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Respiratory disease in calves: Microbiological investigations on trans-tracheally aspirated bronchoalveolar fluid and acute phase protein response

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ABSTRACT

Trans-tracheal aspirations from 56 apparently healthy calves and 34 calves with clinical signs of pneumonia were collected in six different herds during September and November 2002. The 90 samples were cultivated and investigated by PCR tests targeting the species *Histophilus somni*, *Mannheimia haemolytica*, *Pasteurella multocida*, *Mycoplasma bovis*, *Mycoplasma dispar*, and *Mycoplasma bovirhinis*. A PCR test amplifying the *lktC-artJ* intergenic region was evaluated and shown to be specific for the two species *M. haemolytica* and *Mannheimia glucosida*. All 90 aspirations were also analyzed for bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus, and bovine corona virus by antigen ELISA. Surprisingly, 63% of the apparently healthy calves harbored potentially pathogenic bacteria in the lower respiratory tract, 60% of these samples contained either pure cultures or many pathogenic bacteria in mixed culture. Among diseased calves, all samples showed growth of pathogenic bacteria in the lower respiratory tract. All of these were classified as pure culture or many pathogenic bacteria in mixed culture. A higher percentage of the samples were positive for all bacterial species in the group of diseased animals compared to the clinically healthy animals, however this difference was only significant for *M. dispar* and *M. bovirhinis*. *M. bovis* was not detected in any of the samples. BRSV was detected in diseased calves in two herds but not in the clinically healthy animals. Among the diseased calves in these two herds a significant increase in haptoglobin and serum amyloid A levels was observed compared to the healthy calves. The results indicate that haptoglobin might be the best choice for detecting disease under field conditions. For *H. somni* and *M. haemolytica*, a higher percentage of the samples were found positive by PCR than by cultivation, whereas the opposite result was found for *P. multocida*. Detection of *P. multocida* by PCR or cultivation was found to be significantly associated with the disease status of the calves. For *H. somni* a similar association with disease status was only observed for cultivation and not for PCR.

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1. Introduction

Respiratory disease in calves causes great economic losses for the dairy and beef industry worldwide (Snowder et al., 2006). Blom (1982) reported that the annual calf mortality between day 2 and 180 was 7% in Danish dairy

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herds. He reported that on average 4.5% of the calves died due to respiratory disease. A mortality rate in the range 1.5–4.2% has also been reported from other countries (Ames, 1997; Andrews, 2000).

In Denmark, bovine respiratory syncytial virus (BRSV) and coronavirus are the most common viral agents found in relation to calf pneumonia (Larsen et al., 1999). *Histophilus somni*, *Pasteurella multocida*, *Mannheimia haemolytica*, and *Arcanobacterium pyogenes* are the bacteria most commonly isolated from calf pneumonia (Tegtmeier et al., 1999). Several *Mycoplasma* species have been isolated in Denmark from bovine lungs. Friis and Krogh (1983) found *Mycoplasma dispar*, *Mycoplasma bovirhinis*, and *Ureaplasma* spp. to be the most prevalent of these, whereas *Mycoplasma bovis* and *Mycoplasma bovigenitalium* were isolated infrequently.

The aim of the present investigation was to study the presence and interaction of bacteria and virus in the lower respiratory tract in calves with and without clinical symptoms of respiratory disease from six Danish herds. In addition, the serum concentrations of haptoglobin and serum amyloid A (SAA) were determined to describe the correlation between the presence of infectious agents and acute phase protein response. In order to avoid contamination from bacteria resident in the upper respiratory tract, the sampling was performed by trans-tracheal aspiration. This method has been recommended as optimal for evaluation of the microbiological status of the lower respiratory tract in order to elucidate the etiology of pneumonia in an animal (Espinasse et al., 1991; Rebbun, 1995; Virtala et al., 1996; Pommier, 1999; Pommier and Wessel, 2002). Finally, an aim was to evaluate whether PCR tests are suitable for obtaining a reliable and quick diagnosis of pneumonia related to bacterial pathogens.

2. Materials and methods

2.1. Herds and samples

The investigation included six dairy herds, which in previous years had experienced problems with calf pneumonia during the winter period. In September 2002, 56 trans-tracheally aspirated samples were taken from clinically healthy animals in these herds (herds 1, 3, 4, and 6: 10 samples; herd 2: 9 samples; herd 5: 7 samples). In November 34 samples from calves suffering from respiratory distress were taken from 4 of the herds (herd 1: 10 samples; herd 3: 13 samples; herd 5: 6 samples; herd 6: 5 samples). No animals were treated by antibiotics at the time of sampling. In two herds (herds 2 and 4), respiratory disease was not observed and, consequently, no samples were taken. In total, 90 trans-tracheal aspirations were obtained.

The first samples were taken during a warm and dry September from clinically healthy calves (rectal temperatures below 39.5 °C, no nasal discharge, no coughing, and an unprovoked respiration frequency lower than 40 min⁻¹). All calves sampled in November showed clinical symptoms of pneumonic disease. Clinical disease was defined as a rectal temperature above 39.5 °C in

connection with nasal discharge, coughing, or an unprovoked respiration frequency higher than 40 min⁻¹. The age of the calves ranged from 14 days to 4 months. The average age of the clinically healthy calves sampled in September was 2.1 months (S.D. = 1.1) and for the pneumonic calves sampled in November 2.8 months (S.D. = 0.9). Only four of the calves were sampled on both occasions (one calf in each of herd 3 and 5, and two calves in herd 6).

Blood samples were taken from all clinically healthy calves in connection with the first visit in September. In addition, paired blood samples (3 weeks interval) were taken in November–December after onset of clinical disease in the four herds where pneumonia was observed.

An area of 3 cm × 3 cm located 7–10 cm distal to the larynx was shaved and decontaminated with 70% alcohol and iodophors. The calves were sedated by intramuscular injection of 0.1–0.2 mg/kg xylazine. After injection of local analgesic (0.5 ml 2% lidocain), a longitudinal incision of 1 cm was made in the midline directly above the trachea. Perforation of trachea was done with an Intraflon[®] 12G between two cartilage rings. A male dog urinary catheter was inserted into the Intraflon[®] and pushed down into the airway until a slight resistance was felt. Between 20 and 40 ml sterile 0.9% NaCl were injected through the catheter and followed by immediate aspiration. This resulted normally in 5–7 ml aspirated fluid.

One milliliter aspirated fluid was centrifuged for 3 min at 16,000 × g. The supernatant was removed and a loopful (approx. 10 µl) of the sedimented material was used for bacterial cultivation. The growth of the different bacterial species was recorded on a semi-quantitative scale (pure culture, many bacteria in mixed culture, mixed culture, few bacteria in mixed culture, no growth). Bacterial identification was done according to the standard procedures of the laboratory (Tegtmeier et al., 1999). For *M. haemolytica*, the identification was based on the methods given by Angen et al. (2002). The identification of *H. somni* was performed by PCR as described by Angen et al. (1998). Cultivation and identification of *Mycoplasma* spp. were performed according to the procedures described by Friis and Krogh (1983). The samples had been stored at –20 °C for 2 years before cultivation for mycoplasmas was initiated.

The collection of samples in the herds was performed in connection with herd diagnosis by a trained veterinary surgeon according to the Law for veterinarians in Denmark and is thereby accepted by the Danish Animal Experiments Inspectorate.

2.2. PCR detection

DNA was extracted by the addition of 200 µl PrepMan[™] Ultra (Applied Biosystems) to the sedimented trans-tracheal sample according to the manufacturer's recommendations. The DNA preparation was stored at –20 °C until used for PCR analysis. Species-specific detection using previously published methods was performed for *H. somni* (Angen et al., 1998), *P. multocida* (Mifflin and Blackall, 2001), *M. dispar* and *M. bovirhinis* (Miles et al., 2004), and *M. bovis* (Subramaniam et al., 1998) using 2 µl of the extracted DNA per reaction.

For *M. haemolytica*, the *lktA-artJ* intergenic region was used for PCR amplification. From pure cultures, one bacterial colony was resuspended in 100 µl distilled water and lysed by boiling for 10 min. One microliter sample from lysed bacteria or 2 µl of DNA extracted by PrepMan™ Ultra was added to respectively 49 and 48 µl prepared reaction mixture containing 10 mM Tris/HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 100 µM of each dNTP, 65 pmol of each primer (forward primer: GTCCTGTGTTTTCATTA-TAAG; reverse primer: CACTCGATAATTATTCTAAATTAG), and 0.5 U of *Taq* polymerase (Applied Biosystems, Foster City, CA, USA). The reaction mixture was covered with 50 µl of paraffin oil. Samples were subjected to an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min in a thermal cycler. Samples of PCR amplification products (10 µl) were subjected to electrophoresis in a 1.2% agarose gel in Tris/borate buffer according to standard protocols. DNA was visualized by UV fluorescence after staining with ethidium bromide. The PCR test was expected to give a specific amplicon of 385 bp. For evaluation of the species specificity of this test, see supplementary material.

2.3. Detection of viral antigens and antibodies to BRSV

The trans-tracheal aspirations were investigated for the presence of antigens from BRSV, parainfluenza-3 virus (PI-3) and corona virus using the ELISA tests described by Uttenhal et al. (1996). Blood samples from diseased calves were tested for IgG₁ antibodies against BRSV according to Uttenhal et al. (2000).

2.4. Measurement of acute phase proteins

Serum haptoglobin and SAA were determined as earlier described (Heegaard et al., 2000). For these analyses, only the sera from diseased calves in herds 1, 3, and 5 were available in addition to the sera from clinically healthy calves in all herds.

2.5. Statistical methods

Differences in frequencies were evaluated using least-squares test statistics and the relationship between disease

status and test results by a logistic regression model evaluated with the chi-square test using the statistical software package S-Plus 6.1 for Windows (Profession Release 1; Insightful). Data from the acute phase protein measurements were analyzed using a two-sided *t*-test. The similarity of the different populations was tested using the Mann–Whitney non-parametric test as some of the sample sets proved to be not normally distributed.

3. Results

3.1. Clinically healthy animals

Among the 56 clinically healthy calves, potentially pathogenic bacteria were detected either by cultivation or PCR from 68% of the animals (Table 1), this number varied from 29 to 90% between the herds (Table 2). Bacteria were cultivated from 33 samples (59%). From 18 of the calves (32%), *H. somni*, *P. multocida*, *M. haemolytica*, or *A. pyogenes* were isolated in high numbers in pure culture or as the dominating flora in a mixed culture. From three of the calves only few *P. multocida* in pure culture was cultivated. *M. dispar* and *M. bovirhinis* were detected by PCR from 21 and 14% of the samples, respectively. *M. bovis* was not detected. Forty-one percent of the samples tested positive in one or more of the PCR tests applied. By cultivation or PCR, *P. multocida* was detected in 48%, *H. somni* in 20%, and *M. haemolytica* in 23% of the calves. *A. pyogenes* was isolated from three of the calves and *Moraxella* sp. from one calf. From 23 samples (41%) two or more bacterial species were detected. Only one of the samples contained virus antigens (corona virus).

3.2. Diseased animals

Among the 34 diseased animals, potentially pathogenic bacteria were isolated in high numbers in pure culture or as the dominant flora in a mixed culture from all animals (Table 1). *P. multocida*, *H. somni*, and *M. haemolytica* were cultivated or detected by PCR in 82, 41, and 29% of the calves, respectively. *A. pyogenes* was isolated from two of the calves and *Moraxella* sp. from one calf. *M. dispar* and *M. bovirhinis* were both detected from 79% of the calves. *M. bovis* was not detected. From 33 of the samples (97%) two or more bacterial species were detected. A higher

Table 1

Bacteriological investigation of trans-tracheally aspirated bronchoalveolar fluid from clinically normal calves (*n* = 56) and calves with pneumonia (*n* = 34) from six Danish herds.

	Clinically normal calves (% positive)			Diseased calves (% positive)		
	Cultivation or PCR	Cultivation	PCR	Cultivation or PCR	Cultivation	PCR
<i>Pasteurella multocida</i>	48	43	32	82	82	74
<i>Histophilus somni</i>	20	4	20	41	29	38
<i>Mannheimia haemolytica</i>	23	16	23	29	6	29
<i>Arcanobacterium pyogenes</i>	5	5	ND	6	6	ND
<i>Moraxella</i> sp.	2	2	ND	3	3	ND
<i>Mycoplasma dispar</i>	ND	ND	21	ND	ND	79
<i>Mycoplasma bovirhinis</i>	ND	ND	14	ND	ND	79
<i>Mycoplasma bovis</i>	ND	ND	0	ND	ND	0
Total number of positive samples	68	59	41	100	100	97

ND: not done.

Table 2

Herd prevalences (%) of pathogenic microorganisms found in trans-tracheally aspirated bronchoalveolar fluid from clinically normal calves (N) and calves with pneumonia (P) from six Danish herds.

Microorganism present	Herd 1		Herd 2 ^a	Herd 3		Herd 4 ^a		Herd 5		Herd 6	
	N	P	N	N	P	N	N	P	N	P	
	n = 10	n = 10	n = 9	n = 10	n = 13	n = 10	n = 7	n = 6	n = 10	n = 5	
<i>Pasteurella multocida</i>	60	40	44	50	100	50	29	100	50	100	
<i>Histophilus somni</i>	30	90	11	0	0	30	0	50	40	40	
<i>Mannheimia haemolytica</i>	10	30	11	20	31	20	14	17	60	40	
<i>Arcanobacterium pyogenes</i>	0	10	22	0	0	0	0	17	10	0	
<i>Moraxella</i> sp.	0	0	0	0	0	0	14	0	0	0	
<i>Mycoplasma dispar</i>	0	50	44	0	92	20	14	83	50	20	
<i>Mycoplasma bovirhinis</i>	0	70	11	10	85	10	29	67	30	80	
<i>Mycoplasma bovis</i>	0	0	0	0	0	0	0	0	0	0	
No bacteria detected	30	0	22	50	0	20	71	0	10	0	
BRSV-antigen	0	60	0	0	100	0	0	0 ^b	0	0 ^b	
PI-3 antigen	0	0	0	0	0	0	0	0	0	0	
Coronavirus antigen	0	40	0	0	0	0	14	0	0	0	

^a Pneumonic disease not observed during study period.

^b No seroconversion to BRSV detected in herds 5 and 6.

percentage of the samples were positive for all bacterial species investigated in the group of diseased animals compared to the clinically healthy animals, however this difference was statistical significant only for *M. dispar* and *M. bovirhinis* ($p < 0.01$ for both).

3.3. Herd prevalences

Some differences between the herds in the prevalence of the different microorganisms were observed (Table 2). *P. multocida*, *M. haemolytica*, *M. dispar* and *M. bovirhinis* were found in all six herds. *H. somni* was found in all herds except for herd 3. Coronavirus antigen was only detected in herds 1 and 5. BRSV-antigens were detected in 60 and 100% of the calves from herds 1 and 3, respectively (Table 2). In herds 5 and 6, no BRSV-antigens were detected. The test of paired sera from these two herds showed that seroconversion to BRSV had not taken place. *M. bovis* and PI-3 were not detected in any of the herds.

3.4. PCR and cultivation

For *H. somni* and *M. haemolytica*, a significantly higher number of samples were found positive by the PCR tests than by cultivation ($p = 0.04$ and 0.01 , respectively). For *P. multocida*, a higher number of samples were found positive by cultivation than by PCR but the difference was not statistically significant ($p = 0.10$).

There was a significant correlation between detection of *H. somni* by cultivation and pneumonia ($p < 0.01$) but no significant correlation between the presence of pneumonia and detection of *H. somni* by PCR ($p = 0.05$). For *P. multocida* a significant correlation ($p < 0.01$) was found between disease status and both cultivation and PCR, whereas there were no significant correlations observed for the detection of *M. haemolytica* and disease status.

Cultivation for mycoplasma was performed on 10 samples representing each sampling event per herd. *M. dispar* was isolated from five of these samples and *M. bovirhinis* from two samples.

3.5. Acute phase proteins

Comparing the three diseased versus healthy calf populations within each of the herds 1, 3 and 5, statistically significant differences were found for haptoglobin as well as SAA concentrations for all herds with the clear exception of SAA in herd 5 where no difference was found (Fig. 1). Haptoglobin concentrations also showed a less significant increase in herd 5 compared to herds 1 and 3. This increase was, however, significant for all three herds. The SAA concentrations of the healthy populations were generally closer to the values of the diseased calves than what was found for haptoglobin. Thus, for SAA both herd 3 and 5

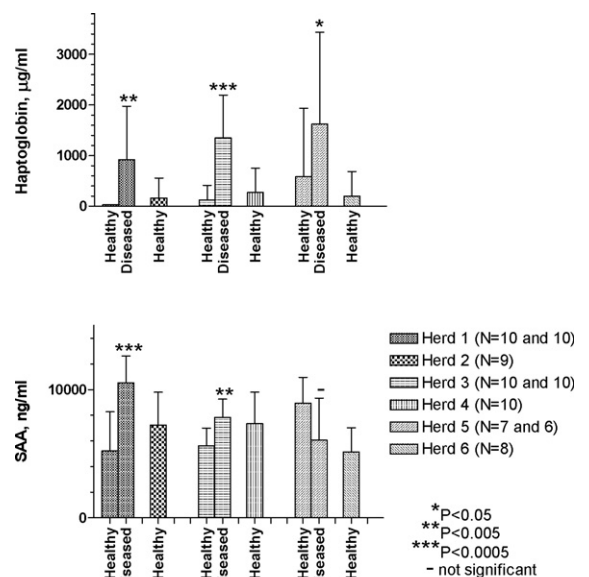


Fig. 1. Concentrations of haptoglobin (top) and SAA (bottom) in the different populations of animals sampled at the different farms as indicated. Mean values and S.D. are given for each population sampled. Significance of differences between healthy and diseased populations within the same herd is indicated by stars as explained in the figure.

diseased populations were not significantly different from the healthy population taken as a whole.

4. Discussion

In the present investigation, 68% of the clinically healthy calves were found to harbor potentially pathogenic bacteria in the lower respiratory tract. Only a limited number of reports have been published on the normal flora of the lower respiratory tract. Viso et al. (1982) sampled 23 healthy animals and found bacteria in 52% of the calves and 83% of these bacteria were identified as “*Pasteurella* sp.”. Espinasse et al. (1991) performed trans-tracheal aspiration on 49 healthy calves. *P. multocida* and *M. haemolytica* were each found in eight of these calves. Virtala et al. (1996) also performed trans-tracheal aspiration on 47 healthy calves and found 55% of these to harbor pathogenic bacteria, finding *Mycoplasma* spp. and *P. multocida* in 47 and 17% of the samples, respectively. Autio et al. (2007) investigated a group of 144 healthy calves by tracheo-bronchial lavage using a double catheter and found pathogenic bacteria (excluding mycoplasmas), *Mycoplasma* spp., and pathogenic bacteria including mycoplasmas in 27, 75, and 83% of the samples, respectively. However, none of these investigations reported the quantity of bacteria isolated in the healthy animals. In the present investigation, a striking observation was that 32% of the healthy calves harbored bacteria in the lower respiratory tract in high numbers without showing clinical disease.

These observations underline the multi-factorial nature of calf pneumonia and supports earlier reports that bacteria seldom act as primary pathogens in relation to calf pneumonia (Tegtmeier et al., 1999, 2000a). Both Baudet et al. (1994) and Babiuk et al. (1988) concluded that virus-infections precede 90% of all bacterial pneumonias.

Samples from clinically diseased animals were only obtained from four of the six herds. Among the calves in herds 1 and 3, BRSV-antigens could be detected in 83% of the diseased calves. In these herds, a viral infection is probably the primary cause for the development of clinical disease. These observations are in accordance with previous results, showing that BRSV plays an important role for the development of calf pneumonia in Denmark (Larsen et al., 1999; Uttenhal et al., 2000). On the other hand, no BRSV-antigens could be detected in herds 5 and 6 and the test of paired sera from these two herds showed that seroconversion to BRSV had not taken place. Consequently, the etiology of pneumonic disease in these two herds was probably dependent on the presence of bacteria alone. However, as a number of different bacterial species were isolated from these herds and no autopsies were performed, firm conclusions about the etiology of the observed pneumonia cannot be drawn.

A general conclusion from the present study is that haptoglobin is the more sensitive indicator of disease in the herds investigated, while neither haptoglobin nor SAA levels were affected by the potentially harmful agents in the lower respiratory tract if animals were

healthy. SAA concentrations of the healthy calves were much closer to those of the diseased populations than what was found for haptoglobin. Of the three herds in which healthy and diseased populations were compared to each other, BRSV could be demonstrated in the diseased subpopulations of herds 1 and 3 but not in the diseased population of herd 5. SAA concentrations in herd 5 did not differ between the diseased and the normal subpopulation. This might indicate that SAA needs virus to be present in order to respond while haptoglobin is fully induced by bacterial infection alone. It has previously been shown that SAA seems to be a more sensitive marker for viral infections (Heegaard et al., 2000) and acute inflammation (Alsemgeest et al., 1994; Horadagoda et al., 1999) compared to haptoglobin. In experimental bacterial infections (intra-tracheal inoculation of *M. haemolytica*), SAA was found to be more rapidly induced than haptoglobin (Horadagoda et al., 1994). The results presented here show that even if SAA is more sensitive and rapidly reacting, haptoglobin might be preferable in the field, its bigger and more prolonged response giving rise to its higher sensitivity in detecting disease.

In the present investigation, a higher percentage of the samples were found positive for *P. multocida*, *M. dispar*, *M. bovirhinis* and *H. somni* in the group of diseased animals compared to the clinically healthy animals (Table 1), this increase was however not statistically significant. In herds 3, 5, and 6, *P. multocida* was found in all diseased animals, indicating a possible role for this organism in the development of pneumonia. Autio et al. (2007) also reported an association between *P. multocida* and clinical respiratory disease among calves in Finland, provided *P. multocida* was found together with other bacterial pathogens. This supports the common opinion that *P. multocida* should be regarded as an opportunistic pathogen (Maheswaran et al., 2002). On the other hand, Nikunen et al. (2007) in another Finnish study found strong indications for *P. multocida* having a pathogenic role, provided other known pathogens were absent. Virtala et al. (1996) found a significant association between clinical disease and increased isolation rate of *P. multocida* and *Mycoplasma* spp., alone or in combination.

In the present investigation we found a significantly higher detection rate of *M. dispar* and *M. bovirhinis* among clinically diseased calves although a high proportion of the healthy calves also harbored these organisms. On the other hand, in two Finnish studies, no association between clinical disease and the presence of *Mycoplasma* spp. was observed (Autio et al., 2007; Nikunen et al., 2007).

M. bovis is in many countries regarded as one of the major causes of respiratory disease in cattle with reports of increasing prevalence (Nicholas and Ayling, 2003). *M. bovis* was not found in any of the six herds in the present investigation. Earlier studies in Denmark have only found a low prevalence of this organism (Friis and Krogh, 1983; Feenstra et al., 1991), so apparently *M. bovis* is still of low importance in connection with pneumonic disease in Denmark.

Cultivation of *Mycoplasma* spp. was attempted from 10 PCR-positive samples. However, due to the long storage

time before analysis, isolation of *Mycoplasma* spp. was only successful from seven of these samples. The cultivation nevertheless confirmed the presence of *M. dispar* and *M. bovirhinis* in these samples as found by PCR.

For *H. somni* and *M. haemolytica*, a significantly higher number of samples were found positive by the PCR tests than by cultivation. For *P. multocida*, a higher number of samples were found positive by cultivation than by PCR, but the difference was not statistically significant. The lower sensitivity of the *P. multocida* PCR compared to cultivation may be due to the large size of the amplicon produced by this method (1250 bp) compared to the *H. somni* and *M. haemolytica* PCRs (both approximately 400 bp). Detection of *P. multocida* by both cultivation and PCR was significantly associated with the disease status of the animal, whereas no such association was observed for *M. haemolytica*. A previous study on *H. somni* demonstrated a higher sensitivity of a species-specific PCR test (Tegtmeier et al., 2000b) compared to bacterial cultivation. However, the present investigation indicates that PCR nevertheless is less suited for prediction of *H. somni*-related pneumonia compared to bacterial cultivation. These observations might be related to the fact that the use of a highly sensitive method such as PCR will also detect the presence of a very low number of bacteria that not necessarily have any correlation with disease. On the other hand, a higher diagnostic value of a PCR test could be obtained if real-time PCR tests were developed, whereby the infectious agents can be quantified and give a result which might better reflect the clinical status of the animal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vet-mic.2008.12.024](https://doi.org/10.1016/j.vet-mic.2008.12.024).

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