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Øystein Angen, John Thomsen, Lars Erik Larsen, Jesper Larsen, Branko Kokotovic, et al.. Respiratory disease in calves: Microbiological investigations on trans-tracheally aspirated bronchoalveolar fluid and acute phase protein response. *Veterinary Microbiology*, 2009, 137 (1-2), pp.165. 10.1016/j.vetmic.2008.12.024 . hal-00485526

HAL Id: hal-00485526

<https://hal.science/hal-00485526>

Submitted on 21 May 2010

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Accepted Manuscript

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

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PII: S0378-1135(08)00610-X
DOI: doi:10.1016/j.vetmic.2008.12.024
Reference: VETMIC 4314

To appear in: *VETMIC*

Received date: 30-10-2008
Revised date: 17-12-2008
Accepted date: 29-12-2008

Please cite this article as: Angen, Ø., Thomsen, J., Larsen, L.E., Larsen, J., Kokotovic, B., Heegaard, P.M.H., Enemark, J.M.D., Respiratory disease in calves: Microbiological investigations on trans-tracheally aspirated bronchoalveolar fluid and acute phase protein response, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.12.024

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

4
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20 **Abstract**

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

44

45 1. Introduction

46 Respiratory disease in calves causes great economic losses for the dairy and beef
47 industry worldwide (Snowder et al., 2006). Blom (1982) reported that the annual calf mortality
48 between day 2 and 180 was 7% in Danish dairy herds. He reported that on average 4.5% of the
49 calves died due to respiratory disease. A mortality rate in the range 1.5-4.2% has also been reported
50 from other countries (Ames, 1997; Andrews, 2000).

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

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

69 2. Materials and methods

70 2.1 Herds and samples

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

79 The first samples were taken during a warm and dry September from clinically
80 healthy calves (rectal temperatures below 39.5°C, no nasal discharge, no coughing, and an
81 unprovoked respiration frequency lower than 40 per min). All calves sampled in November showed
82 clinical symptoms of pneumonic disease. Clinical disease was defined as a rectal temperature above
83 39.5°C in connection with nasal discharge, coughing, or an unprovoked respiration frequency
84 higher than 40 per min. The age of the calves ranged from 14 days to 4 months. The average age of
85 the clinically healthy calves sampled in September was 2.1 months (SD=1.1) and for the pneumonic
86 calves sampled in November 2.8 months (SD=0.9). Only four of the calves were sampled on both
87 occasions (one calf in each of herd 3 and 5, and two calves in herd 6).

88 Blood samples were taken from all clinically healthy calves in connection with the
89 first visit in September. In addition, paired blood samples (3 weeks interval) were taken in
90 November-December after onset of clinical disease in the 4 herds where pneumonia was observed.

91 An area of 3x3 cm located 7 to 10 cm distal to the larynx was shaved and
92 decontaminated with 70% alcohol and iodophors. The calves were sedated by intramuscular

93 injection of 0.1-0.2 mg/kg xylazine. After injection of local analgesic (0.5 ml 2% lidocain), a
94 longitudinal incision of 1 cm was made in the midline directly above the trachea. Perforation of
95 trachea was done with an Intraflon[®] 12G between 2 cartilage rings. A male dog urinary catheter was
96 inserted into the Intraflon[®] and pushed down into the airway until a slight resistance was felt.
97 Between 20 and 40 ml sterile 0.9% NaCl were injected through the catheter and followed by
98 immediate aspiration. This resulted normally in 5 – 7 ml aspirated fluid.

99 One ml aspirated fluid was centrifuged for 3 min at 16.000 g. The supernatant was
100 removed and a loopful (approx. 10µl) of the sedimented material was used for bacterial cultivation.
101 The growth of the different bacterial species was recorded on a semi-quantitative scale (pure
102 culture, many bacteria in mixed culture, mixed culture, few bacteria in mixed culture, no growth).
103 Bacterial identification was done according to the standard procedures of the laboratory (Tegtmeier
104 et al., 1999). For *M. haemolytica*, the identification was based on the methods given by Angen et al.
105 (2002). The identification of *H. somni* was performed by PCR as described by Angen et al. (1998).
106 Cultivation and identification of *Mycoplasma spp.* were performed according to the procedures
107 described by Friis and Krogh (1983). The samples had been stored at -20 °C for 2 years before
108 cultivation for mycoplasmas was initiated.

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

112

113 2.2 PCR detection

114 DNA was extracted by addition of 200 µl PrepMan[™] Ultra (Applied Biosystems) to the sedimented
115 trans-tracheal sample according to the manufacturer's recommendations. The DNA preparation was
116 stored at -20°C until used for PCR analysis. Species specific detection using previously published

117 methods was performed for *H. somni* (Angen et al., 1998), *P. multocida* (Miflin and Blackall,
118 2001), *M. dispar* and *M. bovirhinis* (Miles et al., 2004), and *M. bovis* (Subramaniam et al., 1998)
119 using 2 µl of the extracted DNA per reaction.

120 For *M. haemolytica*, the *lktA-artJ* intergenic region was used for PCR
121 amplification. From pure cultures, one bacterial colony was resuspended in 100 µl distilled water
122 and lysed by boiling for 10 min. One µl sample from lysed bacteria or 2 µl of DNA extracted by
123 PrepMan™ Ultra was added to respectively 49 µl and 48 µl prepared reaction mixture containing
124 10 mM Tris/HCl pH 8.3, 50mM KCl, 2.5mM MgCl₂, 100 µM of each dNTP, 65 pmol of each
125 primer (forward primer: GTCCCTGTGTTTTTCATTATAAG; reverse primer:
126 CACTCGATAATTATTCTAAATTAG), and 0.5 U of *Taq* polymerase (Applied Biosystems,
127 Foster City, CA, USA). The reaction mixture was covered with 50 µl of paraffin oil. Samples were
128 subjected to an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at
129 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min in a thermal cycler.
130 Samples of PCR amplification products (10 µl) were subjected to electrophoresis in a 1.2% agarose
131 gel in Tris/borate buffer according to standard protocols. DNA was visualized by UV fluorescence
132 after staining with ethidium bromide. The PCR test was expected to give a specific amplicon of 385
133 bp. For evaluation of the species specificity of this test, see supplementary material.

134

135 2.3 Detection of viral antigens and antibodies to BRSV

136 The transtracheal aspirations were investigated for the presence of antigens from bovine respiratory
137 syncytial virus (BRSV), parainfluenza-3 virus (PI-3) and corona virus using the ELISA tests
138 described by Uttenthal et al. (1996). Blood samples from diseased calves were tested for IgG₁
139 antibodies against BRSV according to Uttenthal et al. (2000).

140

141 *2.4 Measurement of acute phase proteins*

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

145

146 *2.5 Statistical methods*

147 Differences in frequencies were evaluated using least-squares test statistics and the relationship
148 between disease status and test results by a logistic regression model evaluated with the chi-square
149 test using the statistical software package S-Plus 6.1 for Windows (Profession Release 1;
150 Insightful). Data from the acute phase protein measurements were analyzed using a two-sided t-test.
151 The similarity of the different populations was tested using the Mann-Whitney non-parametric test
152 as some of the sample sets proved to be not normally distributed.

153

154 **3. Results**155 *3.1 Clinically healthy animals*

156 Among the 56 clinically healthy calves, potentially pathogenic bacteria were detected either by
157 cultivation or PCR from 68% of the animals (Table 1), this number varied from 29% to 90%
158 between the herds (Table 2). Bacteria were cultivated from 33 samples (59%). From 18 of the
159 calves (32%), *H. somni*, *P. multocida*, *M. haemolytica*, or *A. pyogenes* were isolated in high
160 numbers in pure culture or as the dominating flora in a mixed culture. From three of the calves only
161 few *P. multocida* in pure culture was cultivated. *M. dispar* and *M. bovirhinis* were detected by PCR
162 from 21% and 14% of the samples, respectively. *M. bovis* was not detected. Forty-one percent of
163 the samples tested positive in one or more of the PCR tests applied. By cultivation or PCR, *P.*

164 *multocida* was detected in 48%, *H. somni* in 20%, and *M. haemolytica* in 23% of the calves. *A.*
165 *pyogenes* was isolated from 3 of the calves and *Moraxella sp.* from one calf. From 23 samples
166 (41%) two or more bacterial species were detected. Only one of the samples contained virus
167 antigens (corona virus).

168

169 3.2 Diseased animals

170 Among the 34 diseased animals, potentially pathogenic bacteria were isolated in high numbers in
171 pure culture or as the dominant flora in a mixed culture from all animals (Table 1). *P. multocida*, *H.*
172 *somni*, and *M. haemolytica* were cultivated or detected by PCR in 82%, 41%, and 29% of the
173 calves, respectively. *A. pyogenes* was isolated from 2 of the calves and *Moraxella sp.* from one calf.
174 *M. dispar* and *M. bovirhinis* were both detected from 79% of the calves. *M. bovis* was not detected.
175 From 33 of the samples (97%) two or more bacterial species were detected. A higher percentage of
176 the samples were positive for all bacterial species investigated in the group of diseased animals
177 compared to the clinically healthy animals, however this difference was statistical significant only
178 for *M. dispar* and *M. bovirhinis* ($p < 0.01$ for both).

179

180 3.3 Herd prevalences

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

188

189 *3.4 PCR and cultivation*

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

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

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

202

203 *3.5 Acute phase proteins*

204 Comparing the three diseased versus healthy calf populations within each of the herds 1, 3 and 5,
205 statistically significant differences were found for haptoglobin as well as SAA concentrations for all
206 herds with the clear exception of SAA in herd 5 where no difference was found (Fig. 1).

207 Haptoglobin concentrations also showed a less significant increase in herd 5 compared to herds 1
208 and 3. This increase was, however, significant for all 3 herds. The SAA concentrations of the
209 healthy populations were generally closer to the values of the diseased calves than what was found
210 for haptoglobin. Thus, for SAA both herd 3 and 5 diseased populations were not significantly
211 different from the healthy population taken as a whole.

212

213 **4. Discussion**

214 In the present investigation, 68% of the clinically healthy calves were found to harbor potentially
215 pathogenic bacteria in the lower respiratory tract. Only a limited number of reports have been
216 published on the normal flora of the lower respiratory tract. Viso et al. (1982) sampled 23 healthy
217 animals and found bacteria in 52% of the calves and 83% of these bacteria were identified as
218 “*Pasteurella* sp.”. Espinasse et al. (1991) performed trans-tracheal aspiration on 49 healthy calves.
219 *P. multocida* and *M. haemolytica* were each found in 8 of these calves. Virtala et al. (1996) also
220 performed trans-tracheal aspiration on 47 healthy calves and found 55% of these to harbor
221 pathogenic bacteria, finding *Mycoplasma* spp. and *P. multocida* in 47% and 17% of the samples,
222 respectively. Autio et al. (2007) investigated a group of 144 healthy calves by tracheobronchial
223 lavage using a double catheter and found pathogenic bacteria (excluding mycoplasmas),
224 *Mycoplasma* spp., and pathogenic bacteria including mycoplasmas in 27%, 75%, and 83% of the
225 samples, respectively. However, none of these investigations reported the quantity of bacteria
226 isolated in the healthy animals. In the present investigation, a striking observation was that 32% of
227 the healthy calves harbored bacteria in the lower respiratory tract in high numbers without showing
228 clinical disease.

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

233 Samples from clinically diseased animals were only obtained from four of the six
234 herds. Among the calves in herds 1 and 3, BRSV antigens could be detected in 83% of the diseased
235 calves. In these herds, a viral infection is probably the primary cause for development of clinical

236 disease. These observations are in accordance with previous results, showing that BRSV plays an
237 important role for development of calf pneumonia in Denmark (Larsen et al., 1999; Uttenthal et al.,
238 2000). On the other hand, no BRSV-antigens could be detected in herds 5 and 6 and the test of
239 paired sera from these two herds showed that seroconversion to BRSV had not taken place.
240 Consequently, the etiology of pneumonic disease in these two herds was probably dependent on the
241 presence of bacteria alone. However, as a number of different bacterial species were isolated from
242 these herds and no autopsies were performed, firm conclusions about the etiology of the observed
243 pneumonia cannot be drawn.

244 A general conclusion from the present study is that haptoglobin is the more sensitive
245 indicator of disease in the herds investigated, while neither haptoglobin nor SAA levels were
246 affected by the potentially harmful agents in the lower respiratory tract if animals were healthy.
247 SAA concentrations of the healthy calves were much closer to those of the diseased populations
248 than what was found for haptoglobin. Of the three herds in which healthy and diseased populations
249 were compared to each other, BRSV could be demonstrated in the diseased subpopulations of herds
250 1 and 3 but not in the diseased population of herd 5. SAA concentrations in herd 5 did not differ
251 between the diseased and the normal subpopulation. This might indicate that SAA needs virus to be
252 present in order to respond while haptoglobin is fully induced by bacterial infection alone. It has
253 previously been shown that SAA seems to be a more sensitive marker for viral infections (Heegaard
254 et al. 2000) and acute inflammation (Alsemgeest et al., 1994; Horadagoda et al., 1999) compared to
255 haptoglobin. In experimental bacterial infections (intratracheal inoculation of *Mannheimia*
256 *haemolytica*), SAA was found to be more rapidly induced than haptoglobin (Horadagoda et al.,
257 1994). The results presented here show that even if SAA is more sensitive and rapidly reacting,
258 haptoglobin might be preferable in the field, its bigger and more prolonged response giving rise to
259 its higher sensitivity in detecting disease.

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

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

277 *M. bovis* is in many countries regarded as one of the major causes of respiratory
278 disease in cattle with reports of increasing prevalence (Nicolas and Ayling, 2003). *M. bovis* was not
279 found in any of the 6 herds in the present investigation. Earlier studies in Denmark have only found
280 a low prevalence of this organism (Friis and Krogh, 1983; Feenstra et al., 1991), so apparently *M.*
281 *bovis* is still of low importance in connection with pneumonic disease in Denmark.

282 Cultivation of *Mycoplasma* spp. was attempted from ten PCR-positive samples.
283 However, due to the long storage time before analysis, isolation of *Mycoplasma* spp. was only

284 successful from seven of these samples. The cultivation nevertheless confirmed the presence of *M.*
285 *dispar* and *M. bovirhinis* in these samples as found by PCR.

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

302

303 **Acknowledgements**

304 The authors want to thank Birgitte Møller, Jannie Jensen, Tamara Plambeck, and Ivan Larsen for
305 skilful technical assistance and Anders Stockmarr for help with the statistical analyses.

306

307 **Figure legend**

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

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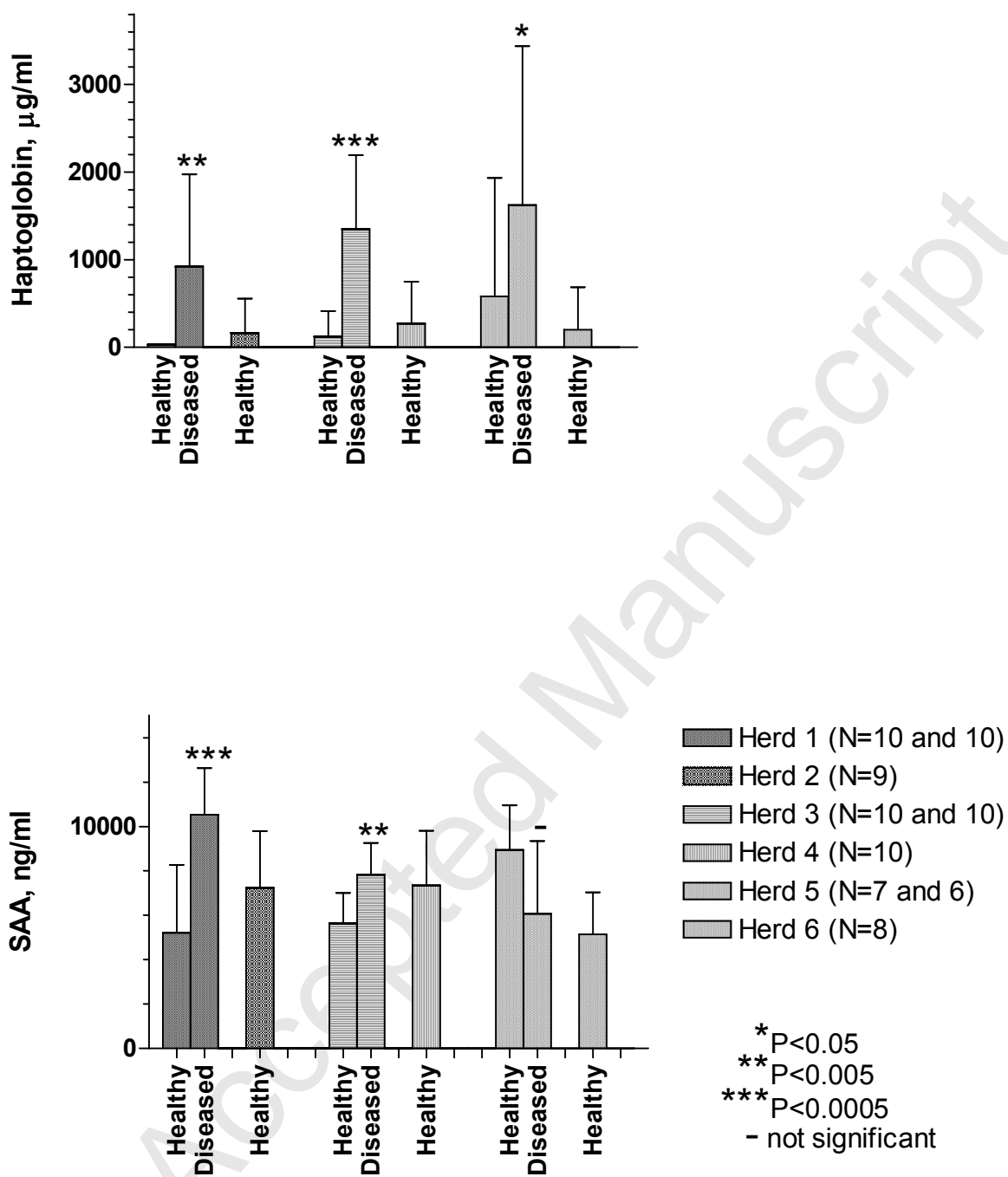


Table 1. Bacteriological investigation of trans-tracheally aspirated bronchoalveolar fluid from clinically normal calves (n=56) and calves with pneumonia (n=34) from 6 Danish herds.

| | 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 sp.</i> | 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 6 Danish herds.

| Microorganism present | Herd 1 | | Herd 2 ^a | Herd 3 | | Herd 4 ^a | Herd 5 | | Herd 6 | |
|---------------------------------|-----------|-----------|---------------------|-----------|-----------|---------------------|----------|----------------|-----------|----------------|
| | N n=10 | P n=10 | N n=9 | N n=10 | P n=13 | N n=10 | N n=7 | P n=6 | N n=10 | P 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 sp.</i> | 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.