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Etiology of respiratory disease in non-vaccinated, non-medicated calves in rearing herds

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Abstract

The aim of this study was to examine the occurrence of bacterial, mycoplasmal and viral pathogens in the lower respiratory tract of calves in all-in all-out calf-rearing units. According to clinical status, non-medicated calves with and without respiratory disease signs were selected of the 40 herds investigated to analyse the micro-organisms present in healthy and diseased calves. Tracheobronchial lavage (TBL) and paired serum samples were analysed for bacteria, mycoplasmas, respiratory syncytial virus (RSV), parainfluenza virus 3 (PIV3), bovine corona virus (BCV) and bovine adenovirus (BAV). *Pasteurella multocida* was the most common bacterial pathogen. It was isolated from 34% of the TBL samples in 28 herds and was associated with clinical respiratory disease ($p < 0.05$) when other pathogenic bacteria or mycoplasma were present in the sample. *Mannheimia* spp. and *Histophilus somni* were rarely found. *Mycoplasma bovis* was not detected at all. *Ureaplasma diversum* was associated with clinical respiratory disease ($p < 0.05$). TBL samples from healthy or suspect calves were more often negative in bacterial culture than samples from diseased calves ($p < 0.05$). No viral infections were detected in six herds, while 16–21 herds had RSV, BCV, BAV or PIV3. In the herds that had calves seroconverted to BCV, respiratory shedding of BCV was more frequently observed than faecal shedding. This study showed that the microbial combinations behind BRD were diverse between herds. *M. bovis*, an emerging pathogen in many countries, was not detected.

Keywords: Bovine respiratory disease; Respiratory pathogens; Etiology

1. Introduction

Bovine respiratory disease (BRD) and diarrhoea are the most common and economically important diseases in calves. Various micro-organisms have been

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shown to be involved in BRD together with predisposing factors (van der Fels-Klerx et al., 2000; Thomas et al., 2002; Härtel et al., 2004; Hägglund et al., 2006). The most important viral agents are respiratory syncytial virus (RSV), parainfluenza virus 3 (PIV3), bovine viral diarrhoea virus (BVDV), bovine corona virus (BCV), bovine adenovirus (BAV) and bovine herpes virus 1, the causative agent of infectious bovine rhinotracheitis (IBR). Finland is free of IBR (Nuotio et al., 2006) and BVD is very rare (Rikula et al., 2005). Thus far, *Mycoplasma bovis* has not been detected in Finnish cattle, and the last case of bovine tuberculosis (*M. bovis*) occurred in 1982. Moreover, prophylactic use of antibiotics is rare. Diseased animals are mostly treated individually, although a vaccine containing killed RSV, PIV3 and *Mannheimia haemolytica* was introduced after this study. This favourable disease situation provides excellent possibilities for investigating the epidemiology of certain respiratory pathogens in calves.

The research on BRD has mainly been focused on feedlot cattle or on calves in dairy herds (Virtala et al., 1996; Thomas et al., 2002; Hägglund et al., 2006). A limited number of reports exist describing the etiology of BRD in all-in all-out calf-rearing units. There is also a shortage of knowledge of co-occurrence of bacteria, mycoplasmas and viruses in other than autopsied calves. Rearing of coeval calves in large groups in an all-in all-out system has increased during the last few years in Finland. Calves originating from several dairy farms are transported into a specialized calf-rearing unit at 1–3 weeks of age. There they are housed in group pens typically containing 20–50 calves. At the age of 3.5–5.5 months, the entire group is moved into a specialized beef-rearing unit. The all-in all-out system enables efficient cleaning and disinfection procedures between rearing groups. On the other hand, mixing of calves from different farms exposes the animals to a heavy infection load, including microbes to which their dams do not have colostral antibodies. BRD is the most common and severe disease in calf-rearing herds.

The aim of this study was to examine the occurrence of bacterial, mycoplasmal and viral pathogens in the lower respiratory tract of calves in all-in all-out calf-rearing units. According to clinical status, non-medicated calves with and without respiratory disease signs were selected to analyse the micro-organisms present in healthy and diseased calves. Both conventional bacteriological methods and PCR detection were used to detect bacterial pathogens, including mycoplasmas (*M. bovis*, *M. bovirhinis*, *M. dispar*) and *Ureaplasma diversum*, and PCR detection was used for viruses (RSV, PIV3, BAV, BCV) in the lower respiratory tract. In addition, serological diagnostics was possible since this study was conducted prior to the introduction of a killed vaccine against a subset of bovine respiratory pathogens.

2. Materials and methods

2.1. Animals and herds

Forty herds with signs of respiratory disease were examined in 38 all-in all-out calf-rearing farms between October 2002 and January 2004. The herds were situated in different parts of Finland. All rearing units had all-in all-out production where calves from several dairy farms were brought to the unit at 13 (median, 5–38) days of age. The units were compartmentalized and the incoming air was taken from an outdoor or an animal-free area. The median herd size in the compartments was 36.5 (range 13–80) calves. The calves were housed in large group pens of 9–51 (median 26.5) calves. The calves were fed with milk replacement either ad libitum by artificial teats (24 herds) or by an automatic milk-feeding system (16 herds). Acidified milk replacement was used in 32 herds. Calves were given free access to hay, concentrate and water. All calves were unvaccinated. The use of animals was approved by the Ethical Committee of the Finnish Veterinary and Food Research Institute.

The 396 calves originated from approximately over 280 different dairy farms (approximately 1.4 calves/single dairy farm) and all the calves in the pens examined (total $n = 1268$) were estimated to originate from 900 dairy herds, which is roughly 5% of all Finnish dairy farms (900/18 000).

2.2. Clinical classification

The herds were first visited on day 17.5 (median, 4–41) post-arrival. In each rearing unit, all animals of the

pen were clinically examined by a veterinarian. The calves were classified into three groups by signs of respiratory disease: diseased, suspect and healthy. Diseased animals had at least three of the following five clinical signs: body temperature $\geq$39.5 °C, respiratory rate $\geq$45 min^{-1}, nasal discharge, coughing or increased respiratory sounds. Healthy animals had body temperature <39.5 °C, respiratory rate <40 min^{-1}, no nasal discharge, no coughing and normal breath sounds. The percentage of calves classified as healthy, suspect or diseased in the pen varied between 0 and 68 (median 25), 23 and 73 (median 50) and 0 and 54 (median 24), respectively.

2.3. Samples and detection of microbes

The herds were sampled during the first visit, and paired serum samples were taken 3–4 weeks later. In each unit, 10 non-medicated calves were selected for sampling according to the clinical status. The aim was to sample animals in different stages of BRD. Tracheobronchial lavage samples (TBL) (Härtel et al., 2004), faecal samples and paired serum samples were collected from a total of 396 animals in 40 herds. 0.5 ml of the TBL was immediately transferred into mycoplasma D medium (Friis and Krogh, 1983). The rest of the TBL samples were analysed for bacteria and viruses.

Conventional aerobic, microaerophilic and anaerobic bacterial cultivation and identification methods were used [Tryptic soy agar with 5% defibrinated bovine blood and fastidious anaerobe agar (Oxoid, Basingstoke, UK)]. Phenotypic characterization of *Mannheimia* strains was performed according to (Angen et al., 2002), *Pasteurella multocida* isolates were tested for indole reaction and *Histophilus somni* was detected by microaerophilic cultivation and PCR (Angen et al., 1998).

The inoculated mycoplasma D medium was subsequently diluted in a 10-fold series up to 10^{-8} in D, F and U media and incubated at 37 °C for up to 14 days. D medium was subcultured onto D agar, and typical colony morphology of *M. dispar* was identified by microscopy (Friis and Krogh, 1983). Identification of *M. bovirhinis* (Bölske, 1988; Kobayashi et al., 1998) and *M. bovis* (Bölske, 1988; Johansson et al., 1996) grown in F broth was done by species specific PCR targeting the 16S rRNA regions. Similarly, *U. diversum* grown in U medium was identified by species specific PCR (Gustafsson et al., 1995; Vasconcellos et al., 2000).

Viral RNA isolation was conducted with a commercial isolation kit (RNeasy Mini Kit, Qiagen, Valencia, USA) according to the manufacturer's instructions. For PIV3 RT-PCR detection, we designed PCR primers (piv3M1 5′GCTCTGTTGAGGCAGCTCTGTTGAGGCAGGATTG, piv3M2 5′ATTGATTGAGGAGCAAGTGCAACC) targeting the membrane protein coding gene (M) cDNA sequence (GenBank AF178654). Subsequent to RNA isolation and synthesis of cDNA, amplification of a 419 bp PCR product was performed using a DYNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) in real-time PCR (DNA Engine Opticon 2, MJ Research, Alameda, CA, USA) (95 °C for 1 min, first 10 cycles: 96 °C for 10 s, 94 °C for 10 s, 45 °C for 20 s, followed by an increase in annealing temperature by 1 °C/cycle until 55 °C, 72 °C for 20 s, data collected at 81 °C, and 25 additional cycles: 94 °C for 10 s, 55 °C for 20 s, 72 °C for 20 s, data collected at 81 °C, 72 °C for 10 min). The PIV3 PCR positive samples were sequenced using a capillary ABI PRISM® 3100-*Avant*™ Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the sequence data obtained was compared to published PIV sequences in GenBank by BLAST search.

RSV was detected by nested RT-PCR targeting the F fusion protein gene (Vilcek et al., 1994). BCV was detected using primers described by Tsunemitsu et al. (1999), and real-time PCR was used to amplify synthesized cDNA (95 °C 1 min, 35 cycles of 96 °C for 10 s, 94 °C for 10 s, 58 °C for 20 s, 72 °C for 20 s, data collected at 81 °C, 72 °C for 10 min). BCV was detected in both TBL and faecal samples. Commercial ELISA tests were used to analyse the paired serum samples for antibodies to RSV (SVANOVA Biotech, Uppsala, Sweden), BCV (SVANOVA Biotech), PIV3 (SVANOVA Biotech) and BAV subgroup I (Institut Pourquier, Montpellier, France).

2.4. Classification of viral infection status

A herd was classified as infected if at least one calf was infected with the virus. A calf was defined as infected when there was seroconversion or the virus was detected by PCR. Seroconversion to PIV3, BCV and RSV was defined according to manufacturers'

instructions, and as a two-fold rise in antibody level in calves with an optical density (OD) value above the cut-off point at the first sampling. Seroconversion to BAV was defined as an increase of two orders of magnitude according to the manufacturer's instructions.

2.5. Statistical analysis

Chi-squared analysis and Fisher's exact test, when appropriate, were applied to bacteriological results. The unpaired *t*-test was used to compare changes in OD values of RSV, PIV3 and BCV antibodies (SPSS version 13.0, SPSS Inc.).

3. Results

No bacterial pathogens were found in 54% of TBL samples (212/396) and no mycoplasma in 27% of samples (108/396). None of the samples harboured *M. bovis* (Table 1). The majority of *P. multocida* isolates (102/136, 75%) were indole-negative. Two or more bacterial species were found in 10% of samples ($n = 40$), and a single bacterial pathogen in 36% ($n = 144$) of samples. Four different bacterial pathogens were detected in three samples. *P. multocida*, *Arcanobacterium pyogenes* and *Fusobacterium* participated in the majority of mixed infections. However, most of the *P. multocida* isolates were from single infections (77%), whereas 74% and 76% of *A. pyogenes* and *Fusobacterium* spp. infections, respectively, were observed in mixed infections. Concurrent presence of mycoplasmas and bacteria was observed in 38% of samples, and the majority of herds were infected with one or more viruses (Table 2).

Isolation of *P. multocida*, *Fusobacterium* spp. or *U. diversum* in TBL samples was associated with clinical respiratory disease ($p < 0.05$) (Table 3). However, no association was found with the isolation of *P. multocida* or *Fusobacterium* and clinical respiratory disease when no other pathogenic bacteria or mycoplasma were present in the sample.

A. pyogenes, *P. multocida*, *M. dispar*, *U. diversum*, *M. bovirhinis* and *Fusobacterium* spp. were detected in more than 50% of herds, whereas *M. haemolytica*, *H. somni* and *Bordetella bronchiseptica* were found in five, two and one herd, respectively (Table 1). The herds infected with *P. multocida* or with various mycoplasma species had more calves (mean 3.3–5.7) infected with

Table 1
Prevalence of respiratory pathogens in samples and herds

Sample	Agent	Total no. of positive samples (%)	No. of positive herds (%)
TBL	*P. multocida*	136 (34)	28 (70)
	M. varigena	8 (2)	8 (20)
	M. haemolytica	7 (2)	5 (13)
	S. suis	22 (6)	13 (33)
	A. pyogenes	31 (8)	21 (53)
	Fusobacterium spp.	34 (9)	21 (53)
	B. bronchiseptica	1 (0.5)	1 (3)
	H. somni	2 (0.5)	2 (5)
	M. dispar	159 (40)	33 (83)
	U. diversum	92 (23)	28 (70)
	M. bovirhinis	181 (45)	32 (80)
	M. bovis	0	0 (0)
	RSV	34 (9)	10 (25)
	BCV	46 (12)	10 (25)
	PIV3	15 (4)	6 (15)
Serum	RSV	76 (19)	15 (38)
	BCV	73 (18)	18 (45)
	PIV3	64 (16)	21 (53)
	BAV	75 (19)	20 (50)
Faeces	BCV	70 (18)	13 (33)

Table 2
Respiratory pathogens in herds

Herd	Bacteria (no. of infected animals)						Mycoplasmas (no. of infected animals)			Viral infection[a]			
	P. multocida	*M. varigena*	*M. haemolytica*	*S. suis*	*A. pyogenes*	*Fusobacterium* spp.	*U. diversum*	*M. bovirhinis*	*M. dispar*	BAV	PIV3	RSV	BCV
1	−	−	−	−	−	−	+ (1)	+ (1)	−	−	−	−	−
2	−	−	−	−	+ (1)	−	+ (2)	+ (9)	−	−	−	−	−
3[b]	−	−	−	−	−	−	−	−	−	−	+	−	−
4	+(2)	−	−	−	−	−	+ (3)	−	−	−	+	+	−
5	+ (10)	−	−	−	+ (2)	+ (3)	+ (8)	+ (9)	−	−	+	+	+
6	−	+ (1)	−	−	+ (1)	+ (1)	−	−	+ (4)	−	+	−	−
7[c]	−	−	−	−	+ (1)	+ (1)	+ (3)	+ (8)	+ (4)	+	+	+	+
8	−	−	−	−	+ (1)	+ (1)	+ (2)	+ (1)	+ (1)	+	+	+	+
9	+ (5)	−	−	−	+ (1)	−	+ (8)	+ (9)	+ (3)	−	+	−	+
10	+ (6)	−	+ (1)	−	+ (1)	−	+ (4)	+ (8)	+ (2)	+	+	+	−
11	+ (7)	−	−	+ (1)	+ (2)	+ (1)	+ (1)	−	+ (5)	+	+	+	+
12	+ (5)	+ (1)	−	−	−	−	+ (4)	+ (10)	+ (8)	−	+	−	−
13	+ (5)	−	+ (1)	−	−	−	+ (9)	+ (7)	+ (4)	−	−	+	+
14	−	−	−	+ (3)	−	−	+ (1)	−	+ (4)	−	−	−	+
15	−	−	−	−	−	−	+ (1)	+ (4)	+ (5)	+	−	−	−
16	+ (1)	−	−	+ (2)	+ (3)	+ (2)	−	+ (1)	+ (5)	+	−	−	+
17	+ (6)	−	+ (2)	−	+ (3)	+ (2)	+ (1)	+ (5)	+ (3)	−	−	+	−
18	+ (4)	−	−	−	+ (1)	+ (1)	+ (3)	+ (5)	+ (2)	−	+	+	+
19	+ (9)	−	−	+ (1)	−	−	+ (5)	+ (10)	+ (3)	−	−	+	+
20	+ (7)	−	−	−	−	−	−	+ (8)	+ (6)	+	+	+	+
21	−	+ (1)	−	−	−	−	+ (3)	+ (8)	+ (7)	+	+	−	+
22	+ (3)	−	−	−	+ (1)	+ (1)	+ (1)	+ (3)	+ (6)	+	−	−	−
23	+ (1)	−	−	+ (2)	−	+ (2)	−	+ (2)	+ (5)	+	−	+	−
24	+ (5)	+ (2)	+ (1)	−	+ (3)	+ (4)	+ (4)	+ (9)	+ (7)	+	+	−	+
25	−	−	−	+ (1)	+ (1)	−	+ (1)	+ (5)	+ (9)	−	−	−	−
26	+ (1)	−	−	+ (5)	−	+ (1)	−	+ (7)	+ (5)	+	+	−	−
27	−	−	−	+ (1)	−	+ (1)	−	−	+ (1)	−	−	−	−
28	+ (3)	−	−	+ (1)	+ (1)	+ (2)	+ (3)	+ (3)	+ (10)	−	−	−	−
29	+ (2)	+ (1)	−	−	−	−	+ (2)	−	+ (7)	+	−	−	−
30	+ (3)	+ (1)	−	−	−	+ (2)	+ (1)	+ (2)	+ (6)	+	−	−	−
31	+ (5)	−	−	−	+ (1)	+ (2)	+ (2)	+ (5)	+ (5)	+	+	+	−
32	+ (4)	−	−	−	−	−	−	+ (7)	+ (2)	−	−	−	−
33	+ (3)	−	−	+ (1)	+ (1)	−	−	+ (2)	+ (4)	−	+	−	+
34	+ (7)	+ (1)	−	−	−	+ (1)	+ (4)	+ (3)	+ (5)	+	+	+	+
35	−	−	−	−	+ (1)	+ (1)	−	+ (5)	+ (4)	+	+	−	+
36	+ (7)	−	−	+ (1)	+ (3)	+ (2)	+ (7)	+ (3)	+ (8)	+	−	+	−
37[c]	+ (9)	−	−	−	−	−	+ (5)	+ (7)	+ (5)	−	−	−	+
38	+ (5)	+ (1)	−	+ (2)	+ (1)	+ (2)	−	−	+ (4)	+	+	−	+
39	+ (4)	−	−	+ (1)	−	−	−	+ (7)	−	−	−	+	−
40	+ (7)	−	+ (2)	−	+ (1)	+ (1)	+ (3)	+ (8)	−	+	+	−	+
Mean[d]	4.9	1.1	1.4	1.7	1.5	1.6	3.3	5.7	4.8				
Minimum	1	1	1	1	1	1	1	1	1				
Maximum	10	2	2	5	3	4	9	10	10				
S.D.	2.5	0.3	1.2	1.2	0.8	0.8	2.3	2.8	2.2				

+: infected; −: not infected.

[a] Herd was classified infected if at least one calf was infected with the virus.

[b] *B. bronchiseptica* ($n = 1$).

[c] *H. somni* ($n = 1$).

[d] Number of positive samples in positive herds.

Table 3
Occurrence of bacterial and mycoplasmal pathogens in different clinical respiratory disease categories

Pathogen	Respiratory disease category (%)		
	Healthy (n = 144)	Suspect (n = 89)	Diseased (n = 163)
No bacterial pathogens or mycoplasmas	17	25	16
No bacterial pathogens	63	58	42[a]
No mycoplasmas	25	37	24
P. multocida	26.4	32.6	42.3[b]
M. varigena	0.7	2.2	3.1
M. haemolytica	1.4	0	3.1
S. suis	5.6	2.2	7.4
A. pyogenes	8.3	6.7	8.0
Fusobacterium	6.9	3.4	12.9[b]
B. bronchiseptica	0	0	0.6
H. somni	0	1.1	0.6
U. diversum	18.1	16.9	30.7[b]
M. bovirhinis	43.8	40.4	49.7
M. dispar	40.3	30.3	45.4

[a] Significantly more prevalent in healthy and suspect categories than in the diseased category ($p < 0.05$).
[b] Significantly more prevalent in diseased than in healthy and suspect ($p < 0.05$).

the respective microbe than the herds infected with *M. haemolytica*, *Mannheimia varigena*, *A. pyogenes*, *Streptococcus suis* and *Fusobacterium* spp. (Table 1). In 19 herds only indole-negative and in four herds, only indole-positive phenotypic variants of *P. multocida* were found. Both variants were detected in five herds.

Most herds (78%) harboured more than one bacterial pathogen (Table 2). A maximum of five different bacterial pathogens were detected in two herds (herds 24 and 38). Concurrent detection of different mycoplasma species was common, and in half of the herds all three mycoplasma species, *M. dispar*, *M. bovirhinis* and *U. diversum,* were found (Table 2). In one herd (herd 15) no bacterial pathogens were detected, but co-existing infections with all three mycoplasmas and adenovirus were observed. All six herds with no viral infections had mycoplasmas and three of them *U. diversum*. Bacterial species prevalent in the virus-negative herds were *P. multocida*, *M. varigena*, *S. suis*, *Fusobacterium* spp. and *A. pyogenes*.

RSV and BCV were detected by PCR in TBL samples of 10 herds and PIV3 in 6 herds (Table 1). The mean number of PCR-positive samples for each virus varied from 2.5 to 4.6 in the herds. Seroconversion to viruses occurred in 16–19% of the animals, whereas 2–11% of animals turned seronegative in sample 2 (Table 4). Calves with a positive BCV, PIV3 or RSV PCR result in the TBL sample had a significantly ($p < 0.05$, t-test) greater increase in the levels of antibodies of BCV, PIV3 or RSV, respectively (shown as change in the OD value), than those with a negative PCR result. However, there were two herds with TBL samples positive either for RSV or BCV, but without a significant rise in antibody level in any of the 10 calves analysed.

For each virus, there were 16–21 herds that could be classified as infected with the virus, PIV3 being the

Table 4
Prevalence of seropositive animals and changes in serological status

Virus	No. of seropositive animals (%)		No. of seroconverted animals (%)	No. of animals turning seronegative[a] (%)
	Sample 1	Sample 2		
RSV	205 (52%)	249 (63%)	76 (19%)	20 (5%)
PIV3	283 (72%)	316 (80%)	64 (16%)	11 (3%)
BCV	226 (57%)	270 (68%)	73 (18%)	8 (2%)
BAV	311 (79%)	301 (76%)	75 (19%)	44 (11%)

[a] Optical density values changed from positive to negative.

Table 5
Occurrence of bovine corona virus (BCV) in tracheobronchial lavage samples (TBL)and faecal samples in herds with and without serological changes

Serological status	Occurrence of BCV		No. of herds
	TBL	Faeces	
No seroconversion	−	−	17
	−	+	4
	+	−	1
	+	+	0
Seroconversion	−	−	8
	−	+	1
	+	−	1
	+	+	8

most widely spread (Table 2). In six herds, no viral infections were detected. A single viral infection occurred in 11 (28%) herds, whereas mixed viral infections occurred in more than half (58%) of the herds. Concurrent infections with two or three viruses were both seen in nine herds. Four concurrent viral infections were found in five herds.

BCV was detected more often in faecal samples ($n = 70$) than in TBL samples ($n = 46$). Eighty-six calves had BCV in TBL or faecal samples, and 30 of these calves had BCV in both TBL and faeces. Seroconversion to BCV was observed more often in herds in which BCV was detected in TBL than in herds with BCV-positive faecal samples (Table 5). BCV was found in faecal samples in 13 herds, and in 8 of these herds BCV was also detected in TBL samples (Table 5). In six herds, BCV was detected in more than half of the faecal samples. In two of these herds, BCV was detected in all 10 TBL samples and in other two herds in 4 TBL samples.

4. Discussion

This field study analysed the etiology of BRD in non-medicated, non-vaccinated calves in all-in all-out calf-rearing units and provides a representative estimate of respiratory infections prevalent in calf-rearing units and on dairy farms in general. *M. bovis* is considered one of the most important causes of respiratory disease in cattle worldwide and an increasing prevalence in several countries has been reported (ter Laak et al., 1992; Brice et al., 2000; Kusiluka et al., 2000; Byrne et al., 2001). *M. bovis* was not found in this study, has never been isolated in the Finnish cattle population, and mastitis caused by *M. bovis* has not been reported in Finland. In a herd with endemic pneumonia every other 5-day-old dairy calf has been shown to shed mycoplasma (Stipkovits et al., 2001), and infected cattle can shed mycoplasma via the respiratory tract for months or even years (Nicholas and Ayling, 2003). Thus, if *M. bovis* existed in Finnish dairy farms, it would appear in calf-rearing herds composed of calves from several farms.

In contrast to *M. bovis*, other mycoplasma species were present in TBL samples in abundance and were found in the majority of herds, and their within-herd prevalence was high. *M. bovirhinis* and *M. dispar* were equally present in both healthy and diseased animals, whereas *U. diversum* was more common among calves with clinical respiratory disease. Our result is supported by studies describing *U. diversum* isolations in pneumonic lungs (Tegtmeier et al., 1999; Kusiluka et al., 2000; Thomas et al., 2002) and reports of lesion production in genotobiotic calves by *U. diversum* (Howard et al., 1976; ter Laak et al., 1993).

An association was observed between *P. multocida* and clinical respiratory disease, except when *P. multocida* was the only bacterial pathogen isolated. These findings support the opinion that *P. multocida* be classified as an opportunistic pathogen. Although commonly isolated, it is not considered to be a primary causative agent of respiratory disease in calves (Maheswaran et al., 2002). However, knowledge of the pathogenesis and level of genetic diversity of bovine *P. multocida* isolates is scant. Davies et al. (2004) found only limited genetic diversity among bovine *P. multocida* isolates originating mainly from clinical cases of pneumonia and mastitis. They suggested that the small number of *P. multocida* clones containing the majority of isolates have increased capacity to cause disease. We detected *P. multocida* in 26% of TBL samples from calves without clinical signs of respiratory disease. It would be interesting to analyse the genetic similarity of *P. multocida* isolates from TBL samples of healthy and pneumonic calves.

Recently, bovine indole-negative *Pasteurella* isolates, formerly classified as *P. canis* biovar 2 or *P. avium* biovar 2, have been demonstrated to belong to *P. multocida* (Christensen et al., 2004). In our study, an

exceptionally high proportion (75%) of *P. multocida* isolates were indole-negative phenotypic variants. There were five herds with both indole-positive and indole-negative variants, but the majority of herds had one or the other phenotype. The role of indole-negative *P. multocida* isolates in calf respiratory disease remains obscure and require elucidation.

H. somni and *Mannheimia* spp., particularly *M. haemolytica*, have been shown to cause serious cases and outbreaks of BRD (Bryson et al., 1990; Tegtmeier et al., 1999). In this study, both pathogens were rare, *Mannheimia* being slightly more common than *H. somni*. Angen et al. (2002) showed that 77% of *Mannheimia* strains of bovine origin belong to *M. haemolytica* and 14% to *M. varigena*. We detected both species rarely, but with equal prevalence. *H. somni* was detected only in two calves, both of which had clinical signs of disease. *H. somni* has not been an uncommon finding in autopsied pneumonic calves in rearing herds in Finland during the last 5 years. These cases, however, have represented fatal respiratory disease, which was rarely seen in our study, explaining the low prevalence of *H. somni* observed.

Viral infections seemed to be endemic in initial dairy herds, with 52–79% of calves having antibodies against viruses in the first serum sample. Maternal antibodies may persist for months and their decline depends on many factors, e.g. infectious pressure (Kimman et al., 1987; van der Poel et al., 1999; Fulton et al., 2004; Step et al., 2005). Commercial antibody ELISAs detect mainly IgG_1 antibodies, which compose the majority of maternally derived antibodies in calves. In case of BCV, RSV and PIV3, the change in OD values of paired samples was statistically higher in calves with PCR-positive TBL samples than in PCR-negative calves. Significant rises in OD values were seen even though the primary samples were positive according to the cut-off value set by the manufacturer. For this reason, a two-fold rise in OD values was defined as seroconversion in cases where already the first sample was positive. For example, in a herd with one animal shedding RSV, all animals were seropositive for RSV in the first sampling. In the second sampling, four animals had a significant rise and others only a minor change in the OD value.

A decrease of antibody level from positive to negative was observed in 2–11% of calves depending on the virus examined, suggesting a decline in maternal antibodies. Maternal antibodies are known to suppress the humoral response to RSV (Kimman et al., 1987; Uttenthal et al., 2000). This phenomenon was seen in some of the herds. For instance, in a herd where four animals shed RSV, nine animals were seropositive and only one seronegative in the first sampling. In the second sampling, the only seronegative animal seroconverted, and OD values decreased for other animals, one animal becoming seronegative. However, in the majority of herds, seroconversion was observed in several calves. In conclusion, paired samples from several calves should be tested by serology to obtain reliable results of infection status of a single calf-rearing herd. The benefits of PCR include its speed and the ease of interpreting results. However, the virus secretion period is often short, which makes optimal sampling time crucial.

In our study using RT-PCR, 12% of calves shed BCV in the respiratory tract and 18% in faeces. Lathrop et al. (2000) found using antigen capture ELISA that 7.3% of feedlot cattle shed BCV via the respiratory tract. Much higher overall shedding rates in feedlots have been observed: 84% nasal and 96% faecal shedding using RT-PCR (Hasoksuz et al., 2002), and 46% and 52%, respectively, using antigen capture ELISA (Cho et al., 2001a). The shedding rates changed over time; shedding peak occurred on day 4 post-arrival and shedding ceased by day 21 (Cho et al., 2001b; Hasoksuz et al., 2002). However, little is known about shedding rates in calves. In experimental infection, BCV could be detected by RT-PCR in nasal samples taken 3–4 days post-infection, and the shedding lasted for 2–10 days (Cho et al., 2001a,b). The sampling time seems to be critical for the detection of virus, since nine herds were defined as infected according to serological findings, but no virus was detected by PCR. The shedding of BCV in faeces was found in four herds without respiratory shedding or seroconversion in the herd. Faecal shedding of BCV in calves with no clinical signs of diarrhoea should be investigated since subclinically infected animals are a possible source of exposure for uninfected animals.

Thirty-five per cent of calves with either TBL or faecal samples positive for BCV had both samples positive. Similar concurrent shedding has also been shown in feedlot cattle (Hasoksuz et al., 2002). In six herds, BCV was detected in more than half of the faecal samples studied, and in four of these herds BCV

was detected in the TBL samples of 4 out of 10 calves. Additional studies should be conducted to characterize similarities between the BCV strains detected in the faeces and respiratory tract of the same calf, and between BCV strains originating from different herds with dissimilar infection rates. Here, the BCV strains shed only in faeces in herds with no clinical signs and no serological findings should be characterized and similarity between those strains and strains associated with calf pneumonia, diarrhoea and winter dysentery should be examined.

We have described the etiological agents of calf respiratory disease in all-in all-out calf-rearing units in an environment where the animals are non-medicated, non-vaccinated and free of IBR, BVD, bovine tuberculosis and, as shown here, free of *M. bovis*. The study produced a large collection of respiratory pathogens. Further studies will be conducted on susceptibility to antimicrobials, on molecular epidemiology of pathogens and on the genetic similarity of *P. multocida* phenotypic variants or respiratory and intestinal BCV strains. Combining the results of the present study with clinical data collected from calves, including levels of acute phase proteins, is anticipated to improve diagnostics and treatment of respiratory disease in calves.

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References

Angen, O., Ahrens, P., Bisgaard, M., 2002. Phenotypic and genotypic characterization of *Mannheimia* (*Pasteurella*) *haemolytica*-like strains isolated from diseased animals in Denmark. Vet. Microbiol. 84, 103–114.

Angen, O., Ahrens, P., Tegtmeier, C., 1998. Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. Vet. Microbiol. 63, 39–48.

Bölske, G., 1988. Survey of Mycoplasma infections in cell cultures and a comparison of detection methods. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 269, 331–340.

Brice, N., Finlay, D., Bryson, D.G., Henderson, J., McConnell, W., Ball, H.J., 2000. Isolation of *Mycoplasma bovis* from cattle in Northern Ireland, 1993 to 1998. Vet. Rec. 146, 643–644.

Bryson, D.G., Ball, H.J., McAliskey, M., McConnell, W., McCullough, S.J., 1990. Pathological, immunocytochemical and microbiological findings in calf pneumonias associated with *Haemophilus somnus* infection. J. Comp Pathol. 103, 433–445.

Byrne, W.J., McCormack, R., Brice, N., Egan, J., Markey, B., Ball, H.J., 2001. Isolation of *Mycoplasma bovis* from bovine clinical samples in the Republic of Ireland. Vet. Rec. 148, 331–333.

Cho, K.O., Hasoksuz, M., Nielsen, P.R., Chang, K.O., Lathrop, S., Saif, L.J., 2001a. Cross-protection studies between respiratory and calf diarrhea and winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. Arch. Virol. 146, 2401–2419.

Cho, K.O., Hoet, A.E., Loerch, S.C., Wittum, T.E., Saif, L.J., 2001b. Evaluation of concurrent shedding of bovine coronavirus via the respiratory tract and enteric route in feedlot cattle. Am. J. Vet. Res. 62, 1436–1441.

Christensen, H., Angen, O., Olsen, J.E., Bisgaard, M., 2004. Revised description and classification of atypical isolates of *Pasteurella multocida* from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of *Pasteurella canis* and *Pasteurella avium*. Microbiology 150, 1757–1767.

Davies, R.L., MacCorquodale, R., Reilly, S., 2004. Characterisation of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. Vet. Microbiol. 99, 145–158.

Friis, N.F., Krogh, H.V., 1983. Isolation of mycoplasmas from Danish cattle. Nord. Vet. Med. 35, 74–81.

Fulton, R.W., Briggs, R.E., Payton, M.E., Confer, A.W., Saliki, J.T., Ridpath, J.F., Burge, L.J., Duff, G.C., 2004. Maternally derived humoral immunity to bovine viral diarrhea virus (BVDV) 1a, BVDV1b, BVDV2, bovine herpesvirus-1, parainfluenza-3 virus bovine respiratory syncytial virus, *Mannheimia haemolytica* and *Pasteurella multocida* in beef calves, antibody decline by half-life studies and effect on response to vaccination. Vaccine 22, 643–649.

Gustafsson, H., Backstrom, G., Bölske, G., Emanuelson, U., 1995. Vaginit hos mjölkkor i sydostra Sverige - resultat av en pilotstudie. SvenskVet. Tidn. 47, 469–473.

Hägglund, S., Svensson, C., Emanuelson, U., Valarcher, J.F., Alenius, S., 2006. Dynamics of virus infections involved in the bovine respiratory disease complex in Swedish dairy herds. Vet. J. 172, 320–328.

Härtel, H., Nikunen, S., Neuvonen, E., Tanskanen, R., Kivelä, S.L., Aho, R., Soveri, T., Saloniemi, H., 2004. Viral and bacterial pathogens in bovine respiratory disease in Finland. Acta Vet. Scand. 45, 193–200.

Hasoksuz, M., Hoet, A.E., Loerch, S.C., Wittum, T.E., Nielsen, P.R., Saif, L.J., 2002. Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in an Ohio feedlot. J. Vet. Diagn. Invest. 14, 308–313.

Howard, C.J., Gourlay, R.N., Thomas, L.H., Stott, E.J., 1976. Induction of pneumonia in gnotobiotic calves following inoculation of *Mycoplasma dispar* and ureaplasmas (T-mycoplasmas). Res. Vet. Sci. 21, 227–231.

Johansson, K.E., Berg, L.O., Bölske, G., Deniz, S., Matsson, J., Persson, M., Pettersson, K., 1996. Specific PCR systems based on the 16S rRNA genes of *Mycoplasma agalactiae* and *Mycoplasma bovis*. In: Mycoplasmas of Ruminants: Pathogenicity, Diagnostics, Epidemiology and Molecular Genetics, European Commission, Brussels, pp. 88–90.

Kimman, T.G., Westenbrink, F., Schreuder, B.E., Straver, P.J., 1987. Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. J. Clin. Microbiol. 25, 1097–1106.

Kobayashi, H., Hirose, K., Worarach, A., Paugtes, P., Ito, N., Morozumi, T., Yamamoto, K., 1998. In vitro amplification of the 16S rRNA genes from *Mycoplasma bovirhinis*, *Mycoplasma alkalescens* and *Mycoplasma bovigenitalium* by PCR. J. Vet. Med. Sci. 60, 1299–1303.

Kusiluka, L.J., Ojeniyi, B., Friis, N.F., 2000. Increasing prevalence of *Mycoplasma bovis* in Danish cattle. Acta Vet. Scand. 41, 139–146.

Lathrop, S.L., Wittum, T.E., Loerch, S.C., Perino, L.J., Saif, L.J., 2000. Antibody titers against bovine coronavirus and shedding of the virus via the respiratory tract in feedlot cattle. Am. J. Vet. Res. 61, 1057–1061.

Maheswaran, S.K., Thumbikat, P., Dileepan, T., 2002. Current knowledge on pathogenesis of lung injury caused by *Mannheimia haemolytica* and *Pasteurella multocida* in the bovine. In: Kaske, M., Scholz, H., Holtershinken, M. (Eds.), XXII World Buiatrics Congress. Recent developments and perspectives in bovine medicine. Hannover, Germany, Klinik für Rinderkrankheiten, Tierarztliche Hochschule Hannover, 2002, pp. 160–167.

Nicholas, R.A., Ayling, R.D., 2003. *Mycoplasma bovis*: disease, diagnosis, and control. Res. Vet. Sci. 74, 105–112.

Nuotio, L., Neuvonen, E., Hyytiäinen, M., 2006. Epidemiology in and eradication of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis from Finland. Acta Vet. Scand., submitted for publication.

Rikula, U., Nuotio, L., Aaltonen, T., Ruoho, O., 2005. Bovine viral diarrhoea virus control in Finland 1998–2004. Prev. Vet. Med. 72, 139–142.

Step, D.L., Confer, A.W., Kirkpatrick, J.G., Richards, J.B., Fulton, R.W., Payton, M.E., 2005. Respiratory tract infections in dairy calves from birth to breeding age: detection by laboratory isolation and antibody responses. Bov. Practition. 39, 44–53.

Stipkovits, L., Ripley, P.H., Varga, J., Palfi, V., 2001. Use of valnemulin in the control of *Mycoplasma bovis* infection under field conditions. Vet. Rec. 148, 399–402.

Tegtmeier, C., Uttenthal, A., Friis, N.F., Jensen, N.E., Jensen, H.E., 1999. Pathological and microbiological studies on pneumonic lungs from Danish calves. Zentralbl. Veterinarmed. B 46, 693–700.

ter Laak, E.A., van Dijk, J.E., Noordergraaf, J.H., 1993. Comparison of pathological signs of disease in specific-pathogen-free calves after inoculation of the respiratory tract with *Ureaplasma diversum* or *Mycoplasma canis*. J. Comp Pathol. 108, 121–132.

ter Laak, E.A., Wentink, G.H., Zimmer, G.M., 1992. Increased prevalence of *Mycoplasma bovis* in the Netherlands. Vet. Q. 14, 100–104.

Thomas, A., Ball, H., Dizier, I., Trolin, A., Bell, C., Mainil, J., Linden, A., 2002. Isolation of mycoplasma species from the lower respiratory tract of healthy cattle and cattle with respiratory disease in Belgium. Vet. Rec. 151, 472–476.

Tsunemitsu, H., Smith, D.R., Saif, L.J., 1999. Experimental inoculation of adult dairy cows with bovine coronavirus and detection of coronavirus in feces by RT-PCR. Arch. Virol. 144, 167–175.

Uttenthal, A., Larsen, L.E., Philipsen, J.S., Tjornehoj, K., Viuff, B., Nielsen, K.H., Nielsen, T.K., 2000. Antibody dynamics in BRSV-infected Danish dairy herds as determined by isotype-specific immunoglobulins. Vet. Microbiol. 76, 329–341.

van der Fels-Klerx, H.J., Horst, H.S., Dijkhuizen, A.A., 2000. Risk factors for bovine respiratory disease in dairy youngstock in The Netherlands: the perception of experts. Livest. Prod. Sci. 66, 35–46.

van der Poel, W.H., Middel, W.G., Schukken, Y.H., 1999. Antibody titer against bovine respiratory syncytial virus in colostrum-fed dairy calves born in various seasons. Am. J. Vet. Res. 60, 1098–1101.

Vasconcellos, C.M., Blanchard, A., Ferris, S., Verlengia, R., Timenetsky, J., Florio Da Cunha, R.A., 2000. Detection of *Ureaplasma diversum* in cattle using a newly developed PCR-based detection assay. Vet. Microbiol. 72, 241–250.

Vilcek, S., Elvander, M., Ballagi-Pordany, A., Belak, S., 1994. Development of nested PCR assays for detection of bovine respiratory syncytial virus in clinical samples. J. Clin. Microbiol. 32, 2225–2231.

Virtala, A.M., Mechor, G.D., Gröhn, Y.T., Erb, H.N., Dubovi, E.J., 1996. Epidemiologic and pathologic characteristics of respiratory tract disease in dairy heifers during the first three months of life. J. Am. Vet. Med. Assoc. 208, 2035–2042.