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Increasing Prevalence of *Mycoplasma bovis* in Danish Cattle

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Kusiluka LJM, Ojeniyi B, Friis NF: Increasing Prevalence of *Mycoplasma bovis* in Danish cattle. Acta vet. scand. 2000, 41, 139-146. – A study on the prevalence of mycoplasmas in pneumonic bovine lungs was performed on material submitted for diagnostic purposes at the Danish Veterinary Laboratory, Copenhagen. Among the 50 examined cases 43 (86.0%) were found to be infected with mycoplasmas. The predominant mycoplasmas were *Ureaplasma* spp. (72.0%), *M. dispar* (48.0%) and *M. bovis* (24.0%). Other mycoplasmas were *M. bovirhinis* (20.0%) and *M. bovigenitalium* (6.0%). Among the infected lungs multiple species infections were predominant (76.7%) over single species infections (23.3%) with *M. dispar-Ureaplasma* (25.6%), *M. bovis-Ureaplasma* (18.6%) and *M. dispar-M. bovirhinis-Ureaplasma* (11.6%) infections being the most frequently encountered combinations. There appears to be an increasing prevalence of *M. bovis* (24.0%) as compared to earlier reports (0.6-2.0%), thus calling for special attention upon this mycoplasma. Pulsed field gel electrophoresis (PFGE) analysis of 11 field isolates of *M. bovis* from 9 different farms revealed different profiles except for 2 isolates which were recovered from the same farm. Because mycoplasmas belonging to the '*M. mycoides* cluster' were not encountered during this study; it appears that the Danish cattle population is still free from this group of mycoplasma in spite of their presence in some other European countries.

pneumonia; electrophoresis.

Introduction

Mycoplasmas are considered to be important causes of losses in the cattle industry worldwide (*Levisohn* 1992, *Whitford et al.* 1994) and *M. bovis,* which was isolated for the first time in Denmark in 1981, is reputed to be the most pathogenic mycoplasma for cattle in areas which are free from contagious bovine pleuropneumonia (*Ter Laak et al.* 1992, *Rodriguez et al.* 1996). *M. bovis* causes mastitis, arthritis, and pneumonia which is most severe in calves and young cattle (*Shimizu* 1982, 1983, *Gourlay et al.* 1989, *Pfutzner & Sachse* 1996). Control procedures for *M. bovis* which involved slaughter of udder-infected animals reduced the prevalence of *M. bovis* in Denmark considerably during some years (*Feenstra et al.* 1991). However, in recent years, there have been no systematic studies to assess the status of *M. bovis* infection in dairy calves in the country. This study was designed to evaluate the aetiological significance of *M. bovis* in pneumonic diseases in Danish dairy calves as a follow-up to previous studies (*Bitsch et al.* 1976, *Friis & Krogh* 1983, *Tegtmeier et al.* 1999) which recorded a very low prevalence of *M. bovis* in pneumonic lungs of Danish cattle. Furthermore, the study was intended to assess the freedom from mycoplasmas belonging to the '*M. mycoides* cluster', es-

pecially *M. mycoides* subspecies *mycoides* (small colony (SC) type), the aetiological agent of contagious bovine pleuropneumonia (CBPP), following the resurgence of this disease in other parts of Europe in the 1990s.

Materials and methods

Source of samples

The study was based on examination of 50 pneumonic lungs of calves of up to about one year old and originating from 43 herds. They were obtained from among the routine diagnostic material submitted to the Danish Veterinary Laboratory, Copenhagen during December 1997 through March 1999. Seriously affected, reasonably fresh lungs were selected, however, irrespective of their various pathological conditions. The samples originated from different veterinary practices in the country but most of them came from Jutland. Clinical information and post mortem findings were collected for cases yielding *M. bovis*. Specimen submission reports indicated that the majority of the calves had a clinical manifestation that was initially dominated by influenza-like symptoms, and which progressively worsened to serious pneumonic syndromes. Most cases exhibited a high frequency of resistance to antibiotic therapy and eventually died.

Media and cultural procedures

Three types of media, selected on the basis of growth requirements of known bovine mycoplasmas, were used for isolation purposes (*Bitsch et al.* 1976, *Friis & Krogh* 1983). A modified Hayflick's medium (*Friis* 1975), herein abbreviated as HAU, containing 15% horse- and swine serum was specially enriched with 0.5 mg/ml L-arginine (Sigma, St Louis), 0.5 mg/ml urea (Sigma, St Louis), 0.2 mg/ml $MgSO_4$ (Sigma, St Louis), pH 7.0 was used for isolation of glucose-, arginine- and urea-metabolising mycoplasmas. A normal medium, originally evolved for *M. hyopneumoniae* and containing 20% horse- and swine serum (NHS-20), pH 7.4 (*Kobisch & Friis* 1996) was used mainly for *M. dispar.* A selective variant of this medium (SB) with 5% rabbit antiserum for *M. bovirhinis* was further used to avoid overgrowth with this rapidly propagating species (*Friis* 1979). For primary propagation attempts all media were supplemented with 0.15 mg/ml of the antibiotic D-cycloserine (Fluka Biochemika, Buchs) in addition to the recipe-included bacitracin and methicillin in NHS-20 and SB while HAU had ampicillin.

Differentiation between arginine- and urea-positive cultures was achieved by subculturing in a modified Hayflick's medium enriched with arginine but no urea, and additionally a NHS-20 medium enriched with urea, $MgSO_4$ and 0.005 mg/ml lincomycin (Fluka Biochemika, Buchs), pH 6.0, herein abbreviated as NHU.

Solid medium was prepared from NHS-20 by using purified agar, L28 (Oxoid, Hampshire, England).

Ten-fold serial dilutions of homogenised pneumonic lung tissue suspensions to 10^{-6} in all 3 liquid standard media were prepared and incubated at 37 °C. Cultures in NHS-20 and SB media were incubated on a rolling drum to promote growth, mainly of *M. dispar.* Broth cultures were examined daily for colour change of phenol red incorporated as a pH indicator for 2 weeks. For acid-producing, glucose-fermenting mycoplasmas growth was evidenced by a distinct colour shift from red to yellow, while a weaker yellow colour change indicated non-glucose fermenting species. Arginine-hydrolysing mycoplasmas and ureaplasmas alkalinised the medium and turned phenol red from yellowish to red purple. Appearance of slight floccular material and weak turbidity in the culture medium was also indicator of mycoplasmal growth.

Positive cultures were subcultured onto solid

Table 1. *Mycoplasma* and *Ureaplasma* infections in pneumonic calf lungs in Denmark in comparison to earlier examinations.

	1976[a]	1983[b]	1993-94[c]	1997-99
Pneumonic lungs:				
No. of lungs examined	50	911	51	50
No. of lungs infected	44 (88.0)	676 (74.2)	43 (84.3)	43 (86.0)
Single infections *	na	na	na	10 (23.3)
Multiple infections *	na	na	na	33 (76.7)
Mycoplasma isolation **				
M. bovis	0	5 (0.5)	1 (2.0)	12 (24.0)
M. dispar	31 (62.0)	458 (50.3)	31 (60.8)	24 (48.0)
Ureaplasma spp.	26 (52.0)	451 (49.5)	35 (68.6)	36 (72.0)
M. bovirhinis	16 (32.0)	286 (31.4)	17 (33.3)	10 (20.0)
M. bovigenitalium	0	24 (2.6)	0	3 (6.0)

Figures indicate absolute values with percentages in brackets.
* percentage of infected lungs; ** percentage of total number of lungs.
1976[a] = data from *Bitsch et al.* (1976) ; 1983[b] = data from *Friis & Krogh* (1983);
1993-94[c] = data from *Tegtmeier et al.* (1999); na = data not available.

medium, as were cultures without evidence of growth after 4 days of incubation. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 in air. They were examined for mycoplasma colonies every second day and discarded if negative after 4-7 days of incubation.

Identification of isolates

Isolates of mycoplasmas were purified by the single colony technique (*Stalheim* 1990) and ureaplasmas in mixed cultures were purified by subcultivation of 10-fold serial dilutions to 10^{-10} in NHU liquid medium. Biochemical tests including sodium polyanethol sulphonate (SPS) and digitonin sensitivity (*Friis* 1975), glucose, arginine and urea metabolism, and film and spots production were used for preliminary characterisation of the isolates (*Erno* 1974, *Stalheim* 1990). Isolates of the classical mycoplasma types were then serotyped by the disc growth inhibition test (Working Group of the FAO/WHO Programme on Comparative Mycoplasmology 1976) using serum-impregnated discs prepared from rabbit hypermimmune sera for the type strains and Danish isolates of known bovine mycoplasmas. The metabolism inhibition test (Working Group of the FAO/WHO Programme on Comparative Mycoplasmology 1975) and epi-immunofluorescence on colonies (*Gardella et al.* 1983) were used as supplementary tests for isolates showing equivocal disc growth inhibition test results. *Ureaplasma* colonies on solid medium were demonstrated by staining with manganous chloride-urea solution 24-48 h after the start of incubation of the plates, in which case the colonies turned brown black (*Razin* 1983). Ureaplasmas were not subtyped to species level because so far only one species, *Ureaplasma diversum,* has been described in cattle (*Whitford et al.* 1994).

Molecular typing of *M. bovis* isolates

Pulsed field gel electrophoresis profiles of 11 field strains of *M. bovis* recovered from the examined lungs were studied and compared to the type strain of *M. bovis* (PG45) using the

Table 2. Mixed *Mycoplasma* and *Ureaplasma* infections in 50 pneumonic calf lungs in Denmark (1997-99).

Mycoplasma/Ureaplasma spp.	No. infected	% infected	
		of total	of positives
M. dispar + *Ureaplasma*	11	22.0	25.6
M. bovis + *Ureaplasma*	8	16.0	18.6
M. dispar + *M. bovirhinis* + *Ureaplasma*	5	10.0	11.6
M. bovirhinis + Ureaplasma	4	8.0	9.3
M. dispar + *M. bovigenitalium* + *Ureaplasma*	2	4.0	4.7
M. bovis + *M. dispar*	1	2.0	2.3
M. bovis + *M. bovirhinis* + *Ureaplasma*	1	2.0	2.3
M. bovigenitalium + *Ureaplasma*	1	2.0	2.3
Mixed infections	33	66.0	76.7
Single infections	10	20.0	23.3

contour-clamped homogenous electric field (CHEF) system (Bio-Rad Laboratories, California). Preparation and digestion of DNA in agarose blocks were carried out as described by *Birren & Lai* (1993). Electrophoresis of the digested DNA was performed using a CHEF-DR® III apparatus (Bio-Rad Laboratories, California). Chromosomal DNA was separated on a 1% agarose (New England Biolabs, Beverly, Massachusetts) in a 0.5x TBE buffer (45 mM Tris, 45 mM borate, 1.0 mM EDTA, pH 8.2) re-circulated at 14 °C. The gels were run at 6 V cm^{-1} for 20 h with a 4- to 40-s pulse ramp time at an included angle of 120°. Testing for effectiveness of restriction endonuclease digestion was carried out using the restriction enzymes *Sma*I, *Bam*HI and *Xho*I (Boehringer Mannheim, Germany), and *Sma*I was selected to produce the final chromosomal DNA digests because it produced more distinct and well-resolved bands. A 48.5 kbp lambda ladder (New England Biolabs, Beverly, Massachusetts) was used as a DNA marker. The gels were stained with 1.0 μg/ml ethidium bromide for 15 min, destained with water for 15 min and the DNA bands were visualized using a UV transilluminator.

Results

The results of the study are summarised in Table 1. *Ureaplasma* spp. (72.0%) was the most frequent species of mycoplasma in the lungs examined, followed by *M. dispar* (48.0%) and *M. bovis* (24.0%); the latter value being represented by 12 isolates from 11 herds. *M. bovirhinis* (20.0%) and *M. bovigenitalium* (6.0%) were the least frequently encountered species. Mixed infections were encountered in 33 (76.7%) of the 43 positive lungs, and single infections in the 10 (23.3%) remaining samples. Among these 43 cases, mixed infections were dominated by *M. dispar-Ureaplasma* (25.6%), *M. bovis-Ureaplasma* (18.6%) and *M. dispar-M. bovirhinis-Ureaplasma* (11.6%) combinations (Table 2). No mycoplasma belonging to the '*M. mycoides* cluster' was isolated during this study despite the special attention paid to this group of mycoplasmas.

Clinical records revealed that in all the herds yielding *M. bovis* serious respiratory tract diseases were encountered, usually as an aggravation of an already existing problem, but also as apparent introduction of a new condition. In all cases, a considerable death ratio was noted among the affected animals. The general picture from the post-mortem examinations was

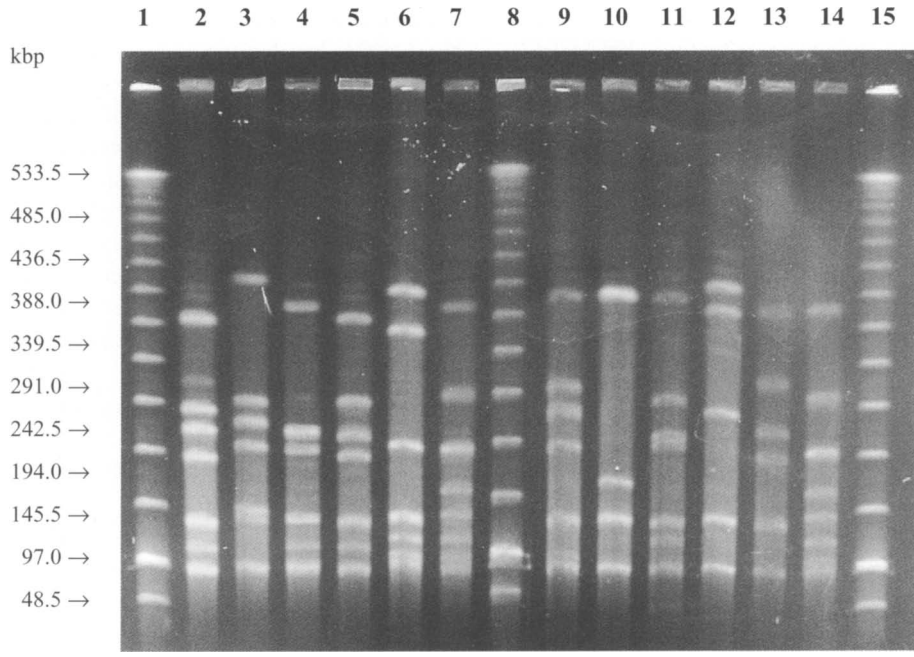

Figure 1. Pulsed field profiles of *Sma*I-digested genomic DNA of the *M. bovis* type strain PG45 (lane 5) and field strains (lanes 2-7 & 9-14) from Danish cattle. Arrows indicate the positions of the DNA fragments of the 48.5-kbp λ ladder molecular weight marker (lanes 1, 8 and 15).

that of a wide-spread, acute, purulent bronchopneumonia sometimes with fibrinous changes.

Analysis by PFGE of the fragments resulting from *Sma*I digestion of genomic DNA from 11 field isolates and the type strain of *M. bovis* (PG45) showed distinct pulsed field profiles (Fig. 1). The type strain PG45 (lane 5) and field strains (lanes 2-7 and lanes 9-14) produced 4-8 resolvable chromosomal DNA fragments ranging from 97 kbp to 412 kbp. The pulsed field profiles of the field isolates were different from the type strain and from each other except for 2 isolates originating from one farm and which revealed identical profiles (lanes 7 and 14).

Discussion

It is evident from this study that mycoplasmas are still prevalent in pneumonic lungs of Danish cattle. The increasing prevalence of *M. bovis,* held together with the persistently high frequency of other species known to be pathogenic for the bovine lung, that is, *M. dispar* and *Ureaplasma* spp., calls for more extensive studies on the aetiological importance of these mycoplasmas for the cattle industry country-wide. However, in evaluating the apparent considerable increase in *M. bovis* infection from 0.6% when it was first demonstrated in the lungs of Danish cattle in 1981 (*Friis & Krogh* 1983) and 2% as reported 5 years ago (*Tegtmeier et al.* 1999) to the current level of 24.0%, it should be noted that the examined 50 lungs is too low a number for true frequency estimations and definite conclusions. In comparison to earlier studies (*Bitsch et al.* 1976, *Friis & Krogh* 1983, *Tegtmeier et al.* 1999), this examination indicates

that *M. bovis* has established itself in the Danish cattle population and that the prevalence is slowly increasing, now up against a level coming nearer to 30% of the examined lungs, which is typical for figures published in other cattle-rearing countries (*Knudtson et al.* 1986, *Rosendal & Martin* 1986, *Gourlay et al.* 1989, *Ter Laak et al.* 1992). This situation might indicate a need for special attention on this mycoplasma with the aim of minimising further spread. The fact that respiratory tract infections can be a source of *M. bovis* infection to the udder, causing a non-curative mastitis (*Jasper et al.* 1987, *Pfutzner & Sasche* 1996) signifies the risk of increasing epidemics of *M. bovis*-induced mastitis in the country, and that the current strategy for control of *M. bovis* in dairy herds by slaughter of udder-infected animals (*Feenstra et al.* 1991) probably underestimates the apparent threat of the respiratory source of *M. bovis*. In view of these observations it is recommendable to continue the monitoring and control of *M. bovis* in mastitic cattle and in other groups of bovine animals, especially calves and young stock.

The heterogeneity of *M. bovis* isolates as revealed by PFGE typing conforms with an earlier study using DNA restriction fragment analysis which also revealed different restriction patterns between isolates from different farms in Denmark (*Feenstra et al.* 1991). Apart from the demonstration that PFGE can reliably be used for genotypic fingerprinting of *M. bovis* isolates, the results also reflect a probable diversity of the sources of *M. bovis* strains currently circulating in the cattle population in Denmark because the origin of the first strains isolated in 1981 has not been traced. Studies in other countries have demonstrated protein, antigenic and genomic heterogeneity of *M. bovis* isolates from different geographical areas (*Sachse et al.* 1992, *Poumarat et al.* 1994). However, further studies are suggested in order to gather more data about the epidemiology of *M. bovis* infections in the Danish cattle population.

The failure to isolate *M. mycoides* subsp. *mycoides* (SC) and other members of the '*M. mycoides* cluster' from the pneumonic lungs implies that Denmark is probably still free from these mycoplasmas and not under special threat from contagious bovine pleuropneumonia (CBPP) if the Danish surveillance system for infectious diseases remains in place. It may further be concluded that mycoplasmas play an important role in the pneumonic syndrome of Danish cattle and that efforts might be instituted to control these infections, especially *M. bovis,* in order to avoid future economic losses as seen in other countries (*Jasper* 1987, *Brown et al.* 1990, *Kirk* 1994).

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Sammendrag

Tiltagende prævalens af Mycoplasma bovis hos kvæg i Danmark.

En undersøgelse over prævalensen af mykoplasmer i pneumoniske kvæglunger blev foretaget på rutinediagnostisk materiale fremsendt til Statens Veterinære Serumlaboratorium, København. Mykoplasmer blev fundet i 43 (86.0%) ud af 50 undersøgte lunger. Hyppigst forekommende art var *Ureaplasma* spp. (72.0%) og derefter *M. dispar* (48.0%), og *M. bovis* (24.0%); andre arter var *M. bovirhinis* (20.0%) og *M. bovigenitalium* (6.0%). Samtidig forekomst af flere arter fandtes i 76.7% af de inficerede lunger med monoinfektion i 23.3%. Den hyppigste kombination var *M. dispar-Ureaplasma* (25.6%), herefter *M. bovis-Ureaplasma* (18.6%) og *M. dispar-M. bovirhinis-Ureaplasma* (11.6%). En forekomst af *M. bovis* på 24.0% viser en tydelig højere prævalens sammenholdt med tidligere undersøgelser på 0.6-2.0%, og påkalder sig derfor ekstra opmærksomhed. Pulsed field gel elektrophoresis (PFGE) af 11 friske isolater af *M. bovis* fra 9 forskellige besætninger viste, at alle besad forskellige profiler på nær hos 2 isolater fra samme ejendom. Mykoplasmer af

gruppen "*M. mycoides* cluster", årsagen til oksens ondartede lungesyge og relaterede lidelser hos andre drøvtyggere, blev ikke påvist i nærværende undersøgelse. Derfor kan man konkludere, at Danmark stadig er fri for disse infektioner på trods af deres forekomst i visse andre europæiske lande.

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