calves than in the healthy calves or contrariwise.

Discussion

Although no significant differences was found between the pathogens isolated in the three outbreak samplings, nothing can be concluded about differences between pathogens in the lungs of veal calves with and without BRD within a barn. There was limited data available, both too few outbreaks and too few pathogens were tested. Because only 4 or 5 of the most common pathogens were tested there were a lot of samples from sick calves that did not test positive for any pathogen. This does not mean that the samples did not contain pathogens; it is possible that they contained other pathogens than the one that were tested.

For example outbreak 1 has only one calf tested positive for BVDV, this calf was a clinically healthy calf. This means that no pathogens were found in the sample obtained by broncho alveolar lavage from the clinically sick calves. This means that none of the tested pathogens, BHV1, PI3V, BRSV, BVDV & *Histophilus somi* can be held responsible for the calves being clinically diseased at moment of clinical observation, there was no significant difference in how many positive results were found for any pathogen between the healthy and the sick calves. But from the other pathogens frequently responsible for BRD nothing can be said. The same applies for outbreak 2 and 3: there was no significant difference in how many positive results were found for any pathogen between the healthy and the sick calves. But from the other frequently pathogens responsible for BRD nothing can be concluded.

When samples are collected there is the possibility that a causing agent is present but that the pathogen is not detected in the sample. For example the pathogen could not be stable during transportation of the sample or the test could not be sensitive enough to find the pathogen. This will be discussed per pathogen in the beneath section.

BHV1

No positive results for BHV1 were found. The excretion of the virus after acute respiratory infection is during 10 to14 days. BHV-1 has an incubation period of 2 to 4 days. Furthermore the virus is easily isolated and has a characteristic cytopathic effect (Biswas, 2013). This means that when the BRD of the calves was caused by BHV-1 it was likely to be found in the broncho alveolar lavage sample.

PI3V

Especially during outbreak 3 positive results for PI3V were found for healthy as well as for sick calves. Clinical signs are shown after 2 days after exposure and last 7 to 10 days.
Virus could be isolated from the calves up to a period of months, though it has not been established if this is one primary infection or if it is a matter of reinfection (Ellis, 2010).
But PI3V is a labile virus during storage and transport, which means that it is unstable during transport and this means that it can be very difficult to isolate. The samples of the broncho alveolar lavage were sent to the CVI overnight or over the weekend , meaning that the virus could have been degenerated. So there is a chance that samples were positive for PI3V but were tested negative in the laboratory. The risk for false negative results is bigger for this virus than for stable viruses (Ellis, 2010).

BRSV

Only during outbreak 3 there were calves that tested positive for BRSV. According to *Brodersen* (*2010*) clinical signs as coughing, fever, nasal discharge and tachypnea occur 3 to 9 days after inoculation with the virus. The virus can be detected in samples of the lung up to 8 days after infection. This means that most of the times the clinical signs are present, the virus can be isolated.
But BRSV is a labile virus, comparable to PI3V, which means that it can be very difficult to detect infectious virus. Therefore there is a higher change of false negative results for this virus. (*Sacco, 2013, Sarmiento-Silva, 2012*)

BVDV

During the outbreak a total of 9 calves were found with BVDV, one calve was tested positive during outbreak 1 and the same calve was tested during outbreak 3 and was positive again. Calves with an acute infection with BVDV excrete only low concentrations virus between 7 and 10 days after infection. Calves can be persistently infected, when gestating (seronegative)

cows are infected during pregnancy (up to day 100-120 of gestation), excrete high concentrations of the virus throughout their life. This means that the chance of finding a calve with an acute infection of BVDV is limited and that is most likely that most of these animals are persistently infected. The persistently infected calves can be distinguished from the acute infected calves by testing for antibodies in the blood. (*Løken, 2013*)
This was not done in this study, which means that we can't be sure that it are persistently infected animals.

Histophilus somi
Histophilus somni can be difficult to find in a sample. It will not survive for a long period of time during transport: the sample must be cooled properly or the bacteria will die. Furthermore, *Histophilus somni* is a slowly growing organism and can be masked by antibiotics or by rapidly growing organisms, especially by Pasteurella. With the use of proper growth media and cultural conditions there is less chance to miss *Histophilus somni* when *Pasteurella* is also found. During this research there has not been tested for *Pasteurella*, so nothing can be said if there could be a chance of more false negatives due to *Pasteurella* overgrow. (*Harris & Janzen, 1989*)

Conclusion

The purpose of this research was to see if there is a difference between the pathogens found in the lungs of veal calves with and without BRD within a farm, to see if the supposed classification of primary and secondary pathogens can be confirmed and to see if the lungs of the sick calves contain more or other pathogens than the lungs of the healthy calves.

For this research so far the samples of the broncho alveolar lavages have only been tested for the following pathogens: BHV1, PI3V, BRSV, BVDV and *Histophilus somi*. There were no significant differences found in positive results between healthy and control calves for these five pathogens. The results show that there is not one of these five pathogens that is more often present in the sick calves than in the healthy calves.

With these result the supposed classification of primary and secondary pathogens cannot be confirmed.

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