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Microbiology of calf pneumonia with major emphasis on mycoplasmal infections

William Urban Knudtson

Iowa State University

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THE MICROBIOLOGY OF CALF PNEUMONIA WITH MAJOR EMPHASIS ON MYCOPLASMAL INFECTIONS

Knudtson, William Urban, Ph.D.
Iowa State University, 1988

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Microbiology of calf pneumonia with major emphasis on mycoplasmal infections

by

William Urban Knudtson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Veterinary Microbiology and Preventive Medicine
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INTRODUCTION

Pneumonia is the most costly disease encountered by beef producers in the United States and results in losses in excess of 400 million (1975) dollars annually. The etiology of bovine infectious respiratory disease is very complex and several bacteria, mycoplasmas and viruses have been associated with the condition. Contagious bovine pleuropneumonia (CBPP), which is caused by *Mycoplasma mycoides* var *mycoides* was eradicated from the United States in 1892. This highly infectious acute to chronic disease (10% to 70% mortality) was introduced into the USA in 1843 and had spread throughout the U.S. cattle population by 1884 prompting the Congress of the United States to establish the Bureau of Animal Industry. Most countries have eliminated CBPP, but the disease is still endemic in parts of Africa. Other mycoplasmas which have been cultured from the respiratory tract of bovines worldwide are: *M. alkalescens*, *M. arginini*, *M. bovigenitalium*, *M. bovirhinis*, *M. bovis*, *M. bovoculi*, *M. californicum*, *M. canadense*, *M. dispar*, *M. gallinarum*, *Mycoplasma* sp. group 7 (Leach) and serogroup L (Al-Aubaudi), *Ureaplasma diversum*, *Acholeplasma axanthum*, *A. laidlawii*, *A. modicum* and *A. oculi*.

About 20 species of bacteria, mollicutes and viruses are associated with the two most common forms of bovine pneumonia, i.e., "shipping-fever" pneumonia and enzootic or "barn" pneumonia. Bacteria implicated are *Pasteurella multocida*, *P. haemolytica*, *Haemophilus somnus*, *Corynebacterium pyogenes*, *Streptobacillus actinoides* and *Chlamydia psittaci*. Mollicutes believed to play a role in pneumonia are *M. bovis*, *M. dispar* and
**Ureaplasma diversum.** Viruses include bovine adenovirus, bovine herpesviruses 1 and 3 (IBR and DN-599/MOVAR), bovine respiratory syncytial virus, parainfluenza type 3 virus, cytopathic and noncytopathic bovine viral diarrhea viruses, coronavirus, enterovirus, parvovirus, reovirus and rhinovirus. The exact mechanism(s) which results in clinical pneumonia has not been determined. The current opinion proposes that stress resulting from various management practices such as vaccination, weaning, castration, dehorning, transportation, irregular feeding and watering, comingling and crowding, as well as climatic changes, affects the calves native and acquired immune systems. This compromises the animals ability to mount an effective immune response and allows microorganisms to establish, replicate and colonize the respiratory tract mucosa.

**Mycoplasma bovis** has been cultured from the respiratory system of both clinically normal and pneumonic calves throughout the world. Interestingly, it was not seen in association with infectious respiratory disease in the United Kingdom and Europe until the mid 1970s and was probably introduced with new breeding stock from Canada. In the United States it is seen in association with bovine arthritis, mastitis, pneumonia and infertility. Under experimental conditions, clinical respiratory disease with gross and microscopic lesions was produced in gnotobiotic calves following challenge with *M. bovis*. However, the mechanism(s) by which this microbe interacts with airway epithelium are not well defined. Since *M. bovis* had been cultured from calves in the United States, it was determined that an *in vitro* study on the pathogenesis of this organism for fetal bovine tracheal explant cultures
would be worthwhile.

The first objective of this study was to identify the mycoplasmal flora present in pneumonic calf lungs (Manuscript 1).

The second objective was to determine whether or not there were significant interactions between Mycoplasma sp. and other pathogens in bovine pneumonia (Manuscript 2).

The third objective was to study the effects of M. bovis on fetal bovine tracheal explant cultures (Manuscript 3).
EXPLANATION OF DISSERTATION FORMAT

This dissertation consists of an introduction, a literature review, 3 separate manuscripts, a general conclusion and a list of references. The doctoral candidate, William Urban Knudtson, is the senior author and principal investigator for each of the manuscripts.
LITERATURE REVIEW

Pneumonia in Calves

Respiratory disease of calves is a major problem for dairy and beef producers throughout the world. Jensen et al. (1976) estimated that respiratory disease accounted for 75% of all diseases and 64% of all mortality in cattle raised in confinement. Ide (1970) categorized calf pneumonia into 4 basic types, i.e., shipping fever pneumonia (acute fibrinous pneumonia), enzootic pneumonia (chronic bronchopneumonia), parasitic pneumonia (Husk) and allergic bronchitis (Fog Fever).

An estimated 15 to 40% of calves entering feedlots in North America and Canada require treatment for infectious respiratory disease. The mortality in affected animals varies from 1 to 5% (Kelly and Janzen, 1986). Pasteurella haemolytica appears to be the major contributor to mortality in this disease (shipping fever) although a number of different microbes are often cultured from clinical specimens (Ide, 1970; Yates, 1982; Houghton and Gourlay, 1984). Stress due to the events (weaning, movement through sale barns, etc.) leading up to and including transportation over long distances to feedlots, results in elevated blood cortisol levels which in turn suppresses the animals ability to mount an effective immune response (Roth and Kaeberle, 1982; Fillion et al., 1984; Roth, 1985). This allows viruses and/or mollicutes to replicate and predispose the lower respiratory tract to colonization by bacteria, i.e., Pasteurella haemolytica and to a lesser degree, Pasteurella multocida and
Haemophilus somnus. Biotype A1 of P. haemolytica liberates a potent cytotoxin which presumably is a major initiator of events leading to acute fibrinous pneumonia (Frank, 1986).

Calves raised in confinement may develop "enzootic" or chronic bronchopneumonia characterized by high morbidity and low mortality (Ide, 1970; Lillie, 1974). High levels of aerosolized microorganisms resulting from elevated relative humidity (>80%) in combination with a lack of maternal passive immunity also contributes to enzootic pneumonia (Fogarty et al., 1986). Stress resulting from crowded rearing conditions was reported to induce an increase in blood cortisol levels (Quaassdorff et al., 1985; Danzer and Mormede, 1983). Presumably events similar to those leading to pneumonia in shipped cattle can play a role in bronchopneumonia of confined calves. Bacteria, mycoplasmas and viruses are frequently cultured from the lungs of calves with this condition.

Allergic broncho-alveolitis (Farmer's Lung) develops almost exclusively in housed cattle which are sensitized by repeated exposure to moldy hay containing spores of the thermophilic actinomycete Micropolyspora faeni (Selman et al., 1977). Precipitating antibodies are produced against enzyme-like substances which are released into the tissue when the inhaled spores germinate. Thereafter, inhalation of plant material and dust containing preformed enzyme-like substances results in complement-mediated Type III hypersensitivity and clinical signs of respiratory distress which can develop 6 to 10 hours after exposure.

Fog fever (atypical interstitial pneumonia) is usually seen in adult beef cattle but can occur in calves 6 months of age or older (Selman et
This is a metabolic disease associated with ingestion of lush grass containing L-tryptophan which is converted in the rumen to 3-methyl-indole. This compound is metabolized in the lung by a mixed function oxidase system resulting in pneumotoxicity. Acute respiratory distress and death develop within 2 weeks after animals are moved either from confinement or dry pasture to a lush pasture. Bovine respiratory syncytial virus also has been associated with atypical interstitial pneumonia in recently weaned calves (Frey, 1983).

Parasitic bronchitis or "Husk" generally affects calves which have been put on pasture for the first time. The etiologic agent associated with this condition is the lungworm Dictyocaulus viviparus. Infective larvae, which develop from eggs deposited in feces, migrate onto grass where they are ingested by calves. The larvae penetrate the wall of the intestine, travel through the lymphatic system and eventually locate in the pulmonary alveoli and then in the bronchioles causing bronchitis and pneumonia (Selman et al., 1977).

Pierson and Kainer (1980) estimated that 90% of pneumonias could be classified as either bronchial, interstitial or metastatic. In their scheme, bronchial pneumonia would include "shipping fever" and "enzootic" pneumonia. Interstitial pneumonia which is less common than bronchial pneumonia would include the following: pulmonary adenomatosis or atypical interstitial pneumonia in feedlot cattle, fog fever or acute pulmonary emphysema in pastured cattle, farmers' lung in housed cattle and pulmonary emphysema in recently weaned calves. Breeze et al. (1978) recommended that these entities be collectively termed "acute respiratory distress
syndrome". Metastatic pneumonia results from Fusobacterium necrophorum-induced caudal vena cava thrombosis following a sudden change in feed from roughage to concentrate. Septic emboli then detach from the thrombus and eventually lodge in pulmonary vessels producing embolic aneurysms, the rupture of which leads to exsanguination.

Mixed Respiratory Infections in Calves


Experimental infection with a single agent generally does not result
in clinical disease (Yates, 1982). Synergistic responses where the additive effect of combined infections was greater than that produced by either agent acting alone has been demonstrated for the following: *P. haemolytica* and *M. bovis* (Houghton and Gourlay, 1983; Gourlay and Houghton, 1985), *P. haemolytica* and BHV-1 or PI3 or BVD viruses (Jericho and Langford, 1978; Jericho et al., 1982; Potgieter et al., 1984), *P. multocida* and PI3 virus or BHV-1 (Hetrick et al., 1963; Jericho and Carter, 1985) and *Haemophilus somnus* and BRSV (Potgieter et al., 1988).

However, Lopez et al. (1982) reported that sequential challenge of calves with either BVD virus or *M. bovis* followed by *P. haemolytica* did not influence clearance of *P. haemolytica* from the lung. Combined infection with *M. bovis* and *C. pyogenes* in gnotobiotic calves did not produce clinical signs of respiratory disease or result in significant macroscopic pneumonia (Houghton and Gourlay, 1984). Also, exposure of calves to *M. dispar* followed in 2 to 4 weeks by *H. somnus* did not influence the degree of pneumonia produced over that seen in calves challenge with only *H. somnus* (Krough et al., 1986).

Synergism and microbial interaction(s) in relationship to disease of the bovine respiratory tract have been reviewed by Yates (1982), Jakab (1984) and Roth (1984). Several of the microbes listed above were demonstrated by various *in vitro* and *in vivo* assays to suppress certain functions of immune regulatory cells (alveolar macrophages, neutrophils and lymphocytes).
Taxonomy of the Class Mollicutes

Mollicutes (mollis=soft, cutes=skin) are the smallest procaryotes capable of replication in an acellular environment. Unlike other procaryotes, they not only lack a rigid cell wall but have no precursors for cell-wall peptidoglycan synthesis. The organisms are bound by a phospholipid bilayer (plasma) membrane which may be coated with a thin layer of electron dense material (Razin, 1978). Edward (1974) has indicated that absence of a cell wall contributes to the ability of these organisms to grow into solid medium producing the typical "fried-egg" appearance characteristic of all mycoplasma colonies. General characteristics of Mollicutes are summarized in Table 1. The class

Table 1. General characteristics of the class Mollicutes

1. Growth in an acellular medium.
2. Lack of cell wall or peptidoglycan precursors.
3. A minimum reproductive unit size of about 0.3 micrometers.
4. Growth requirement for sterol by most species.
5. Typical colony morphology (fried egg-like) when grown on solid medium.
6. Organisms surrounded by plasma membrane and sometimes coated with a thin layer of electron dense material.
7. Resistant to penicillin and other antibiotics whose action is directed against synthesis of peptidoglycan.
8. Small genomes and low G+C ratio for DNA.

aAdopted from Razin (1978), Tully (1978) and Archer and Daniels (1982).
Table 2. Taxonomy of the Mollicutes\textsuperscript{a}

<table>
<thead>
<tr>
<th>Class: Mollicutes</th>
<th>Order: Mycoplasmatales</th>
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<tr>
<td></td>
<td>Family I: Mycoplasmataceae</td>
</tr>
<tr>
<td></td>
<td>Sterol required for growth</td>
</tr>
<tr>
<td></td>
<td>Genome size about $5 \times 10^8$ daltons</td>
</tr>
<tr>
<td></td>
<td>NADH oxidase localized in cytoplasm</td>
</tr>
<tr>
<td>Genus I: Mycoplasma (over 70 species)</td>
<td>Do not hydrolyze urea</td>
</tr>
<tr>
<td>Genus II: Ureaplasma (2 species with serotypes)</td>
<td>Hydrolyzes urea</td>
</tr>
<tr>
<td></td>
<td>Family II: Acholeplasmataceae</td>
</tr>
<tr>
<td></td>
<td>Sterol not required for growth</td>
</tr>
<tr>
<td></td>
<td>Genome size about $10^9$ daltons</td>
</tr>
<tr>
<td></td>
<td>NADH oxidase localized in the membrane</td>
</tr>
<tr>
<td>Genus I: Acholeplasma (10 species)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Family III: Spiroplasmataceae</td>
</tr>
<tr>
<td></td>
<td>Helical organisms during some phase of growth</td>
</tr>
<tr>
<td></td>
<td>Sterol required for growth</td>
</tr>
<tr>
<td></td>
<td>Genome size about $10^9$ daltons</td>
</tr>
<tr>
<td></td>
<td>NADH oxidase localized in cytoplasm</td>
</tr>
<tr>
<td>Genus I: Spiroplasma (10 species; 23 serotypes)</td>
<td></td>
</tr>
<tr>
<td>Genus of uncertain taxonomic position</td>
<td></td>
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<tr>
<td>Anaeroplasma (2 species)</td>
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<tr>
<td>Asteroplasma (1 species)</td>
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\textsuperscript{a}Adopted from Chanock and Tully (1980) and Razin and Freundt (1984).
Mollicutes has been differentiated into one order, Mycoplasmatales consisting of 3 families (Mycoplasmataceae, Acholeplasmataceae, Spiroplasmataceae). There are about 100 species distributed among 5 genera in the class Mollicutes (Table 2). Most recently, a fourth family, Anaeroplasmataceae, was proposed to accommodate those species in the genus Anaeroplasma (Stephans et al., 1985; Christiansen et al., 1986).

In this review the trivial names mycoplasma, ureaplasma and acholeplasma will be used when reference is made to the corresponding genus; mollicutes will be used to denote all members of the Class (Subcommittee, 1979). The focus of the remainder of the review will be on those mollicutes associated with bovine infectious respiratory disease.

Mollicutes in the Respiratory Tracts of Animals

Mollicutes are part of the autochthonous microbial flora of moist mucosal surfaces in most animals. In particular, the respiratory systems of many animals are colonized by these microorganisms (Cassell et al., 1985). Some mollicutes have demonstrated a predilection for either upper or lower respiratory tract epithelium (Gourlay and Howard, 1982). These microbes tend to exhibit a high degree of species-specificity although there are examples of an organism being isolated from a number of host species, e.g., M. arginini, A. laidlawii and A. axanthum (Gourlay and Howard, 1982; Orning et al., 1978). However, for the most part, relatively few of the currently recognized mollicutes have been
demonstrated to be primary respiratory pathogens. The occurrence of mollicutes in animals, plants and arthropods was thoroughly reviewed by several authors in the publication edited by Razin (1981). Gourlay and Howard (1982) reviewed reports concerning the ubiquity of mollicutes in the respiratory systems of animals. Stalheim (1983) surveyed the literature pertaining to mycoplasmal infections in ruminants. Howard (1984) reported that ureaplasmas were present in the respiratory tract of several animals species.

Mollicutes in the Respiratory Tracts of Calves

Isolation of pleuropneumonia-like organisms from the respiratory tracts of calves with "shipping fever" pneumonia in the early 1950s sparked renewed interest in these organisms and the role they play in infectious respiratory disease (Carter, 1954a,b; Carter and McSherry, 1955). In the ensuing years at least 17 species of acholeplasma, mycoplasma and ureaplasma have been isolated from the bovine respiratory tract (Gourlay and Howard, 1979, 1982; Beer et al., 1986; Liberal and Boughton, 1987).

Probably the most frequent mollicute isolated from the bovine respiratory tract is M. bovirhinis. It has been isolated from cattle worldwide and would appear to be very common in the upper respiratory tract of normal calves although original isolates made by Harbourne et al. (1965) were from nasal swabs as well as lungs of calves with pneumonia. Springer et al. (1982) isolated M. bovirhinis from 31% of nasal swabs taken from calves free of respiratory disease. Friis and Krough (1983)
cultured the organism from 78% of nasal swabs vs. 31.4% of lungs examined; most of the samples were from diseased animals. Thomas and Smith (1972) sampled the nose, trachea, bronchus and lungs of 20, 22 and 28 nonpneumonic calves aged 1-2 days (group A), 3-4 months (group B) and 10 months and older (group C) respectively. Over 90% of nasal swabs from group B yielded *M. bovirhinis* whereas 10% of group A and 18% from group C were positive. Thirty-six percent of tracheas from the group B calves were colonized with the microbe vs 10% and 18% for groups A and C. Only one of the 70 calves which was from group B, yielded *M. bovirhinis* from the lung. Friis (1981) observed a similar pattern of tissue colonization in 2 calves which were challenged with the organism. Hamdy and Trapp (1967b) isolated *M. bovirhinis* from 25% of nasal swabs taken from preweaned calves and from 48% of nasal swabs sampled after weaning. However, it should be noted that *M. bovirhinis* also can occur in a high percentage of lungs from calves with pneumonia. Kuniyasu et al. (1977) isolated it from 89% of one group of 18 calves which also were heavily colonized with *M. dispar* and *U. diversum*. Viring et al. (1986) examined the lower respiratory tracts of 18 normal and 46 pneumonic calves from 21 farms for the presence of microorganisms. *Mycoplasma bovirhinis* was isolated from 83% of the normal calves and 93.5% of those with pneumonia. The pneumonic calves were colonized with *M. dispar* (30%) and *U. diversum* (63%); normal calves also yielded *M. dispar* (17%) and *U. diversum* (28%). The percent recovery of *M. bovirhinis* in select surveys of pneumonic calves ranged from 4% to 43% of samples examined (Nicolet and de Meuron, 1970; Bitsch et al., 1976; Muenster et al., 1979; Zalewska-Schonthaler,
isolated *M. bovirhinis* from 4% of 52 buffaloes which had pneumonia. 

*Mycoplasma arginini* has been cultured from the respiratory tract of many animals including cattle (Gourlay and Howard, 1979). Muenster et al. (1979) cultured the organism from 37% of 153 pneumonic calves; 45% of calves with pneumo-enteritis yielded the microbe (Nicolet and de Meuron, 1970). Jurmanova and Krejci (1971) reported that culture of 200 samples of nasal and eye swabs from 1 to 4-week-old pneumonic calves yielded 63 isolates of *M. arginini* and 80 isolates of *M. bovirhinis*; *M. arginini* also was isolated from 17/28 pneumonic calf lungs. Dellinger et al. (1977) found that the organism persisted for several years in the nasal passages of animals from a large dairy in California. Taoudi et al. (1987) isolated *M. arginini* from 3 calves with nasal discharge; however, attempts to culture the organism from 50 pneumonic calf lungs yielded negative results. Al-Aubaidi and Fabricant (1968), Gourlay et al. (1970), Kuniyasu et al. (1977), Bitsch et al. (1976), Zalewska-Schonthaler (1980) and Friis and Krough (1983) also failed to culture *M. arginini* from pneumonic calf lungs.

*Mycoplasma bovoculi* is an etiologic agent of infectious bovine conjunctivitis (Rosenbusch and Knudtson, 1980) and is associated with infectious bovine keratoconjunctivitis (Rosenbusch, 1983) but is rarely isolated from the respiratory tract. Friis and Krough (1983) isolated the organism from the nasal passages of 3 calves with pneumonia. Jericho and Carter (1985) recorded the isolation of *M. bovoculi* from the respiratory tract (exact site not identified) of 7 calves which had been
experimentally infected with Pasteurella multocida.

Al-Aubaidi (1970) and Cottew (1970) indicated that M. bovigenitalium had been cultured from the bovine respiratory tract. In surveys of calves with pneumonia, the organism has been recovered from approximately 10% of the specimens examined (Pignatelli, 1978; Zalewska-Schonthaler, 1980; Friis and Krough, 1983; Westermilies, 1987). Sood et al. (1986) cultured the organism from 2/52 buffaloes with purulent bronchopneumonia. Muenster et al. (1979), Bitsch et al. (1976) and Gourlay et al. (1970) failed to isolate the organism from pneumonic lung tissues and/or nasal swabs.

Mycoplasma bovis, which was called M. bovimastitidis and M. agalactiae subsp bovis prior to 1976, is frequently cultured from pneumonic calf lungs (Gourlay and Howard, 1979, 1982). Muenster et al. (1979) cultured M. bovis from 37% of 153 pneumonic calves; Pignatelli (1978) isolated M. bovis from 35% of 571 intensively reared veal calves with pneumonia. Eighty-one percent of 29 animals from 16 herds with a history of pneumonia and arthritis yielded M. bovis from lung tissues (Langford, 1977). Bocklisch et al. (1983) reported that 83% of isolates cultured from 426 calves were identified as M. bovis. The organism was also cultured from 8% of 52 pneumonic buffaloes examined by Sood et al. (1986). The first recorded isolations of this species in the United States were from the lung of a pneumonic calf and from an animal with mastitis (Langer and McEntee, 1961). Jasper (1967) mentioned that strains of mycoplasma (later identified as M. bovis) isolated from calves with respiratory disease and supplied by McKercher and Kennedy were identical to those associated with mastitis in California. Thomas et al. (1975)
recorded the first isolation of *M. bovis* in England; Friis and Krough (1983) cultured the organism from only 0.5% of 911 pneumonic lung specimens examined between 1974 and 1981 and indicated that the organism was introduced into Europe about 1980. Taoudi et al. (1987) isolated *M. bovis* from 16% of 50 pneumonic lungs taken from animals in Morocco. Although *M. bovis* has been isolated from the upper respiratory tract of normal healthy calves (Bennett and Jasper, 1977; Springer et al., 1982; Boothby et al., 1983; Kirchhoff and Binder, 1986; Liberal and Boughton, 1987), it is not routinely cultured from normal lung tissue (Collier, 1968; Langford, 1977).

Since *M. dispar* was first reported in 1969, it has been isolated from normal and pneumonic calves worldwide (Gourlay and Howard, 1982; Friis and Krough, 1983). This fastidious organism requires specialized growth medium for isolation from clinical specimens (Gourlay and Leach, 1970; Bitsch et al., 1976). The colony morphology on primary isolation is unique to all other mollicutes save *M. ovipneumoniae*. The colonies are slightly raised with an "oily" or "lacy" surface vs the typical "fried-egg" appearance of conventional mycoplasmas (Gourlay and Leach, 1970). Typical mycoplasmal-like colonies do develop on solid medium after the organism has been subcultured.

Thomas and Smith (1972) reported that *M. dispar* was common in the respiratory tract of non-pneumonic calves 3 to 4 months of age but not in 2-day-old calves or those 10 months and older. Gourlay et al. (1970) isolated *M. dispar* from the lungs of 60% of 45 apparently normal calves and from only 30% of 20 calves which had pneumonia. Gourlay and Howard
(1979) also found a high percentage (93%) of *M. dispar* in nasopharyngeal samples collected from 1 to 8-week-old apparently normal calves. The lungs of 94% of 18 pneumonic calves were colonized with the organism (Kuniyasu et al., 1977). As mentioned previously, this group of calves also was heavily colonized with *M. bovirhinis* (89%) and *U. diversum* (89%). Neither age or breed had any influence on the isolation of *M. dispar* from calves (50% were positive) with clinical signs of respiratory disease (Friis and Krough, 1983). Bitsch et al. (1976) and Muenster et al. (1979) isolated the organism from 62% and 56% of pneumonic lungs respectively.

*Ureaplasma diversum* (Howard and Gourlay, 1982) is frequently cultured from the lungs of calves with pneumonia, often in combination with *M. dispar* (Gourlay and Howard, 1982). This species, which has 3 distinct serotypes, has been cultured from about 50% of the lung samples examined in various surveys, although higher rates have been reported (Gourlay et al., 1970; Bitsch et al., 1976; Gourlay and Howard, 1979; Muenster et al., 1979; Friis and Krough, 1983). Recently, Piolaszek (1986) reported that 47.5% of pneumonic calves or calves in close contact with pneumonic calves yielded *U. diversum* from nasal swabs; similar results were obtained with lung samples. *Ureaplasma diversum* was not cultured from nasal swabs or lung tissue from healthy calves. Gourlay and Howard (1979) also stated that this species was rarely cultured from normal calves.

*Mycoplasma alkalescens, M. californicum, M. canadense* and *M. gallinarum* are not commonly isolated from bovine respiratory tracts. *Mycoplasma alkalescens* (Leach, 1973) was originally isolated from the external nares of cattle in Australia (Hudson and Etheridge, 1963).
Pignatelli (1978) indicated that *M. alkalescens* was isolated from 1.8% of 571 samples taken during a survey of respiratory disease in veal calves. However, samples of synovial fluid were included in the survey and the author did not identify the sources from which *M. alkalescens* was isolated. This organism also has been associated with arthritis and synovitis in calves (Gourlay and Howard, 1979). Beer et al. (1986) cultured *M. californicum* from the lungs of a cow which had therapy-resistant chronic mastitis and from the lungs of a calf which also had polyarthritis. Dellinger et al. (1977) isolated *M. canadense* from nasal passages of an undefined number of calves. Viring et al. (1986) cultured *M. canadense* from the lower respiratory tract of one of 46 pneumonic calves; it was not isolated from 18 healthy animals. Gourlay and Howard (1979) discussed the fact that 3 organisms cultured from the bovine respiratory tract and classified as serotype I by Al-Aubaidi (1970) were later identified as *M. gallinarum*. They also indicated that there had not been any further reported isolations of this avian species from the bovine. However, the organism recently was isolated from sheep with rhinotracheitis at a laboratory which did not have the strain in its stock collection and did not process clinical material from birds (Singh and Uppal, 1987). The identity of the isolate was confirmed at the Mycoplasma Reference Laboratory in England. This leads one to speculate that *M. gallinarum* is not as species-specific as had been assumed.

Two additional organisms not frequently seen in association with calf pneumonia are *Mycoplasma* species group 7 (Leach, 1967) and serogroup L (Al-Aubaidi, 1970). These isolates are closely related to each other as
well as M. mycoides subsp mycoides (Askaa et al., 1978) but have yet to be classified. Langer and Carmicheal (1963) and Hamdy and Trapp (1967a) isolated Mycoplasma species group 7 from the respiratory tract of calves. Pignatelli (1978) reported that the microbe had been cultured from 3 of 326 pneumonic calves. Recently, Alexander et al. (1985) described an outbreak of mastitis in dairy cows and polyarthritis and pneumonia in calves associated with this mollicute. Mycoplasmas were isolated from joint fluids and synovial membranes of 10 calves with polyarthritis and from the apical and cardiac lobes of another calf with pneumonia. Mycoplasma species serogroup L (Al-Aubaidi, 1970) was initially isolated from tissues other than the respiratory tract of a calf which had bronchopneumonia and suppurative fibrinous arthritis (Moulton et al., 1956).

Acholeplasmas have been cultured from the respiratory tract of bovines but there is no valid evidence to suggest that these organisms contribute to disease (Gourlay and Howard, 1979). Acholeplasma laidlawii is the most common species and it has been isolated from the upper respiratory tract of normal (Liberal and Boughton, 1987) and pneumonic (Taoudi et al., 1987) calves. Zalewska-Schonthaler (1980) cultured A. laidlawii from 1% of 263 pneumonic calf lungs. Sood et al. (1986) cultured the organism from 6 lung specimens collected from 52 pneumonic buffaloes. Bitsch et al. (1976), Muenster et al. (1979), Friis and Krough (1983) and Thomas et al. (1982) failed to culture acholeplasmas from the lungs of pneumonic calves. Gourlay and Howard (1979) indicated that a strain of A. axanthum had been cultured from bovine nasal discharge.
Original isolations of this organism were from contaminated cell cultures. *Acholeplasma modicum* has been cultured from pleural exudate, peribronchial lymph nodes and lung material from calves with respiratory disease (Gourlay and Howard, 1979). Recently, Liberal and Boughton (1987) reported that *A. oculi* had been isolated from the upper respiratory tract of apparently normal calves. Rae et al. (1987) then reported that this acholeplasma had been cultured from a number of clinical specimens taken from diseased bovines, including nasal discharge of 5 calves with respiratory disease. Most recently, *A. oculi* was isolated from the amniotic fluid of a clinically normal human (Waites et al., 1987). Prior to these recent reports, the organism had only been isolated from horses and goats (Gourlay and Howard, 1979).

Pathogenicity of Bovine Mycoplasmas in the Respiratory Tract

The mechanisms by which mollicutes may express virulence were reviewed by Gabridge et al. (1985). The exact mechanism(s) by which bovine mycoplasmas and ureaplasmas contribute to disease are not well defined. Several of these organisms possess capsules which have been associated with cytadsorption, virulence and hemagglutination (Tajima et al., 1985). A thick, galactose-based polysaccharide material (galactan) encapsulates *M. mycoides* subsp *mycoides*. Intravenous injection of this material into calves produced changes in blood pressure and hemorrhages and mimicked the action of the biogenic amine, 5-hydroxytryptophan and indicated that the galactan could bind to erythocytes or vascular tissue causing release of biogenic amines in the lung (Razin, 1978). Also,
diffusible substances produced by \textit{M. mycoides} subsp \textit{mycoides} were shown to induce edema and proliferation of connective tissue leading to encapsulation of organisms and isolation from host cellular immune defenses. \textit{Mycoplasma dispar} also is encapsulated (Howard and Gourlay, 1974) and Almeida and Rosenbusch (1987) reported that the capsular material was produced in response to interaction with live bovine cells and speculated that this (capsule formation) may be an initial event in natural infection. In addition, a membrane receptor which may partially be composed of sialic acid was described for \textit{M. dispar} (Howard et al., 1974) and could play a role in attachment of the organism to ciliated epithelium (Thomas and Howard, 1974; Thomas et al., 1987). Geary et al. (1981) have described a toxic glycoprotein purified from \textit{M. bovis} that produced mastitis characterized by influx of eosinophils when infused into the bovine mammary gland. The relevance of such a toxin to bovine respiratory disease is not known. Stalheim and Gallagher (1977) reported that cytopathic effect induced in bovine oviduct organ cultures by bovine ureaplasmas (\textit{U. diversum}) was due to ammonia produced during metabolism of urea by the enzyme urease. However, in studies using fetal bovine tracheal explants, Thomas and Howard (1974) reported that bovine ureaplasmas replicated in the cultures but did not induce ciliostasis or cytopathic effects. \textit{Ureaplasma urealyticum} possesses an immunoglobulin A (IgA) protease which cleaves the IgA molecule into 2 fragments and it was suggested that this activity could facilitate colonization of mucosal surfaces. Although strains of \textit{U. diversum} did not produce a specific IgAase activity, \textit{U. urealyticum} did cause complete degradation of both
Many mollicutes produce hydrogen peroxide as an end product of cell respiration and Gabridge et al. (1985) suggested that this molecule could potentially mediate damage to host cell membranes even though nonvirulent strains of mollicutes also produced hydrogen peroxide. Alternative to direct peroxidation of membrane lipids, Almogar et al. (1986) reported that \textit{M. pneumoniae} infection inhibited host intracellular catalase activity which could lead to oxidative damage to inner-cell wall components as a consequence of increased host-generated hydrogen peroxide and superoxide anion. An intact host-cell glutathione/redox cycle precluded increased intracellular buildup of the toxic oxidants.

Reproduction of disease and achievement of the criteria of Koch's postulates have been difficult at best when working with mollicutes. Recognizing that \textit{M. mycoides subsp mycoides} is probably the most virulent organism in the class, only in recent years was classical CBPP produced following experimental aerosol challenge. The pathogenicity of mollicutes associated with the bovine respiratory tract are examined in this section.

\textbf{Mycoplasma mycoides subsp mycoides}

Direct contact between animals infected with \textit{M. mycoides subsp mycoides} and non-infected animals results in classic CBPP. Also, indirect contact with urine-contaminated hay and placentas from infected animals apparently can spread the disease (Gourlay and Howard, 1979). Early attempts to reproduce CBPP by aerosol challenge resulted in the development of pneumonic lesions which were not characteristic of those
observed under field conditions. Lloyd and Etheridge (1983) exposed 3 groups of bovines to aerosols of particle sizes 12, 6 or 2 micrometers containing *M. mycoides* subsp *mycoides*. Although pneumonia was induced by each aerosol, only one animal from the group exposed to particle sizes of 12 micrometers developed lesions consistent with those seen in CBPP.

The type strain of *M. mycoides* subsp *mycoides* (PG1) produced complete and partial ciliostasis in fetal mouse and chicken embryo tracheal explants respectively. The organisms remained viable in the mouse explant cultures for 8 days and were still viable at 21 days post-inoculation of the chicken explant cultures (Araake, 1982).

*Mycoplasma bovigenitalium*

Four gnotobiotic calves were exposed intratracheally to 2 strains of *M. bovigenitalium* and examined 3 weeks later (Gourlay et al., 1979). Although clinical signs were not observed, macroscopic pneumonia involving 3% to 8% of total lung surface was seen in 3 calves challenged with one of the strains. Peribronchiolar and perivascular lymphocytic infiltrates (cuffing) and catarrhal bronchiolitis with sloughing of bronchiolar epithelium were seen in histologic sections of lung. Culture of lung washings yielded *M. bovigenitalium* (10^4 & 10^5 organisms/ml). The second strain used in this study did not colonize the lung. These data demonstrated that strains of *M. bovigenitalium* can vary in their ability to colonize and produce disease in the bovine lung.

Afsher (1967) reported that infection of cell culture monolayers with *M. bovigenitalium* produced cytopathic effects; sterile spent medium from
these infected monolayers also produced cytopathic effects when used on fresh monolayers.

**Mycoplasma bovis**

*Mycoplasma bovis* is second to *M. mycoides* subsp *mycoides* in pathogenicity for cattle and has long been associated with bovine infertility and mastitis (Gourlay and Howard, 1979, 1982; Thomas et al., 1986). Only recently has it been reported to produce pneumonia under controlled conditions. Four conventional calves each challenged intratracheally with $6.8 \times 10^{10}$ *M. bovis* organisms did not develop consistent macroscopic changes but did develop mild to severe peribronchiolar lymphoid hyperplasia 7 days after infection (Lopez et al., 1986). Culture of lung tissues from each calf yielded *M. bovis*. Two 5-month-old conventional calves were inoculated endobronchially with homogenate of pneumonic lung containing *M. bovis* (Thomas et al., 1975). Clinical signs, e.g., polyarthritis, pyrexia, dyspnea, developed and at necropsy (8 days post-infection), extensive consolidation of one diaphragmatic lobe and fibrinous adhesions were observed in each calf. Culture of lung homogenates yielded $10^{7}$ and $10^{8}$ colony forming units (cfu) of *M. bovis* organisms per gram (g$^{-1}$) of tissue. Gourlay and Houghton (1985) produced macroscopic pneumonia involving 2% to 60% of the lung surface in 20 conventional calves challenged with *M. bovis* and *P. haemolytica*. Peribronchiolar lymphoid hyperplasia and focal areas of necrosis were observed in the lung parenchyma in 4 animals. Titers of *M. bovis* organisms ranged from $10^{3}$ to $10^{9}$ cfu g$^{-1}$ of lung tissue. Gilmore
et al. (1986) have confirmed these findings and used this \textit{M. bovis}/\textit{P. haemolytica} challenge system to study the effects of oxytetracycline therapy in calves with pneumonic pasteurellosis. Recently, Brys (1987) reported that nasal discharge developed 3-5 days after intranasal instillation of \textit{M. bovis} in 19 conventional calves. Infected calves shed the organism for up to 3 weeks post challenge. Macroscopic changes and catarrhal bronchopneumonia were present in all but 3 calves at necropsy. No changes were seen in 4 calves inoculated with sterile broth. Nineteen calves were exposed by intratracheal inoculation and 7 calves by endobronchial inoculation of \(2 \times 10^8\) \textit{M. bovis} organisms (Pfutzner et al., 1983). Pneumonia was evident in several calves and \textit{M. bovis} was cultured from lung tissues of 15 of the calves; however, \textit{P. haemolytica} also was isolated from the calves. Seven conventional Zebu bulls were challenge-exposed with \(3.5 \times 10^8\) \textit{M. bovis} organisms by multiple intranasal instillation; 3 control bulls received 5 intranasal inoculations with sterile broth (Onoviran, 1972). Clinical signs observed after challenge were a febrile response in 2 animals and nasal discharge (3 animals). At necropsy (3-5 weeks post challenge), gross changes were seen in 3 bulls; however, microscopic changes were not described. Culture of various tissues yielded \textit{M. bovis}; no changes were seen in control animals. The author concluded that this strain of \textit{M. bovis} was able to colonize respiratory tissues without producing disease.

In unpublished reports, Frey indicated that 6 cesarian-derived, colostrum-deprived calves raised as gnotobiotics to 4 weeks of age developed various clinical signs of pneumonia following aerosol exposure
to *M. bovis* either 7 days before bovine respiratory syncytial virus challenge, or 5 days after challenge with bovine rhinovirus. Further details were not described and no mention of control calves was made. (Progress reports to North Central-107 Technical Committee, Manhattan, KS, September 6-7, 1978; Fargo, ND, September, 5-6, 1979).

Since pathogenic microorganisms would not be present in gnotobiotic animals, results from challenge studies would presumably be more significant than those obtained using conventional animals. Also, prior to 1972, mycoplasma cultures were not generally "cloned" and challenge inocula quite probably contained more than a single species. Gourlay et al. (1976) reported that endobronchial inoculation of a cloned *M. bovis* isolate induced subclinical pneumonia with macroscopic lesions involving 6% to 14% of the lung surface and arthritis in gnotobiotic calves. Microscopically, peribronchiolar lymphocytic cuffing ranging from simple infiltration of lymphocytes to extensive lymphoid hyperplasia was observed. Also, focal areas of coagulative necrosis surrounded by lymphocytes were observed in the lung parenchyma. Although 1/5 control calves inoculated with sterile broth had a macroscopic lesion, no significant cellular response was observed. Whereas endobronchial exposure resulted in pneumonic changes, intranasal exposure of 2 gnotobiotic calves to *M. bovis* failed to induce any changes in respiratory tract tissues (Houghton and Gourlay, 1983). Knudtson (1980) also reported that the colonization pattern of *M. bovis* in conventional calves with pneumonia was different from that of *M. dispar*. In addition to colonization of bronchiolar epithelium, *M. bovis* antigen was detected by
immunofluorescence within alveoli and in focal areas of necrosis in the parenchyma of the lung. Recently, Thomas et al. (1986) infected 7 gnotobiotic calves intratracheally with M. bovis producing clinical signs (inappetence and increased respiration) and gross pneumonia involving 4% to 37% of the lung surface. Microscopic examination of lung tissues revealed foci of coagulative necrosis surrounded by mononuclear cells, suppurative bronchiolitis and only limited peribronchiolar lymphoid hyperplasia. Alveolitis was not a consistent lesion seen in the M. bovis-challenged calves. Immunoperoxidase staining revealed M. bovis antigen within and surrounding the areas of focal necrosis. The authors suggested that this very characteristic lesion was similar to peribronchiolar lymphoid hyperplasia (cuffing), which is common to a number of mycoplasmal infections including those associated with M. hyopneumoniae, M. pneumoniae and M. pulmonis. Also, Howard et al. (1986) reported that antibodies specific for M. bovis were synthesized by the lymphocytes associated with the lesion. Culture of lung homogenates from the 7 calves revealed M. bovis titers ranging from $10^6.6$ to $10^8.8$ cfu g$^{-1}$ of tissue.

Fetal bovine tracheal explants infected with M. bovis did not develop ciliostasis; however, the organism did establish and replicate in explant subepithelium (Thomas et al., 1987). Cytopathic effects induced in fetal bovine kidney and turbinate cell monolayers by cultures of M. bovis were serum-dependent (Hirth et al., 1970). Geary et al. (1981) have reported that M. bovis possesses an extractable toxin which had biologic activity. Thomas et al. (1987) have indicated that they were unable to duplicate the
results of Geary et al. using a different isolate of *M. bovis*. Also, they were unable to demonstrate toxin activity in sterile, cell-free filtrates of spent medium from explant cultures that had been infected with *M. bovis*.

**Mycoplasma dispar**

Endobronchial challenge exposure with cloned cultures of *M. dispar* resulted in pneumonic lesions in 12/25 conventional calves. However, 2/9 control calves inoculated with sterile broth also had pneumonic lesions (Gourlay and Thomas, 1969, 1970). Subclinical but macroscopic pneumonia was produced in naturally-born, colostrum-deprived calves exposed to aerosols of *M. dispar* (Friis, 1980). Two strains of the organism produced lesions in one 3-week-old calf each while a third *M. dispar* isolate produced lesions in a 3-month-old calf. Microscopic examination of lung sections revealed catarrhal bronchiolitis, cuffing and mononuclear cell infiltration of alveolar walls (alveolitis). The lumen of the air passages was often filled with neutrophils and cellular debris.

*Mycoplasma dispar* was isolated from lung and pharyngeal tissues of all animals sampled. No control calves were included in the study. Riberio (1979) reported that endobronchial inoculation of 4 conventionally-reared calves with *M. dispar* produced mild inflammatory changes in the airway epithelium. Culture of lung tissues from these calves yielded negative results but high titers of the organism were cultured from the trachea and nasopharyngeal mucosae. Culture of tissues from 2 calves inoculated with sterile broth yielded negative results.
Proliferative interstitial pneumonia was produced in 1 and 2-day-old cesarean-derived or colostrum-deprived calves inoculated intratracheally either with *M. dispar* broth culture or lung homogenate containing *M. dispar* (St. George et al., 1973). Explants of trachea prepared from challenged calves were collected at necropsy, placed in suitable culture media and monitored for the presence of *M. dispar*. Two of the explant cultures yielded *M. dispar*. No lesions were observed nor organisms cultured from the lungs of 2 control calves inoculated intratracheally with sterile mycoplasma broth.

Howard et al. (1976) reported that macroscopic but subclinical pneumonia involving 2-5% of the lung was produced in gnotobiotic calves following endobronchial challenge with a cloned culture of *M. dispar*. Histologic examination of lung sections revealed peribronchiolar lymphocytic infiltration in one calf, catarrhal bronchiolitis in 2 calves and round cell infiltration in each calf. The organism was isolated from lung tissues of each calf and ranged in titers from $1 \times 10^4$ to $1 \times 10^6$ ccu/ml. Also, 4 additional calves were exposed to a combination of *M. dispar* and *U. diversum*. The colonization of *M. dispar* and microscopic lesions in the lung were essentially the same as described for calves challenged with only *M. dispar*. Round cell proliferation was seen in histologic sections of lung from 1 of 3 control calves inoculated endobronchially with sterile broth; no mycoplasmas were cultivated from lung homogenates. An additional 10 gnotobiotic calves were challenged intratracheally with the same strain of *M. dispar* (Gourlay et al., 1979). Clinical signs were not observed but macroscopic pneumonia (0-17%) was
present in 8 animals. Microscopic lesions present in the lungs were interstitial alveolitis, round cell infiltration and catarrhal bronchiolitis in 100%, 70% and 30% of the calves respectively. One animal exhibited cuffing pneumonia. All lung homogenates yielded *M. dispar* on culture examination. It is interesting that in these calves alveolitis was the most consistent lesion induced, whereas in clinical cases of calf pneumonia associated with *M. dispar*, peribronchiolar lymphocytic infiltrations are frequently observed (Pirie and Allen, 1975; Tinant et al., 1979).

Thomas and Howard (1974) reported that *M. dispar* colonized the ciliated epithelium of fetal bovine tracheal explant cultures producing ciliostasis and cytopathic effects. The cytopathic effects were dependent on the presence of serum in the explant medium and the number of organisms in the inoculum. Thomas et al. (1987) confirmed the pathogenicity of *M. dispar* for fetal bovine tracheal explants.

**Ureaplasma diversum**

Sixteen conventionally reared calves were exposed by endobronchial inoculation of broth cultures of *U. diversum* (T-mycoplasmas) and necropsied 4 weeks post-challenge (Gourlay and Thomas, 1969, 1970). Six calves developed clinical pneumonia; macroscopic changes were observed in 14 animals and ureaplasmas were cultured from 13. Clinical signs developed in 1/9 controls inoculated with sterile broth; 2 calves had macroscopic lesions but ureaplasmas were not isolated from the lungs. However, it should be mentioned that in 12 of 16 calves sampled, 9 yielded
mycoplasmas and ureaplasmas from pre-challenge swabs taken from the lower respiratory tract. The presence of these organisms prior to inoculation of the challenge did not correlate with development of lesions or influence the titer of ureaplasmas isolated at necropsy.

Four-week-old conventional calves were challenge exposed to three strains of *U. diversum* (5 calves/strain) originally cultured from pneumatic calf lung, nasal mucosa and normal bull semen respectively. Each calf received 10.0 ml intratracheally and 5.0 ml intranasally of sterile broth or broth culture containing 1 X 10^5 ureaplasma organisms. Five calves inoculated with sterile broth served as controls. Clinical signs and macroscopic lesions were observed in 10/10 and 9/10 calves exposed to the lung and nasal strains respectively and *U. diversum* was reisolated from lung tissues. Two calves exposed to the semen isolate developed clinical signs but had no gross changes and ureaplasmas were not cultured from the lung specimens. No changes were observed in control calves and ureaplasmas were not cultured from their tissues. The authors did not provide data relating to microscopic examinations of respiratory tract tissues (Truzczynsk and Pilaszek, 1984).

Six colostrum-deprived calves isolated immediately after birth were exposed to aerosols of broth culture of cloned and pooled crude isolates of *U. diversum* which originated from the lung of a calf with pneumonia (Friis, 1981). No respiratory distress was observed in any of the calves; culture of larynx, trachea and right cardiac lobe of one calf challenged with 2 cloned isolates yielded *U. diversum*. No control animals were included in the study.
Two gnotobiotic calves inoculated endobronchial with *U. diversum* developed gross changes (10% of total lung area) and microscopic pneumonia characterized by round cell infiltration and peribronchiolar lymphoid hyperplasia (Howard et al., 1976).

Fetal bovine tracheal explants inoculated with *U. diversum* did not develop any changes, i.e., ciliostasis, however the organisms did replicate in explant culture medium (Thomas and Howard, 1974). Kotani and McGarrity (1986) infected HeLa and CV-1 cell cultures with *U. diversum* and showed that the organism would replicate and remain viable for 48 hours; no cytopathic effects were described.

**Bovine Respiratory Tract Mollicutes of Questionable Pathogenicity**

*Mycoplasma alkalescens*

No changes were observed in 2 gnotobiotic calves during a 3 week period after intratracheal challenge with 2 strains of *M. alkalescens*. Culture of lung washings taken 3 weeks post challenge yielded the organism, indicating that *M. alkalescens* could colonize the bovine lung without producing any significant lung pathology (Gourlay et al., 1979).

*Mycoplasma arginini*

Two gnotobiotic calves were inoculated intratracheally each with a different strain of *M. arginini*. Clinical signs were not observed and no changes were seen in the lungs at necropsy (3 weeks post challenge); histologic examination of the lung also yielded negative results. The organisms were cultured from lung washings of the 2 calves indicating that
the strains of *M. arginini* used were able to colonize the lung but did not induce pneumonia (Gourlay et al., 1979).

Reduced ciliary activity was observed in chicken tracheal explant cultures 8 days after inoculation with an isolate of *M. arginini* which originated from the nasal cavity of a horse with acute rhinitis (Moorthy and Spradbow, 1985). Ciliostasis and loss of cilia also were produced in explant cultures prepared from neonatal kid and fetal lamb tracheas following inoculation with ovine strains of *M. arginini* (Jones, 1985).

**Mycoplasma bovirhinis**

Challenge exposure of neonatal colostrum-deprived or conventional calves with strains of *M. bovirhinis* commonly has failed to induce clinical pneumonia or gross and microscopic changes in the respiratory tract (Hamdy et al., 1958; Dawson et al., 1966; Gourlay and Howard, 1979; Friis, 1981). However, scattered pneumatic lesions were produced in 2 calves exposed by aerosol and intranasal inoculation (Hamdy and Trapp, 1967a). Jurmanova et al. as cited by Gourlay and Howard (1979) challenged conventional calves endobronchially with *M. bovirhinis* alone or in combination with *Pasteurella haemolytica* or parainfluenza 3 virus. Clinical signs developed in 2 calves exposed endobronchially only to *M. bovirhinis*, and at necropsy one calf had pneumonia; the organism was not recovered from lung tissues. However, it was isolated from other organs and the upper respiratory tract. In combined infection with the bacterium or the virus, 4 of 5 calves developed gross pneumonia but again, *M. bovirhinis* was not recovered from lung tissue.
Gourlay et al. (1979) exposed gnotobiotic calves to 2 strains of *M. bovirhinis*. Although macroscopic pneumonia (6%) developed in one calf and the organism was reisolated from lung tissue, a concurrent *E. coli* infection was determined to be the major contributor in development of the lesion. Another calf challenged with the same strain of *M. bovirhinis* showed no changes; the authors concluded that *M. bovirhinis* had little, if any, pathogenicity for the bovine lung.

Thomas and Howard (1974) reported that *M. bovirhinis* did not induce changes in fetal bovine tracheal explants, but did replicate and maintain itself in the explant culture.

**Mycoplasma species Group 7**

Hamdy and Trapp (1967a) challenged 6 colostrum-deprived calves at 3-weeks of age by intranasal and intratracheal inoculation with an organism identified as *Mycoplasma* sp. group 7 by Al-Aubaidi (1970). No clinical signs were observed but 2 calves posted 7 days later had pericarditis and mycoplasmas were cultured from pericardial fluid. Scattered mild pneumonic lesions were seen in 2 calves posted 4 weeks after challenge; no changes were seen in the 2 calves examined 2 weeks after challenge.

**Mycoplasma verecundum**

No changes were seen in 2 gnotobiotic calves challenged with $10^{10}$ *M. verecundum* organisms (Gourlay et al., 1979).
**Mycoplasma canadense**

*Mycoplasma canadense* did not colonize nor induce lesions in the lungs of 2 gnotobiotic calves that had been challenged intratracheally with the organism (Gourlay et al., 1979).

**Acholeplasma species**

Gourlay and Howard (1979) concluded that bovine acholeplasmas were not pathogenic to cattle. Thomas and Howard (1974) had showed that *A. laidlawii* was capable of replicating in fetal bovine tracheal explant cultures without producing any cytopathic effects. There is some evidence that *A. oculi*, an organism only recently cultured from calves, may be pathogenic in sheep and goats (Cottee, 1979). This mollicute also has been isolated from equines and shown to replicate and produce cytopathic effects in chicken-embryo tracheal explants (Moorthy and Spradbrow, 1985).
PART I  IDENTIFICATION OF MYCOPLASMATALES IN PNEUMONIC CALF LUNGS
IDENTIFICATION OF MYCOPLASMATALES IN PNEUMONIC CALF LUNGS

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SUMMARY

Lungs from 153 calves with clinical signs of pneumonia were examined post-mortem for the presence of mycoplasmas and ureaplasmas during a 38-month period. Sixty-two percent of the cases were submitted during the months when wide fluctuations in climatic conditions occur. However, the presence of mollicutes in the lungs was not seasonal suggesting that infection by these organisms possibly could have been related to management-related factors. Indirect fluorescent antibody tests (IFAT) and culture were used and mycoplasmas and/or ureaplasmas were detected in 63% of the lungs examined. Mycoplasma dispar was detected in 39%, M. bovis in 36%, U. diversum in 22% and M. bovirhinis in 8.5% of the lungs. Thirty percent of the lungs were infected with more than one species; the most frequent combination was M. bovis, M. dispar and U. diversum (10.5%). Detection of M. bovis by culture and IFAT agreed 100% of the time indicating that IFAT could replace culture for routine detection of this microbe. Mycoplasma arginini, M. bovigenitalium and acholeplasmas were not cultured. Mycoplasma dispar was shown to remain viable for up to 15 days post mortem in apical and cardiac lobes held at 4 C and also was detected by IFAT in the same tissues for 49 days.
INTRODUCTION

Of the 20 or so members of the Mycoplasmataceae which have been recovered from cattle, 13 have been from the respiratory tract (Gourlay and Howard, 1982). Although the upper respiratory tract of both normal and pneumonic calves is frequently colonized with mycoplasmas (Hamdy and Trapp, 1967; Nawakowski, 1971; Pignatelli, 1978; Gourlay and Howard, 1978; Springer et al., 1982), the percentage of pneumonic calves which harbor mycoplasmas in their lungs is greater than that of non-pneumonic calves (Bitsch et al., 1976; Gourlay and Leach, 1970; Harbourne et al., 1965; Thomas and Smith, 1972; Muenster et al., 1979).

Of the 13 species of Mycoplasmataceae cultured from bovine lungs, Mycoplasma bovigenitalium, M. bovis, M. dispar and U. diversum have produced subclinical pneumonia when endobronchially and/or intratracheally introduced into gnotobiotic calves (Gourlay et al., 1976; Howard et al., 1976; Gourlay et al., 1979). Pneumonia was not produced in gnotobiotic calves by intratracheal inoculation of M. arginini, M. alkalescens, M. bovirhinis, M. canadense or M. verecundum; M. mycoides subsp mycoides was not included in the experiments (Gourlay et al., 1979).

Mycoplasma bovis is seldom isolated from clinically normal calves (Bennett and Jasper, 1977; Springer et al., 1982); however, in separate surveys Muenster et al. (1979) and Pignatelli (1978) isolated the organism from a high percentage of pneumonic calves. Gourlay and Howard (1982) reported that M. dispar and U. diversum were frequently isolated from pneumonic calves while M. bovigenitalium was rarely cultured from the
bovine respiratory tract.

As part of a microbial survey of lungs from pneumonic calves immunofluorescence and/or culture were used to detect mycoplasmas and ureaplasmas. The results of these examinations are the subject of this report.
MATERIALS AND METHODS

Indirect Fluorescent Antibody Test (IFAT)

Lyophilized horse and rabbit anti-bovine mycoplasma sera (A/S) were obtained from Dr. O. H. V. Stalheim, National Animal Disease Center, Ames, IA and Dr. H. Erno, World Health Organization Reference Center, Aarhus, Denmark. Rabbit A/S to M. bovirhinis and M. dispar were received from Dr. J. G. Tully, National Institutes of Health, Beltsville, MD. A working dilution of 1:100 of horse and rabbit anti-mycoplasma species A/S yielded excellent fluorescence and this dilution was used routinely. Frozen lung sections (6 to 8 micrometers) were fixed in cold acetone for 8 minutes and either stained immediately or stored at -70 C. In the staining process sections were flooded with horse A/S to mycoplasmas and incubated in a moist chamber for 60 minutes at 37 C. The sections were rinsed for 5 minutes in each of 3 PBS washes followed by 2 minutes in distilled water. The sections were then allowed to air-dry before being flooded with fluorescein-conjugated goat anti-horse gamma globulin (Cappel Laboratories, Cochranville, PA). The sections were incubated for 25-30 minutes followed by rinses with PBS and water. Glass coverslips were applied with pH 8.6 glycerol-carbonate bicarbonate buffer (9:1) and the sections were examined with an epi-illumination fluorescence microscope. Tissues were routinely screened for M. arginini, M. bovigenitalium, M. bovirhinis, M. bovis and M. dispar. Specificity of the IFAT reaction was demonstrated by a blocking test whereby preincubation of lung sections
with rabbit A/S to mycoplasma species was able to block the reaction. Normal rabbit serum did not prevent fluorescence.

The effect of long term storage at 4 C on fluorescence also was evaluated. Apical and cardiac lobes of lungs from calves experimentally infected with *M. dispar* were used as positive controls. Pieces of tissue were cut from the lobes every 7 days and examined by the IFAT. Also, bronchial exudates were cultured on a modified Friis agar as described below.

Survey of Pneumonic Calves

**Specimens**

Calves with clinical signs of pneumonia or dead as result of pneumonia were submitted to the Iowa Veterinary Diagnostic Laboratory for evaluation. The animals ranged in age from 1 week to 11 months with the majority of them 2-4 months of age. Most were of Holstein or Hereford breed from confined dairy and small feedlot operations. Portions of apical and cardiac lobes and other areas of the lung with gross lesions were removed for mycoplasma and *U. diversum* examinations.

**Preparation of inoculum**

A 1:10 (w/v) suspension of lung material representative of the submitted sample was prepared in modified Bovarnick's solution (Rosenbusch and Knudtson, 1980) using a Ten-Broeck grinder. The suspension was centrifuged at 150 x g for 8 minutes and the supernatant fluid removed.
Following inoculation of broth media, the remaining supernatant fluid was stored at -73 C.

**Fresh yeast extract**

Yeast extract (HYE) was prepared essentially according to Herderschee (1963). Briefly, 1 liter of water, pH 4.5 was heated to 80 C in a water bath and 1 kg Fleischmann's dry yeast Type 20-40 was added with stirring. The mixture was held at 80 C for 20 minutes being certain the pH remained at 4.5. After cooling, the mixture was transferred to 250 ml tubes and centrifuged at 3500 x g for 30 minutes. The supernatant fluids were pooled and centrifuged at 6000 x g for 30 to 60 minutes. Following clarification through Whatman's GF/A paper the extract was sterilized by filtration and stored at -73 C. The HYE was used in all media at a final concentration of 2%.

**Microbial inhibitors**

The following aqueous stock solutions (100X) were prepared: bacitracin 2%, staphcillin 2%, thallium acetate 1% and penicillin G 100,000 units/ml. The solutions were filter sterilized and stored at -73 C as 5 ml aliquots.

**Mycoplasma media**

A modification of Friis medium (Friis, 1975) with components of M-96 medium (Frey et al., 1973) was prepared as a 2X stock solution (2 X FM). Brain-heart infusion 32.8 g, PPLO broth base without crystal violet, 34.8 g and 2 bottles of 1X desiccated Hanks' balanced salts (Difco
Laboratories, Detroit, MI) were added to 1 liter of distilled/deionized water and allowed to mix for 1 hour. After mixing, the following components were added: 10.6 ml of 1% calf DNA, 1.65 ml of 1% phenol red solution, 0.4 g L-arginine, 0.45 g L-glutamine and 1490 ml water. This mixture was stirred continuously for 30 minutes, prefiltered, sterilized by filtration and stored at 4 C.

Broth medium for isolation was prepared by combining the following sterile components: 50% 2X FM, 20% heat-inactivated fetal bovine serum, 2% HYE, 25% water and 1% each of bacitracin, staphcillin and thallium acetate. Following adjustment of the medium to pH 7.5 with sterile 1N NaOH, 1.8 ml was dispensed into 13 x 100 mm sterile tubes and stored at 4 C.

Solid FM medium was FM broth plus 0.6% Agarose ME (FMC Corporation, Rockland, ME) and 0.01% DEAE dextran (Sigma Chem. Co., St. Louis, MO) as described by Friis, 1975.

**Ureaplasma media**

Modified Hayflick's liquid medium was used as described by Livingston and Gauer (1976) except HYE replaced the yeast extract component. FM broth modified to contain 20% horse serum, 1% penicillin G, and 0.8% urea and adjusted to pH 6.0 also was used for primary isolation. Broth medium was dispensed into 13 x 100 mm tubes and stored at 4 C for a maximum of 2 weeks before discarding if not used.

Hayflick's solid medium was modified to contain 0.6% Agarose ME, 0.01% DEAE dextran and 0.02 M 2-(N-morpholine) ethane sulfonic acid (MES).
The agarose medium was discarded if not used within 7 days; solid FM was not used for primary isolation of ureaplasmas.

Shepard's urease reagent was used to differentiate mycoplasmas from ureaplasmas on solid medium (Livingston and Gauer, 1976).

**Isolation procedures**

Bronchial exudate was obtained from cut sections of lungs and streaked onto solid FM which was incubated in a CO₂ (7%) incubator for 30 minutes prior to inoculation. Following inoculation, the plates were incubated at 37 C in a high humidity CO₂ incubator and examined daily. After 5 days those not showing growth were incubated an additional 20 days and examined periodically.

Serial ten-fold dilutions of lung inoculum were prepared to 10⁻⁹ in broth. The dilution cultures were incubated on a roller drum (8 rev/hr) at 37 C and observed daily for signs of a pH change and/or turbidity. When growth was evident, the culture was streaked onto FM agar and incubated as described. Representative colonies which developed on agar were cloned 3X before identification by growth inhibition and growth precipitation tests according to Erno and Jurmanova, 1973. All isolates were tested for sensitivity to 5% sodium-polyanethol sulfonate and 1.5% digitonin (Freundt et al., 1973). Mycoplasmas are susceptible to the lytic action of these 2 compounds while acholeplasmas are not.

When growth was evident in broth used to isolate ureaplasmas, a drop was streaked onto Hayflick's agar and incubated as described. After development of colonies, the surface of the agar was flooded with
Shepard’s urease reagent. Colonies which developed a dark brown to black precipitate were considered *U. diversum*. 
RESULTS

Indirect Immunofluorescent Antibody Test

Figure 1 illustrates the pattern of fluorescence observed on the bronchiolar epithelial surface in sections containing M. dispar. A similar pattern was observed in sections from one lung colonized with M. bovirhinis. Mycoplasma bovis organisms were present on the epithelial surface as well as within neutrophil-filled exudate present in bronchiolar lumens (Figures 2 and 3). In some instances, accumulation of numerous M. bovis organisms within bronchioles and alveoli resulted in areas of intense fluorescence (Figure 4).

A gradual decrease in the intensity of fluorescence for M. dispar was observed in tissues held at 4 C. Three lungs were positive for 15 days post-mortem; 2 remained positive for 21 days. One lung remained positive for 4 weeks while another which was in an advanced state of decay was positive through the 7th week. Immunofluorescence was correlated with culture of M. dispar from bronchial exudates up to 14 days post-mortem; M. dispar was not cultured from tissue stored at 4 C beyond 15 days post-mortem.

Detection of Mycoplasmatales

During the time of this survey (38 months), 153 calves representing 131 herds were examined for the presence of Mycoplasmatales. The isolation rates per sampling period are given in Table 1.
Figure 1. An immunofluorescent positive bronchiole. The zone of fluorescence is confined to the bronchiolar epithelium colonized with *Mycoplasma dispar*. x250

Figure 2. An immunofluorescent positive bronchiole. The bright particles within the bronchiolar lumen are *Mycoplasma bovis* organisms. x400
Figure 3. An immunofluorescent positive bronchiole. The lumen is occluded with neutrophils and *M. bovis* organisms. x250

Figure 4. Focal areas of immunofluorescence in lung colonized with *Mycoplasma bovis*. The bright particles represent individual *M. bovis* organisms. x130
Table 1. Sampling period distribution of individual calves and herds which were positive for Mycoplasmataceae during a 38-month survey

<table>
<thead>
<tr>
<th>Sampling Period</th>
<th>Animals Positive</th>
<th>Herds Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>I</td>
<td>19/31b</td>
<td>17/27 63</td>
</tr>
<tr>
<td>II</td>
<td>63/97</td>
<td>54/81 67</td>
</tr>
<tr>
<td>III</td>
<td>14/25</td>
<td>14/23 61</td>
</tr>
<tr>
<td>Survey Total</td>
<td>96/153</td>
<td>85/131 65</td>
</tr>
</tbody>
</table>

*aSampling Periods: I (June-September); II (October-January); III (February-May).

*bNumber positive/number samples.

Approximately 63% of all samples examined were positive for ureaplasmas or mycoplasmas either by IFAT and/or culture; more than one species was cultured from 30% of the animals examined (Table 2). *Mycoplasma dispar* was detected in 39% of the specimens and was the only mycoplasma detected in 26 samples. The correlation between isolation and IFAT was 82%; in 9 instances it was detected only by IFAT and repeated
Table 2. Pattern of distribution of mycoplasmas and ureaplasma demonstrated by culture and indirect immunofluorescence in 96 of 153 pneumonic calf lungs

<table>
<thead>
<tr>
<th>Organism(s)</th>
<th>Number of Calves</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>dispar</td>
<td>26</td>
<td>17</td>
</tr>
<tr>
<td>dispar/bovis</td>
<td>9</td>
<td>5.9</td>
</tr>
<tr>
<td>dispar/ureaplasma</td>
<td>4</td>
<td>2.6</td>
</tr>
<tr>
<td>dispar/bovirhinis</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>dispar/bovis/bovirhinis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>dispar/bovis/ureaplasma/bovirhinis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>dispar/bovis/ureaplasma</td>
<td>16</td>
<td>10.5</td>
</tr>
<tr>
<td>bovis</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>bovis/bovirhinis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>bovis/ureaplasma</td>
<td>10</td>
<td>6.5</td>
</tr>
<tr>
<td>ureaplasma</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ureaplasma/bovirhinis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>bovirhinis</td>
<td>6</td>
<td>3.9</td>
</tr>
</tbody>
</table>
attempts to isolate the organism from the original lung specimens stored at 4 C or lung inoculum stored at -73 C were unsuccessful. Two specimens from which M. dispar was isolated were negative by IFAT. The titer of M. dispar organisms ranged from $5 \times 10^4$ to $5 \times 10^7$ per gram (g$^{-1}$) of lung tissue. In some instances, M. dispar was not cultured from $10^{-1}$ or $10^{-2}$ dilution cultures but was present at $10^{-3}$ and beyond. Thirty-six percent of the samples yielded M. bovis which was isolated in combination with other mycoplasmas and/or ureaplasmas in 63% of those samples. The correlation between isolation and IFAT was 100%. Titers of M. bovis ranged from $5 \times 10^4$ to $5 \times 10^9$ organisms g$^{-1}$ of lung tissue.

Ureaplasma diversum was cultured from 22% of the samples. After examining 55 specimens for ureaplasmas, no advantage was observed in using 2 media for primary isolation. Thereafter, only the Hayflick's medium was used to culture U. diversum. Detection of U. diversum colonies on solid medium was made easier by addition of MES buffer which enhanced the size of the colonies on the agar surface. The titer of U. diversum organisms in pneumonic lung tissues was essentially the same as for M. bovis.

Mycoplasma bovirhinis was isolated from 8.5% of the calves examined, in 6 instances as a pure culture. The only lung positive for M. bovirhinis by both IFAT and culture also was positive by both IFAT and culture for M. dispar.

Approximately 11% of the lungs were infected with M. bovis, M. dispar and U. diversum. Other combinations exceeding 1% were as follows: M. bovis and U. diversum, 6.5%; M. bovis and M. dispar, 6%; M. dispar and U. diversum, 2.6%; M. dispar and M. bovirhinis 2%. 
None of the specimens were positive by culture or IFAT examination for *M. arginini* or *M. bovigenitalium*. Attempts to isolate acholeplasmas also yielded negative results.
DISCUSSION

In this study, we found that mycoplasmas and ureaplasmas were present in 63% of the pneumonic calf lungs examined during a 38-month period. A majority of cases (63%) was submitted during a period from October through January when extremes in climatic conditions are experienced in the Northern Plains States, U.S.A. However, the incidence of mycoplasmas detected in pneumonic lungs did not change dramatically from one sampling period to another. Thus, we conclude that the role of mycoplasmas in pneumonia of calves is not seasonal but may be the result of management-related factors.

The overall isolation rate in this survey was similar to the 60% and 68% reported by Nawakowski (1971) and Gourlay et al. (1970). In contrast, percentages of 81, 88, and 88 were reported by Pignatelli (1978), Bitsch et al. (1976) and Muenster et al. (1979). There are several factors which could contribute to the differences in isolation percentages. The age of calves sampled has been shown to influence the rate of isolation of mycoplasmas from lung specimens. Thomas and Smith (1972) found that 60% of lungs from non-pneumonic calves between 3 and 4 months of age obtained at abattoirs were infected with M. dispar, M. bovirhinis and Acholeplasma laidlawii while calves 1-2 days of age and 10 months or older were relatively free of mycoplasmas. Culture of lungs from calves 2-10 weeks of age obtained from herds free of clinical signs of pneumonia failed to yield any mycoplasmas and ureaplasmas (Gourlay et al., 1970; Gourlay and Leach, 1970; Harbourne et al., 1965).
Muenster et al. (1979) increased the percentage of mollicutes identified by using a direct fluorescent antibody technique to stain mycoplasma colonies which developed on agar. Also, Frey's M-96 medium was used for isolation of mycoplasmas other than M. dispar and a special broth was used to support growth of M. dispar. Bitsch et al. (1976) also used more than one medium to isolate mycoplasmas. The use of fluorescent antibody to identify colonies on agar surfaces and a additional media may have increased our isolation percentage. However, we previously had demonstrated that the modified Friis medium supported the growth of reference strains and was used to isolate M. bovigenitalium, M. arginini, M. bovoculi, M. dispar, M. bovis, M. bovirhinis and U. diversum from various clinical specimens (W. U. Knudtson, unpublished data, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa, 1979).

It is possible that certain enzymes or other toxic factors present in the lung inoculum may have reduced the viability of some mycoplasmas (Kaklamanis et al., 1969). A dilution effect was noted in some M. dispar cultures where growth was detected at 10^-3 and beyond, but not in 10^-1 or 10^-2 dilution cultures. St. George et al. (1973) also observed a dilution effect where the lowest dilution of lung material from an experimentally infected calf did not yield M. dispar whereas it was cultured from higher dilutions of inoculum. Taylor-Robinson and Chen (1983) reviewed the topic concerning growth inhibiting factors in animal tissues. The exact nature of the inhibitory substance(s) is not known although lysolethicin is one candidate based on the finding that addition of ammonium reineckate to culture media overcomes the inhibitory activity. Ammonium reineckate is
known to complex with quaternary ammonium compounds such as choline, forming an insoluble precipitate. These authors also speculated that antibiotic residues and antibodies in animal tissues could influence the growth of mycoplasmas (mollicutes).

Geographical locations and management practices may also be important factors in determining rates of isolation of the mollicutes (Roe, 1982). The individual mollicute isolation percentages with 3 surveys, namely those of Gourlay et al., 1970; Bitsch et al., 1976 and Muenster et al., 1979 vary only slightly. In comparison, our percentages were lower except for *M. bovis* which correlated with the 34% reported by Muenster et al., 1979. *Mycoplasma bovis* was not cultured from calves in Denmark (Bitsch et al., 1976) or in England prior to 1974 (Gourlay et al., 1970; Thomas et al., 1975) and from only 1% of calves with bronchopneumonia examined during a study in Poland (Zalewska-Schonthaler, 1980). Frey (1973) showed that 27% of calves sampled from herds throughout Iowa, U.S.A., exhibited a four-fold increase in indirect hemagglutination titer to *M. bovis* while experiencing outbreaks of pneumonia. Bennett and Jasper (1977) showed that 34% of calves from dairy herds with histories of mastitis associated with *M. bovis* carried the organism in nasal cavities whereas the nasal cavities of only 6% of calves in herds without mycoplasmal mastitis were colonized with the microbe. *Mycoplasma bovis* was isolated from 2 calves which were free of clinical signs of pneumonia but originated in herds with a history of pneumonia (Springer et al., 1982). Boothby et al. (1983) found the prevalence of *M. bovis* in a group of non-pneumonic calves to be higher than that previously reported by Bennett and Jasper (1977).
Thirty calves obtained from a commercial calf-rearing facility were twice shown to be free of *M. bovis* by nasal swab cultures. After shipment and relocation, 53% of the calves yielded *M. bovis* from lung lavage cultures and 20% from nasal swab cultures. Although lung lavage cultures were not prepared prior to shipment, it is possible they would have yielded *M. bovis*. Gourlay and Thomas (1970) and Thomas and Smith (1972) reported that culture of nasal cavities was not a reliable method for determining the mycoplasmal status of conventional calves. Thus, it is possible that stress from shipment could have exacerbated a persistent *M. bovis* infection of the respiratory tract resulting in increased replication of the microbe and shedding from the external nares. The respiratory tract of calves could indeed be the natural reservoir for *M. bovis* as suggested by Boothby et al. (1983). However, it is our contention that the organism is not commonly found in the respiratory tract of normal calves and should be considered of primary importance when isolated from calves with pneumonia.

Our data suggest that *M. bovirhinis* possesses limited tropism for the bovine lung and reflects the opinions of Gourlay et al. (1979) and Friis (1981). Although cultured from 8.5% of the lungs examined, which is in agreement with Zalewska-Schonthaler (1980), it was detected in tissue by immunofluorescence in only one instance; the same tissue also was colonized with *M. dispar*. It is possible that *M. bovirhinis* colonized the lung tissue secondarily to *M. dispar*. Gourlay et al. (1979) suggested that upon challenge of a gnotobiotic calf with *M. bovirhinis*, the microorganism colonized lung tissue previously damaged by *Escherichia*
coli; no lesions developed nor was *M. bovirhinis* cultured from 2 additional *E. coli*-free gnotobiotic calves following challenge with the mycoplasma. Also, Thomas and Howard (1974) showed that *M. bovirhinis* did not produce cytopathic effects in bovine fetal tracheal explants although it did establish and replicate in the explant culture. Thus, in certain situations, *M. bovirhinis* may colonize the bovine lung and may or may not contribute to a disease process.

Although *M. dispar* and *U. diversum* are frequently isolated from the same pneumonic lungs and both were shown to produce subclinical pneumonia in gnotobiotic calves, their significance in respiratory disease remains unclear (Gourlay et al., 1970; Howard et al., 1976; Gourlay and Howard, 1978). *Mycoplasma dispar* also has been cultured from clinically normal calves while *U. diversum* is rarely isolated from such animals (Gourlay and Thomas, 1970; Gourlay and Howard, 1979).

Several investigators have used immunofluorescence to detect mycoplasmas in bovine tissues (Karbe and Helmboldt, 1968; Masiga and Stone, 1968; Perreau et al., 1969; Riberio, 1979; Tinant et al., 1979; Friis, 1980; Rosenbusch, 1983). In this study, the IFAT was used to detect *M. bovirhinis*, *M. bovis* and *M. dispar* in frozen thin sections of pneumonic calf lungs. Furthermore, we showed that the IFAT conducted on cryostat sections of pneumonic lung tissues could be used in place of culture for routine detection of *M. bovis*. Its value also was demonstrated in those 9 instances where *M. dispar* was not cultured from the tissues but was detected by IFAT. However, based on 2 instances reported by Riberio (1979) where organisms were isolated (< 50 organisms
g⁻¹ lung tissue) but not detected by IFAT, it would appear that both culture and immunofluorescence should be used for routine detection of this very fastidious mollicute. Also, the finding that *M. dispar* could be detected by culture and IFAT in tissue held at 4°C for 15 and 49 days respectively after the animals were killed is important to the veterinary diagnostician since not all submitted specimens arrive promptly to the laboratory.
REFERENCES


PART II MICROBIOLOGY OF CALF PNEUMONIA: A VETERINARY DIAGNOSTIC LABORATORY SURVEY
MICROBIOLOGY OF CALF PNEUMONIA: A VETERINARY DIAGNOSTIC LABORATORY SURVEY

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SUMMARY

Microorganisms were detected by immunofluorescence and culture in 90% of lung specimens taken from 83 pneumonic calves. A single microbial species was detected in 18% of the cases; 71% of the specimens yielded 2 or more microorganisms. Bacteria were cultured from 73.5% of the lungs, mycoplasmas and ureaplasmas 59% and viruses 43%. Pasteurella multocida was the most common bacterium 39%, followed by P. haemolytica 31%, Haemophilus somnus 12% and Escherichia coli 7%. Both pasteurellae were present in 8% of the lungs. Mycoplasma dispar was identified in 40%, M. bovis 34%, Ureaplasma diversum 24% and M. bovirhinis 7% of lungs respectively. Approximately 11% of the lungs harbored M. bovis, M. dispar and U. diversum. One sample yielded a chlamydial isolate. Noncytopathic bovine viral diarrhea virus (BVD) was the most frequent isolate (27%) followed by bovine herpesvirus-1 (BHV-1) 16%, parainfluenza-3 virus (PI3) 2.4%, cytopathic BVD virus 2.4% and BHV-3 (Movar/DN599) 1%. Pasteurella multocida was strongly associated with the mycoplasmas and U. diversum (P=.0006 to .0487); however, viruses were not associated with bacteria or mollicutes. These data lead to speculation that infection with M. bovis and U. diversum could possibly predispose to colonization by P. multocida but not to BVD or BHV-1 viruses. The data did not support a hypothesis that virus infection would predispose the lung to infection by mollicutes.
INTRODUCTION

Bovine infectious respiratory disease which encompasses shipping fever pneumonia, pasteurellosis and enzootic or "cuffing" pneumonia is a major cause of morbidity and mortality in North American feedlot and confinement facilities (Jericho, 1979; Martin 1983). Mixed microbial infections involving bacteria, mycoplasmas and viruses are commonly observed in bovine pneumonia and account for yearly producer losses estimated at 224 to 750 million dollars (U.S.) (Anonymous, 1981; Loan, 1984). Stress resulting from fluctuations in temperature and relative humidity, weaning, transportation, handling, confinement, or other management-related causes is thought to initiate the disease process by compromising host native and acquired immune defense mechanisms (Irwin et al., 1979; Roth and Kaeberle, 1982; Roth, 1984; Jones, 1987). This in turn predisposes to viral/mycoplasma infection and enhances conditions for bacterial, mainly pasteurellae, replication and colonization (Jensen et al., 1976; Irwin et al., 1979; Martin, 1979).

Except with infectious bovine rhinotracheitis virus (BHV-1), initial experimental attempts to produce clinical pneumonia using a single microbial species generally were unsuccessful (Yates, 1982). This led to a series of reports whereby sequential challenge of calves with one of several respiratory viruses (BHV-1, BRSV, BVD, PI3) followed 4 to 6 days later by either aerosol, endobronchial or intratracheal inoculation with Pasteurella haemolytica or P. multocida or Haemophilus somnus produced clinical pneumonia (Hetrick et al., 1963; Jericho and Langford, 1978;
Jericho et al., 1982a; Potgieter et al., 1984; Jericho and Carter, 1985; Potgieter et al., 1988). Clinical pneumonia and pneumonic consolidation also were produced in conventionally-reared calves (3 to 8 weeks old) challenged intranasally (IN) with Mycoplasma bovis one day before IN and IT challenge with a 6 hour culture of P. haemolytica. Severe clinical disease and pneumonic consolidation of 50% and 64% of total lung surface was produced in 2 two-week-old gnotobiotic calves following the same challenge protocol (Gourlay and Houghton, 1985). Furthermore, mixed infection resulting from the simultaneous inoculation of both microbes produced disease which was greater than could be accounted for by the additive effects of each single infection (Gilmore et al., 1986; Houghton and Gourlay 1983). These findings supported a hypothesis that synergism between microbes is probably an important factor in bovine pneumonia (Irwin et al., 1979; Jericho et al., 1982a,b; Yates, 1982; Houghton and Gourlay 1983).

Previously we reported that mycoplasmas and U. diversum (T-strain mycoplasmas) were detected in lung tissues from 96 of 153 (64%) pneumonic calves which had been submitted to a veterinary diagnostic laboratory (Knudtson et al., PART I). Attempts were made to demonstrate other microorganisms in the lung tissues from 83 of those calves and determine if there were significant interactions between them. These results as well as the corresponding mycoplasma data from PART I are presented in this report.
MATERIALS AND METHODS

Animals

Holsteins and Herefords with clinical signs of pneumonia and between 1 week and 11 months of age were examined post-mortem.

Specimens

Portions of apical and cardiac lobes were routinely submitted; other areas of the lungs with gross lesions also were included for examination. A 1:10 (w/v) suspension representative of the submitted sample was prepared in modified Bovarnick's buffer (Rosenbush and Knudtson, 1980) using a Ten-Broeck grinder. Following centrifugation at 150 x g for 8 minutes, the supernatant was used to inoculate various media and cell cultures or stored at -73 C until such time when microbiological examination could be completed.

Bacteriologic Examination

Sheep blood agar (5%) was streaked with bronchial exudate obtained from cut surfaces of the lung specimens. A Staphylococcus aureus nurse colony was applied to the agar and the plates were incubated in a high-humidity incubator with free-flowing CO₂ (7%). Colonies were picked from the agar and identified using standard techniques (Carter, 1984). Plates not showing growth at 24 to 48 hours were held an additional 4 days before discarding.
Mycoplasma and Ureaplasma Examination

Media formulations, isolation procedures and the indirect fluorescent antibody technique (IFAT) are described in detail (Knudtson et al., PART I). Briefly, mycoplasmas were isolated using a modification of Friis' medium (MF) which contained components of Frey's M-96 medium. Modified Hayflick's medium was used to isolate ureaplasmas. Serial ten-fold dilution cultures of lung inoculum were utilized in the isolation of mycoplasmas and ureaplasmas. Broth cultures as well as bronchial exudates were streaked for isolated colonies on solid MF medium and the plates were incubated as described. Isolated colonies were cloned 3X prior to identification by growth inhibition and growth precipitation tests (Erno and Jurmanova, 1973). Shepard's urease reagent was used to confirm the presence of U. diversum (Shepard and Howard, 1970). The IFAT was used to detect M. bovirhinis, M. bovis and M. dispar in frozen lung sections.

Chlamydia Examination

Six-day-old developing chicken embryos were inoculated with 0.5 ml of lung inoculum which had been diluted 1:1 in Bovarnick's buffer containing the following inhibitors: vancomycin, 1.0 mg/ml (mg ml^-1); streptomycin, 1.0 mg ml^-1; Fungizone, 0.013 mg ml^-1. Three blind subpassages were made with pooled yolk-sac membranes of surviving embryos at 14 day intervals before a sample was considered negative for chlamydiae. The Gimenez stain was used to detect elementary bodies in yolk-sac impression smears of infected embryos.
Virologic Examination

Two serial ten-fold dilutions of lung inoculum representing $10^{-2}$ and $10^{-3}$ dilutions of lung tissue were inoculated into 2 tubes each of bovine embryonic kidney and lung cell monolayers and observed daily for cytopathic effects. Three blind passages of each culture were made at 7 day intervals before samples were considered negative. All virus isolates were identified by immunofluorescence using conjugates received from the National Animal Disease Center, Ames, Iowa.

Statistical Analysis

The significance of association between microbes detected in lung homogenates was determined by chi-square analysis of 2 X 2 contingency tables using one degree of freedom.
RESULTS

Microbes were identified in 90.4% of 83 pneumatic calf lungs; 9.6% were sterile by the methods of detection used in this study (Table 1a and 1b). A single microbial species was detected in 26.5% of the samples and 20.5% yielded 2 species. The remainder of the specimens (43.3%) harbored 3 or more detectable microorganisms. The major groups identified were bacteria 73.5%, mycoplasmas, including U. diversum 59%, viruses 43.4% and chlamydia 1%.

Pasteurella multocida and P. haemolytica were isolated from 39% and 31% of the samples respectively; 8% yielded both microbes. No attempt was made to determine the biotype of the P. haemolytica isolants; P. haemolytica occurred in combination with other bacteria in only 2 instances. Twelve percent yielded Haemophilus somnus which occurred in combination with P. multocida in 7% of the samples; it was not associated with P. haemolytica. Other bacteria isolated were E. coli 7%, alpha-haemolytic streptococci 4% and C. pyogenes 4%.

Mycoplasmas and U. diversum were detected in 59% of the samples and the distribution was as follows: M. dispar 40%, M. bovis 34%, U. diversum 24% and M. bovirhinis 7%. Mycoplasma dispar was detected in combination with M. bovis and/or U. diversum in 23% of the samples. The three microbes occurred in combination in 10.8% of the samples. Mycoplasma dispar and M. bovis were the only mycoplasmas detected in 15% and 10% of the samples respectively. Mycoplasma arginini, M. bovigenitalium and acholeplasmas were not detected in the samples. However, in one instance
Table 1a. Abbreviations for Tables 1b and 2

C = Corynebacterium
Ch = Chlamydia
E = Escherichia
H = Haemophilus
M = Mycoplasma
P = Pasteurella
U = Ureaplasma
BHV-1 = Bovine herpes virus-1 (Infectious bovine rhinotracheitis virus)
BHV-3 = Bovine herpes virus-3 (Movar/DN599)
CBVD = Cytopathic bovine viral diarrhea virus
NBVD = Noncytopathic bovine viral diarrhea virus
PI3 = Parainfluenza type 3 virus

(calf #75), nasal swabs taken from a live calf yielded *Acholeplasma laidlawii*, *M. arginini*, *M. dispar* and *M. bovis*.

Viruses were isolated from 43.4% of the samples. Noncytopathic BVD virus was the most frequent isolant (27%) followed by BHV-1 (16%), cytopathic BVD (2.4%), PI3 (2.4%), and BHV-3 1%. Bovine RSV was not isolated from lung tissue but it was cultured from nasal swabs taken from calf number 75 before it was killed. BHV-1 and NBVD virus were isolated in combination from 3.7% of the samples. Bovine adenoviruses, coronavirus and rhinoviruses were not isolated from the lungs of calves in this study.
Table 1b. Microbes detected by culture and indirect fluorescent antibody examinations in 75 of 83 pneumonic calf lungs

<table>
<thead>
<tr>
<th>Calf</th>
<th>Bacteria</th>
<th>Mycoplasmataceae</th>
<th>Viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>P. multocida, H. somnus</td>
<td>M. dispar</td>
<td>NBVD</td>
</tr>
<tr>
<td>2.</td>
<td>P. haemolytica</td>
<td>M. dispar, M. bovis, M. bovirhinis</td>
<td>BHV-1</td>
</tr>
<tr>
<td>3.</td>
<td>P. haemolytica</td>
<td>M. dispar, M. bovirhinis</td>
<td>NBVD</td>
</tr>
<tr>
<td>4.</td>
<td>P. haemolytica</td>
<td>M. dispar, M. bovirhinis</td>
<td>BHV-1</td>
</tr>
<tr>
<td>5.</td>
<td>P. multocida</td>
<td>M. bovis, M. dispar, U. diversum</td>
<td>NBVD</td>
</tr>
<tr>
<td>6.</td>
<td>P. multocida</td>
<td>M. dispar, U. diversum</td>
<td>NBVD</td>
</tr>
<tr>
<td>7.</td>
<td>P. multocida, C. pyogenes</td>
<td>M. bovis</td>
<td>BHV-1</td>
</tr>
<tr>
<td>8.</td>
<td>P. multocida, H. somnus</td>
<td>M. dispar, U. diversum</td>
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</tr>
<tr>
<td>9.</td>
<td>P. multocida, P. haemolytica</td>
<td>M. dispar</td>
<td>NBVD</td>
</tr>
<tr>
<td>11.</td>
<td>P. multocida, P. haemolytica</td>
<td>M. bovis, M. dispar, M. bovirhinis</td>
<td>NBVD</td>
</tr>
<tr>
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<tr>
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<td>NBVD</td>
</tr>
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<tr>
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<td>M. dispar</td>
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<tr>
<td>No.</td>
<td>Bacterial Species</td>
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<tr>
<td>-----</td>
<td>-------------------------------------------------------</td>
<td>------------------------------------------------</td>
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<td>31</td>
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<td>M. dispar</td>
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<tr>
<td>32</td>
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<td>M. dispar</td>
<td></td>
</tr>
<tr>
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<td><em>P. multocida</em></td>
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<td>M. bovis</td>
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<td><em>P. haemolytica, Streptococcus sp</em></td>
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<td>74</td>
<td><em>H. somnus</em></td>
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<td>75</td>
<td><em>P. multocida, H. somnus</em></td>
<td>M. bovis, U. diversum</td>
<td></td>
</tr>
</tbody>
</table>

*aNasal swabs taken just prior to killing this animal yielded the following: M. bovirhinis, M. bovis, M. argini, M. dispar, P. multocida, Bacillus sp, Micrococcus sp and respiratory syncytial virus.*
One chlamydial isolant was isolated and presumed to be *Chlamydia psittaci*; however, serology was not performed to confirm the identity.

Significant associations between isolants are listed in Table 2. The most significant association established was between *M. bovis* and *U. diversum* (P=.0001) followed by *M. bovis* and *P. multocida* (P=.0006). Although the association between *P. haemolytica* and NBVD was significant (P=.0270), no significance was established between *P. multocida* and NBVD.

Table 2. Probability values for significance of association between microbes detected in pneumonic lungs^a^

<table>
<thead>
<tr>
<th>Organisms</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em></td>
<td></td>
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<tr>
<td><em>U. diversum</em></td>
<td>.0001</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
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<tr>
<td><em>P. multocida</em></td>
<td>.0006</td>
</tr>
<tr>
<td><em>U. diversum</em></td>
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<td><em>M. dispar</em></td>
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<tr>
<td><em>U. diversum</em></td>
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<tr>
<td><em>P. haemolytica</em></td>
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</tr>
<tr>
<td>NBVD</td>
<td>.0270</td>
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<tr>
<td><em>M. dispar</em></td>
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<tr>
<td><em>P. multocida</em></td>
<td>.0487</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
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<tr>
<td>NBVD</td>
<td>.0490</td>
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<td><em>H. somnus</em></td>
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<tr>
<td>NBVD</td>
<td>.0520</td>
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</table>

^aProbability values for other associations between microbes not included in the table all exceeded P>.0520. See Table 1a for nomenclature.
DISCUSSION

The intent of this survey was to examine lung specimens in a manner consistent with that utilized by a diagnostic laboratory. It is important to recognize that the samples represented a very small "window" relative to all cases of calf pneumonia and that other microorganisms could have been present but were not detected by our methods. For example, respiratory syncytial virus (BRSV) was not cultured from any of the lung specimens, although the virus was present as evidenced by culture from a nasal swab. Immunofluorescent staining of lung sections for BRSV would have increased detection of virus (Baker et al., 1986). Also, the use of L-cells would probably have enhanced the isolation of chlamydia (Ronsholt, 1981). The isolation rates for different classes of organisms are in general agreement with other reported surveys of this nature (Bitsch et al., 1976; Reggiardo, 1979; Friis and Krough, 1983). No unusual or uncommon respiratory pathogens were identified during this study. However, in an elegant study by Thomas et al. (1982) in which nasopharyngeal and bronchial washings from calves experiencing acute pneumonia were inoculated intratracheally into gnotobiotic calves, the only microbe detected which was not previously isolated from pneumonic calf lungs was a coronavirus.

The bacteria detected are very similar to those isolated from pneumonic calves from the same geographical area in 1948 by Kenzy and suggests that these organisms are stable in the bovine population. Pasteurella multocida (39%), P. haemolytica (31%) and Haemophilus somnus
(12%) were the most frequently isolated bacteria. Although these organisms can be cultured from the upper respiratory tract and tonsils of calves without clinical signs, they are not commonly isolated from normal lung tissue (Collier, 1968; Frank et al., 1987). *Pasteurella haemolytica* is a major cause of acute fibrinous pneumonia and death of transported calves (Frank, 1986). This organism produces an exotoxin (leukotoxin) which is cytotoxic for bovine alveolar macrophages and neutrophils (Frank et al., 1987). Antibody against the leukotoxin in addition to a somatic component of the bacterial cell confers some protection against challenge with cytotoxin-producing strains of *P. haemolytica* (Shewen and Wilkie, 1984). The strains of *P. multocida* associated with bovine respiratory disease are most often capsule type A; only recently have type D strains been recognized in calves. Ryu et al. (1984) reported that a high molecular weight substance associated with the hyaluronic acid capsule of type A *P. multocida* inhibited "in vitro" phagocytosis of *Staphylococcus aureus* by bovine neutrophils. Recently, weakly toxigenic strains of capsule type D were isolated from pneumonic calf lungs (Kielstein, 1986). Viring et al. (1986) also isolated type D strains from the lower respiratory tract of both normal and pneumonic calves; however, their toxigenic capabilities were not determined. Certain isolates of type D *P. multocida*, some of which were associated with porcine pneumonia, produce a potent toxin which induces permanent turbinate atrophy in swine (Rutter, 1983). One can speculate that such a toxin could play a role in the pathogenesis of pneumonia in calves associated with type D *P. multocida*. It is not uncommon to find a higher incidence of *P. multocida* vs *P.
haemolytica in surveys involving young calves. Ishino et al. (1979) cultured P. multocida and P. haemolytica from 56% and 11% respectively of 131 calves with varying degrees of pneumonia. Viring et al. (1986) also detected a higher percentage of P. multocida vs P. haemolytica in pneumonic calf lungs (41% vs 28%). A high molecular weight substance from H. somnus was shown to inhibit ingestion of S. aureus by bovine neutrophils (Hubbard et al., 1986). Also, breakdown products of ribonucleic acid (guanine and adenine) released into culture supernatants by H. somnus were shown to inhibit the iodination of protein by bovine neutrophils (Chiang et al., 1986).

Previously we reported that M. dispar, M. bovis and U. diversum were the most common mollicutes detected in pneumonic calf lungs examined in Iowa (Knudtson et al., PART I). Mycoplasma bovis is probably the most important of the 3 species based on (1) pathogenicity studies in gnotobiotic calves (Gourlay et al., 1979; Thomas et al., 1986) and (2) its association with sporadic but severe pneumonia in the field (Thomas et al., 1982). The highly significant association between this mycoplasma and U. diversum (P=.0001) or P. multocida (P=.0006) leads one to speculate that M. bovis could possibly predispose to infection by U. diversum and P. multocida. There also was a significant association between M. bovis and noncytopathic BVD virus (P=.049). Gourlay et al. (1979) showed that both M. dispar and U. diversum produced subclinical but macroscopic pneumonia in gnotobiotic calves. Although the significance of association between M. dispar or U. diversum and P. multocida was not nearly as dramatic as with M. bovis, it was significant (P<.05). Since M. dispar produced
subclinical but macroscopic and/or microscopic pneumonia in gnotobiotic (Gourlay et al., 1979) and conventionally-reared calves (Riberio, 1979; Friis, 1980) and destruction of cilia in vitro (Thomas and Howard, 1974), it is not unreasonable to speculate that this mollicute could condition the respiratory tract to infection by other microorganisms. Although U. diversum also produced subclinical but macroscopic pneumonia in gnotobiotic calves, its role in pneumonia remains to be determined (Howard et al., 1976).

The viruses isolated have been shown to inhibit or alter a number of neutrophil and alveolar macrophage functions critical in averting disease (Roth, 1984; Bielefeldt-Ohmann and Babiuk, 1986; Espinasse, 1986; Liggit et al., 1986). Included are: inhibition of phagosome-lysosome fusion in alveolar macrophages by PI3 virus, inhibition of ingestion of S. aureus and protein iodination in neutrophils by BVD viruses, altered chemotactic ability of neutrophils and alteration in production of soluble mediators by alveolar macrophages induced by BHV-1 and inhibition of alveolar macrophage ingestion of S. epidermidis by BRSV. Also, in the case of PI3 virus, infection altered the rheologic properties of the mucus escalator of the respiratory tract through the action of neuraminidase (Friberg et al., 1973). The significance of coronavirus in pneumonia of calves is unknown. Sizov et al. (1985) reported that mild clinical signs were produced in calves following intratracheal inoculation of coronavirus which had been isolated from one calf in a herd experiencing acute respiratory disease.

We have shown that lungs from calves experiencing acute to chronic
respiratory disease are colonized by a variety of bacteria, mollicutes and viruses. In the United States, USDA licensed bacterins and vaccines against these agents except for the mollicutes are available and used routinely by producers. During the past 5 years a quadrivalent vaccine containing inactivated components of BRSV, PI3 virus, M. dispar and M. bovis has been used with success in the United Kingdom (Howard et al., 1987). Good protection was afforded when the disease outbreak was caused by agents associated with the vaccine components. The data from our survey revealed significant interactions between M. bovis and/or M. dispar and P. multocida leading to speculation that infection with these mollicutes could possibly predispose to secondary infection by other microbes, especially P. multocida, and would seem to make them viable candidates for inclusion in vaccines/bacterins intended for use in prevention of bovine respiratory disease.
REFERENCES


PART III THE EFFECT OF MYCOPLASMA BOVIS ON BOVINE FETAL TRACHEAL EXPLANT CULTURES
THE EFFECT OF MYCOPLASMA BOVIS ON BOVINE FETAL TRACHEAL EXPLANT CULTURES

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SUMMARY

Mycoplasma bovis and Mycoplasma bovoculi were examined for ability to replicate in bovine fetal tracheal explant cultures (TEC) and to produce changes in ciliary activity. Both microorganisms replicated to highest titers in TEC containing 4% fetal bovine serum (FBS). Fifty-five TEC from six tracheas were used to evaluate M. bovis induced ciliostasis. Ciliostasis was observed as early as 8 hours post-inoculation (PI) and only in TEC supplemented with FBS or dialyzed FBS. The average time to complete ciliostasis was 62 ± 22 hours PI. Under similar culture conditions, M. bovoculi did not affect ciliary activity of TEC.

The addition of catalase to TEC did not inhibit or alter the course of M. bovis-induced ciliostasis. Also, addition of formaldehyde-killed M. bovis organisms to TEC did not affect ciliary activity. Microscopic examination of thin sections from normal and infected explants revealed a transition from ciliated columnar to non-ciliated squamous epithelium in the explants exhibiting ciliostasis. Microorganisms were not observed on the surface of ciliated epithelial cells and immunofluorescence revealed specific staining in only 2 of the 55 TEC. These data suggest that a soluble toxin could be responsible for the ciliostasis. However, sterile spent medium had no effect on fresh tracheal explants and no changes were seen in TEC cultured in parabiotic chambers.

Although the mechanism of M. bovis-induced ciliostasis could not be determined, viable organisms and a component of FBS were required to produce this effect in the model described.
INTRODUCTION

Several mollicutes are associated with infectious respiratory disease in calves but only *Mycoplasma bovis*, *M. dispar* and *Ureaplasma diversum* are thought to play a role in the disease process (Gourlay and Howard, 1979). Under experimental conditions, only *M. bovis* was capable of producing clinical pneumonia when introduced into the respiratory tract of gnotobiotic calves (Gourlay et al., 1979; Thomas et al., 1986). Gross changes involving up to 37% of total lung surface were observed in gnotobiotic calves inoculated intratracheally with *M. bovis*. Microscopic changes seen were multi-focal areas of coagulative necrosis in the lung parenchyma and some perivascular and peribronchiolar lymphoid accumulations. Immunoperoxidase staining of the lesions revealed numerous *M. bovis* organisms in association with macrophages and lymphocytes (Howard et al., 1986). Knudtson et al. (PART I) used an immunofluorescent antibody test to show that *M. bovis* colonized the lung parenchyma as well as ciliated epithelium in the lungs of conventionally-reared calves with pneumonia.

Tracheal explant cultures (TEC) have served as useful in vitro models for evaluating virulence of respiratory pathogens, including mollicutes (Rossi and Kiesel, 1977; Williams and Gallagher, 1978; Collier, 1979; Gabridge, 1979; Araake, 1982; Bemis and Wilson, 1985; Jones et al., 1985; Moorthy and Spradbrow, 1985; Mylotte et al., 1985; Hingley et al., 1986). Thomas and Howard (1974) and Howard and Thomas (1974) reported that *M.*
dispar was cytopathic for fetal bovine TEC and that the effect was dependent on the presence of serum in the explant medium and the number of organisms in the inoculum. No changes were observed in ciliary activity of TEC inoculated with U. diversum, M. bovirhinis or Acholeplasma laidlawii. Recently, Thomas et al. (1987) showed that M. bovis penetrated between the ciliated epithelial cells of bovine fetal TEC and replicated in the sub-epithelial tissue without affecting ciliary activity.

In preliminary studies we had found that Pseudomonas aeruginosa, Pasteurella multocida, Haemophilus pleuropnuemoniae and M. bovis cultured from the respiratory tracts of livestock produced ciliostasis in fetal bovine and porcine TEC. In this report we describe the changes produced in fetal bovine TEC by M. bovis and M. bovoculi. The latter organism is a cause of bovine infectious conjunctivitis (Rosenbusch and Knudtson, 1980) and has been associated with infectious bovine keratoconjunctivitis (Rosenbusch, 1983). This organism was recently cultured from the respiratory tracts of calves with naturally-occurring pneumonia (Friis and Krough, 1983). Jericho and Carter (1985) reported that M. bovoculi had been isolated from the respiratory tract of calves that had been challenged with Pasteurella multocida and bovine herpesvirus-1.
MATERIALS AND METHODS

Tracheal Explant and Mycoplasma Culture Media

Tracheal explant cultures were maintained in filter-sterilized Eagle’s minimal essential medium (MEM) (GIBCO, Grand Island, NY) at pH 7.2 modified to contain sodium bicarbonate 3.0 mg/ml (mg ml⁻¹), lactalbumin hydrolysate (1.0 mg ml⁻¹), L-glutamine (0.292 mg ml⁻¹), N-2-hydroxyethylpiperazine-N’-2-ethane sulphonic acid (12 mg ml⁻¹) and penicillin G (1000 units ml⁻¹). The MEM was supplemented with 4% (vol/vol) heat-inactivated bovine virus diarrhea virus-free fetal bovine serum (FBS) for evaluation of mycoplasmal ciliostasis activity.

An aliquot of FBS was filtered under nitrogen pressure through a 10,000 dalton pore size (PM/10) membrane filter (Amicon, Danvers, MA), filter sterilized through a 220 nm membrane and frozen at -20 C. The ultrafiltrate was incorporated into MEM at 4% in certain studies.

A modification of Friis medium (MF) previously described in detail (Knudtson et al., PART I) and containing 4% FBS and 100 units ml⁻¹ of penicillin G, but without thallium acetate was used to cultivate mycoplasmas.

Mycoplasma Cultures

Mycoplasma bovis isolate 1315 was initially cultured from a pneunonic calf lung (Knudtson et al. PART I); strain 277HL of M. bovoculi originated from the conjunctiva of a calf with conjunctivitis (Rosenbusch and
Knudtson, 1980). Both organisms had been cloned 3 times by passing broth cultures through 800 nm membrane filters prior to identification by growth inhibition using antiserum provided by the World Health Organization's Mycoplasma Reference Laboratory in Arhus, Denmark. Following identification the organisms were expanded in MF broth (36 hour culture), dispensed into cryotubes (1.8 ml) and stored at -70 C.

Fetal Bovine Tracheal Explant Cultures

Tracheal explant cultures were prepared from fetal bovine respiratory systems harvested at 4-6 months of gestation. Tracheas were excised from fetuses at the abattoir, placed in Hank's balanced salts solution (HBSS) containing mycostatin (1 mg ml⁻¹) and penicillin (1000 units ml⁻¹) and transferred on ice to the laboratory. As much of the extraneous tissue as possible was removed from the outside of the trachea. Tracheas were rinsed 3 to 4 times in the HBSS, placed in a large petri dish and submerged in MEM. Transverse sections each containing one cartilage ring were prepared using sterile scissors and forceps. Explants were placed in a flask containing MEM and rinsed several times. Each explant was then placed in a sterile, tissue culture grade, plastic screw-cap tube (16mm x 120mm) containing 4.0 ml of MEM and incubated at 36 C on a roller-drum (15 revolutions per hour). After 5 days incubation, TEC were vigorously vortexed and rinsed three times in MEM prior to addition of fresh MEM with or without FBS. This treatment was usually sufficient to free the explant of mucus and cellular debris which tended to accumulate in the explant.
lumen during the first 5 days in culture making visualization of ciliary activity difficult.

Inoculation of Tracheal Explant Cultures

The ability of *M. bovis* to replicate in fetal bovine TEC medium (MEM) was determined by establishing a growth curve using the following conditions: (1) MEM, explant and 4% FBS, (2) MEM, explant and 4% ultrafiltrate of FBS, (3) MEM without 4% FBS but containing explant, (4) MEM without explant but containing 4% FBS and (5) MEM without explant or serum. Each explant culture was inoculated with 0.2 ml of a standard inoculum containing $3.8 \times 10^6$ colony forming units (cfu) per ml ($\text{ml}^{-1}$) of *M. bovis* organisms; controls received 0.2 ml of sterile MF broth. A growth curve for *M. bovoculi* was not established since it was known that the organism replicated in fetal bovine turbinate and kidney cell monolayers.

Six replicates, each representing a single trachea and totaling 55 TEC in tubes were used in determining the ciliostasis producing capability of *M. bovis*; 6 explants from each of tracheas 1, 3 and 4 were used to study the effects induced by *M. bovoculi* infection. Five additional explants from each trachea were inoculated with 0.2 ml of sterile MF broth and incubated in parallel with infected explants. Following inoculation of explants with either standard inoculum or 24 to 36 hour culture in MF broth, serial ten-fold dilution cultures were prepared and aliquots spread onto solid MF medium to determine the cfu ml$^{-1}$ of mycoplasmal organisms in the inoculum.
Evaluation of Ciliary Activity

A subjective scoring system was established for evaluation of the ciliostasis of the TEC. An inverted microscope at magnification of X100 was used to grade ciliary activity of the entire inner circumference of each TEC. Noninoculated TEC were used as a point of reference and the time at which ciliary activity was reduced by 25%, 50%, 75% and 100% was recorded. Explants were routinely examined every 4 to 8 hours unless otherwise specified.

Studies on Mechanisms of Mycoplasma Induced Ciliostasis

Parabiotic chamber cultures

Explants which had been in culture for 5 days were rinsed as described and transferred to sterile parabiotic chambers (Bellco Glass Co., Vineland, NJ) and sufficient MEM with or without FBS was added to cover each explant (one per chamber). The 2 chambers of each unit were separated by sterile 220 nm or 450 nm membrane filters. One chamber of each unit was inoculated with 0.2 ml of standard inoculum or a 24 hour culture of mycoplasma; the other chamber was inoculated with 0.2 ml of sterile MF broth. Each unit was incubated at 36°C and observed every 8 to 12 hours for 5 days; the units were gently agitated at each observation period. The cfu ml⁻¹ of organisms in inocula were determined as previously described. Twelve explants were used in studies with M. bovis; 8 were used for M. bovoculi.
Filtrate of spent explant culture medium

Spent medium from explants showing complete inhibition of ciliary activity was prefilled twice through a 450 nm membrane, filter sterilized through a 220 nm membrane and dispensed into sterile tubes (4.0 ml per tube). Medium from control TEC was treated in the same manner. Fresh explants were added to the sterile media, incubated at 36 C and ciliary activity was observed at 8 to 12 hours for 7 days. Culture medium from a minimum of 3 M. bovis-infected TEC from each of the 6 replicates (see Table 1) was tested for toxic activity using fresh explants. Spent medium from 4 M. bovoculi infected TEC also was examined.

Catalase medium

Sixteen explants from each of 2 tracheas were placed in 4 groups and used to evaluate the effect of catalase on M. bovis-induced ciliostasis. Catalase (Sigma Chemical Co., St. Louis, MO, USA) was added to MEM to yield final concentrations of 0, 5, 10 and 15 mg ml⁻¹. Catalase was not used in TEC infected with M. bovoculi.

Formaldehyde inactivation

Mycoplasma bovis cultures containing approximately 1 X 10⁹ cfu ml⁻¹ were treated by exposure to formaldehyde (0.25% final concentration) at room temperature for 4 hours followed by 18 hours at 4 C. The organisms were harvested by centrifugation, washed 3 times and resuspended in MEM at one tenth the original volume and checked for sterility by culture in MF broth and solid media. Four tracheal explants each in 5.0 ml of MEM containing 4% FBS were inoculated with 0.1, 0.5 and 1.0 ml of treated
organisms and the ciliary activity of the explants monitored for 5 days. Inactivated *M. bovoculi* organisms were not examined.

**Saturation studies**

A 48 hour-old culture of *M. bovis* was concentrated by centrifugation to approximately $1 \times 10^{10}$ cfu ml$^{-1}$. One ml of the culture was added to each of 10 fresh explants. Ten explants also were added to tubes containing 48 hour-old cultures that were not concentrated. Five explants were placed in sterile MF broth. Following incubation at 36 C for 2 to 8 hours, explants were rinsed in 3 changes of MEM and their ciliary activity recorded. Explants from each group were examined by fluorescent and light microscopy. *Mycoplasma bovoculi* was not examined by this procedure.

**Microscopic Examinations**

Cryostat sections of whole TEC were prepared and examined by the indirect fluorescent antibody technique (IFAT) using previously described protocols and reagents (Knudtson et al., PART I). Pieces of TEC mucosae were excised from the cartilage, fixed in gluteraldehyde, dehydrated, sectioned at 3.0 micrometers, stained with toluidene blue and examined by light microscopy.
RESULTS

Mycoplasmal Growth in Tracheal Explant Culture Medium

*Mycoplasma bovis* replicated in TEC without serum but the maximal titer achieved was generally one log less than that in TEC supplemented with FBS (Figure 1). Growth in TEC supplemented with ultrafiltrate of FBS was essentially the same as that in MEM without FBS. Growth did not occur in MEM with or without FBS in the absence of explants. The titer of organisms remained essentially the same in MEM containing FBS but decreased in MEM without FBS. Although a growth curve was not established for *M. bovoculi*, a similar pattern of growth was observed (data not shown).

Effects of Mycoplasmal Organisms on Explant Ciliary Activity

Only *M. bovis* was capable of inducing ciliostasis in fetal bovine TEC. *Mycoplasma bovoculi* had no effect on the ciliary activity of explants maintained in culture for 14 days either in culture tubes or parabiotic chambers.

Listed in Table 1 are the average hours at which 25%, 50%, 75%, and 100% ciliostasis were observed during 6 replicates. *Mycoplasma bovis* reduced or totally eliminated ciliary activity within 1 to 5 days after inoculation. The mean time to complete ciliostasis appeared to be independent of the titer of organisms in inocula used to infect explants.
Figure 1. Growth of *Mycoplasma bovis* in fetal bovine tracheal explant culture medium supplemented with or without fetal bovine serum (FBS) or ultrafiltrate of FBS (PM10) and with or without explants.
Table 1. Percent ciliostasis produced in fetal bovine tracheal explant cultures following inoculation with *Mycoplasma bovis* organisms

<table>
<thead>
<tr>
<th>Trachea No.</th>
<th>No. Explants</th>
<th>CFU Inoculum(^a)</th>
<th>CFU at 100% Cilio(^b)</th>
<th>25% Avg ± SEM(^c)</th>
<th>50% Avg ± SEM</th>
<th>75% Avg ± SEM</th>
<th>100% Avg ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>6.5</td>
<td>8.4</td>
<td>11.6 ± 3.9</td>
<td>19.1 ± 3.9</td>
<td>27.3 ± 4.1</td>
<td>36.0 ± 2.8</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>6.6</td>
<td>8.2</td>
<td>13.8 ± 4.6</td>
<td>30.2 ± 9.1</td>
<td>66.4 ± 14.6</td>
<td>97.4 ± 8.2</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>6.0</td>
<td>9.1</td>
<td>13.0 ± 2.7</td>
<td>22.8 ± 5.3</td>
<td>35.4 ± 5.7</td>
<td>47.6 ± 6.3</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>6.1</td>
<td>7.8</td>
<td>14.0 ± 4.7</td>
<td>25.8 ± 6.4</td>
<td>37.6 ± 4.5</td>
<td>69.0 ± 9.5</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>6.6</td>
<td>8.3</td>
<td>10.3 ± 2.0</td>
<td>22.3 ± 3.5</td>
<td>35.4 ± 5.9</td>
<td>46.3 ± 4.2</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>6.6</td>
<td>7.9</td>
<td>10.9 ± 3.1</td>
<td>28.4 ± 5.5</td>
<td>48.0 ± 6.8</td>
<td>69.3 ± 11.9</td>
</tr>
<tr>
<td>Means</td>
<td></td>
<td>6.4</td>
<td>8.3</td>
<td>12.4 ± 4.0</td>
<td>24.9 ± 7.1</td>
<td>42.2 ± 15.1</td>
<td>62.0 ± 22.1</td>
</tr>
</tbody>
</table>

\(^a\)Number of organisms cfu ml\(^{-1}\) (10\(^n\)) in explant medium at time zero.

\(^b\)Number of organisms cfu ml\(^{-1}\) (10\(^n\)) in explant medium at 100% ciliostasis (n=4).

\(^c\)Avg ± SEM = Average hours to designated percent ciliostasis plus or minus standard error of the mean.

\(^d\)Five additional explants were prepared from each trachea and incubated (not inoculated) in parallel with those infected with *M. bovis*.
For instance, the mean time required in explants from trachea 5 was approximately one half that for explants from trachea 2, even though they were inoculated with approximately the same number of organisms.

**Mechanism of Mycoplasma Induced Ciliostasis**

**Parabiotic chambers**

Complete ciliostasis was produced by *M. bovis* in time ranges similar to those in tube cultures which contained serum. None of the noninfected explants separated by 220 nm or 450 nm membranes from *M. bovis* infected explants showed changes in ciliary activity and no mycoplasmas could be cultured from them. No changes were observed in ciliary activity of TEC infected with *M. bovoculi*.

**Spent culture medium**

Fresh tracheal explants added to sterile spent explant culture medium from both *M. bovis* and *M. bovoculi*-infected TEC showed no changes during incubation at 37 C for 5 days. Mycoplasmas were not cultured from the media or explants at the end of the incubation period. Since ciliostasis was not observed in explants infected with *M. bovoculi*, no further studies in TEC were conducted.

**Catalase-containing medium**

Addition of catalase to the TEC medium to yield final concentrations between 5 and 15 mg ml\(^{-1}\) did not preclude development of ciliostasis in TEC inoculated with *M. bovis*. 
Inactivated *Mycoplasma bovis*

Formaldehyde inactivated *M. bovis* organisms added to fresh TEC to approximate $2 \times 10^8$, $9 \times 10^8$ and to $1.7 \times 10^9$ cfu ml$^{-1}$ had no effect on TEC ciliary activity.

**Saturation studies**

The ciliary activity of TEC placed in MF broth cultures of *M. bovis* for 2 hours was unchanged; no mycoplasmas were observed by IFAT and no microscopic changes were seen in the explants.

**Microscopic Findings**

Immunofluorescent staining of cryostat sections from *M. bovis* infected TEC revealed few organisms in the damaged ciliated epithelium. Only 2 *M. bovis* infected TEC prepared from the same trachea and sampled at 36 and 48 hours PI yielded positive results by IFAT (Figures 2 and 3). A diffuse pattern of fluorescence was observed in the disrupted epithelial layer and could be blocked by pretreatment with a different species anti-*M. bovis* serum, thus indicating the fluorescence was specific. The pattern of fluorescence appeared to follow the perimeter of epithelial cells in one section (Figure 3).

Following inoculation of TEC, ciliated epithelial cells became swollen and disorganized prior to sloughing from the underlying tissue. Microscopic examination of thin sections revealed a transition from the ciliated columnar epithelium in noninfected TEC (Figures 4 and 5) to a non-ciliated squamous epithelium in infected TEC (Figures 6 and 7).
However, attempts to demonstrate organisms in association with ciliated epithelium yielded negative results.
Figure 2. Cryostat section of *Mycoplasma bovis* infected fetal bovine tracheal explant stained by indirect immunofluorescence using equine anti-*M. bovis* serum. Note the diffuse staining in the epithelium. x250

Figure 3. Serial section from same explant as Figure 2, but counter-stained with Azo-black. Note both diffuse and particulate staining, especially the staining which appears between cells (arrows). x250
Figure 4. Thin section of normal fetal bovine tracheal explant stained with toluidine blue and showing ciliated epithelium. x250

Figure 5. Thin section of normal fetal bovine tracheal explant stained with toluidine blue showing ciliated epithelium. x700
Figure 6. Thin section from fetal bovine tracheal explant 36 hours after infection with Mycoplasma bovis. The ciliated epithelial layer is degenerated, showing enlarged and hyperchromatic nuclei and very few cilia. Toluidine blue, x250.

Figure 7. Thin section from fetal tracheal explant showing the transition to non-ciliated squamous epithelium 48 hours after infection with Mycoplasma bovis. Toluidine blue, x250.
DISCUSSION

*Mycoplasma bovis* replicated to highest titer in fetal bovine TEC supplemented with serum. The inoculum remained at a constant titer in MEM containing serum but lost viability in MEM alone or without serum. Howard and Thomas (1974) reported similar findings in TEC infected with *M. dispar*.

Although both *M. bovis* and *M. bovoculi* replicated in TEC containing serum, only *M. bovis* produced ciliostasis. No changes were observed in TEC devoid of serum. This serum-dependent inhibition of ciliary activity varies from one mycoplasma species to another. Both *M. dispar* and *M. pneumoniae* required serum, whereas *M. mycoides* var. *capri*, *M. hyorhinis*, and *M. gallisepticum* produced changes in explant ciliary activity in the absence of serum (Cherry and Taylor-Robinson, 1970, 1971; Collier, 1979). A decrease in ciliary activity of approximately 25% was observed as early as 8 hours post-inoculation of *M. bovis* organisms; complete ciliostasis usually was evident after 2 to 3 days incubation. These observations are in conflict with those described by Thomas et al. (1987) who reported that *M. bovis* infiltrated between the columnar epithelium without inducing any changes in ciliary activity. In their study, organisms were seen in interepithelial spaces but did not appear to affect adjacent epithelial cell membranes; numerous organisms also had accumulated in the lamina propria near the basement membrane. Some portions of the ciliated epithelium become detached only after 14 to 22 days in culture. Also, Thomas et al. make no mention of studies in explants which did not contain serum.
The relative virulence of the strains of *M. bovis* used to infect the explants could have accounted for the differences observed in the 2 studies. Strain Ab/1 produced pneumonia in gnotobiotic calves (Thomas et al., 1986); the ability of strain 1315 to produce pneumonia is not known, but it did produce purulent arthritis when introduced into the tarsal joint of a 4 month-old calf (W. U. Knudtson and R. Rosenbusch, unpublished data, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa, 1983).

The numbers of organisms used to infect explants in this study did not appear to influence the development of ciliostasis. Thomas et al. reported a similar finding for the *M. bovis*-induced cytopathology. However, the inoculum titer dramatically influenced the rate at which ciliostasis developed in *M. dispar*-infected explant cultures (Howard and Thomas, 1974; Thomas et al., 1987). Regardless of these observations, one must consider that the much higher titer of organisms in the inoculum used in this study (10^{6.4} vs 10^{4.3} in Thomas et al.) may have initiated an effect on the explants, leading to ciliostasis rather than invasion and colonization of the lamina propria as reported by Thomas et al.

Attempts to elucidate the mechanism by which *M. bovis* mediates ciliostasis were unsuccessful. Non-viable *M. bovis* organisms did not induce ciliostasis in TEC and no changes were observed in fresh explants placed in filter-sterilized spent explant medium. Cherry and Taylor-Robinson (1971) reported that spent medium from chicken embryo TEC infected with *M. gallisepticum* was toxic to fresh explants. Thomas et al. (1987) also were not able to demonstrate toxic activity in sterile spent
explant medium from *M. bovis* infected TEC. Attempts to demonstrate a toxin in this study using parabiotic chambers also yielded negative results. Furthermore, Howard and Thomas (1974) were not able to demonstrate a toxin in spent medium from *M. dispar* infected TEC which showed ciliostasis. The inability to consistently demonstrate *M. bovis* organisms in association with cilia, as well as microscopic findings in which degeneration and exfoliation of the mucosae preceded transition to squamous epithelium, suggested that a toxin could have been associated with the ciliostasis.

What then are the factors that could account for these findings? It is possible that a toxin was present in the cultures used to inoculate the TEC. However, no changes were seen in explants placed in MF broth cultures or in a concentrated suspension of *M. bovis* organisms. If a toxin had been liberated into the TEC medium, it could have been bound to membrane filters used (1) to sterilize spent TEC culture medium and (2) to separate infected from noninfected explants in the parabiotic chambers. Buttery et al. (1980) failed to demonstrate toxin activity *in vivo* using diffusion chambers inoculated with $2.5 \times 10^6$ *M. bovis* organisms prior to implantation into the oblique abdominal muscle of cattle. The titer of *M. bovis* in the chambers after 14 days was $2.5 \times 10^8$; no difference in tissue response was observed between chambers with *M. bovis* and chambers with sterile medium. It is known that many mollicutes liberate hydrogen peroxide during growth and this could potentially mediate host cell death through peroxidation of membrane components. Cherry and Taylor-Robinson (1970) reported that addition of catalase to TEC prevented development of
ciliostasis in chicken embryo tracheal explants by \textit{M. mycoides} var. \textit{capri}. In the model described here, catalase had no effect on ciliostasis produced by \textit{M. bovis}. Howard and Thomas (1974) reported similar findings in explants infected with \textit{M. dispar}. Also, if a toxin was produced only during early log phase growth, it could have been metabolized or denatured by autolytic enzymes released by degenerating epithelial cells. As an alternative to release of a toxin, it is possible that the interaction of a factor(s) from FBS and those liberated into the explant medium by \textit{M. bovis} resulted in physical interference, i.e., cross linking of cilia, or perturbation of the cell membrane, leading to cell death.

Geary et al. (1981) reported that a complex polysaccharide purified from \textit{M. bovis} organisms produced mastitis characterized by eosinophils and polymorphonuclear leukocytes. However, the material was not cytotoxic for BK cells. Mosher et al. (1968) had reported that infusion of a cell-free intracytoplasmic material (ICM) from sonicated \textit{M. bovis} organisms into the bovine mammary gland produced acute mastitis with the predominate cell type being eosinophils. Jasper (1979) later indicated that the ICM failed to produce cytotoxic effects in several bioassays, including BK cells, an observation that was confirmed by Geary et al., 1981. However, it was of interest to note that serum-dependent cytopathic effects were produced in BK cells infected with viable \textit{M. bovis} organisms (Hirth et al., 1970); spent culture fluids did not induce changes when placed on fresh BK monolayers (Rovozzo et al., 1963).

Thus it would appear that the ciliostatic effects associated with \textit{M. bovis} infection in this study were dependent on (1) viable organisms and
(2), a component of FBS in excess of molecular weight 10,000. The fetal bovine TEC system would appear to be an alternative method for studying the virulence mechanisms of *M. bovis* and other mollicutes associated with pneumonia in calves.
REFERENCES


GENERAL CONCLUSIONS

This work involved (1) the identification of microorganisms (especially mollicutes) associated with infectious respiratory disease in calves and (2) an in vitro investigation into the effects of Mycoplasma bovis infection in fetal bovine tracheal explant cultures. Pneumonic calves examined in Iowa, USA, harbored essentially the same microorganisms in their lungs as calves from other beef producing areas of the world. Approximately 90% of the samples yielded microorganisms (bacteria 74%, mollicutes 63%, viruses 43%). Pasteurella multocida 39%, P. haemolytica 31% and H. somnus 12% were the most frequent bacteria cultured. Of the recognized bovine mollicutes, M. dispar 40%, M. bovis 34%, Ureaplasma diversum 24% and M. bovirhinis 7% were isolated from calves with pneumonia; M. arginini, M. bovigenitalium and acholeplasmas were not detected in lung specimens. Noncytopathic bovine viral diarrhea (BVD) virus 27% and bovine herpesvirus-1 (BHV-1) 16% were the most common viruses isolated. Significant interactions between species from different classes were determined. The interaction between M. bovis or U. diversum and P. multocida was highly significant (P=.0001 and .0006) leading to the suggestion that the 2 mollicutes could possibly predispose calves to infection with the bacterium.

Immunofluorescent (IF) examination of cryostat sections of lung tissues revealed that M. dispar and M. bovis differed in the manner in which they colonized lung tissues of conventionally-reared calves with pneumonia. Mycoplasma dispar preferentially colonized ciliated epithelium
whereas in addition to colonizing ciliated epithelium, *M. bovis* also was detected in the lung parenchyma. Furthermore it was determined that IF could replace culture for routine detection of *M. bovis*. In addition, *M. dispar* remained viable in tissue held at 4°C for 14 days; whereas the organisms could be detected by IF in cryostat sections from the same tissues for up to 7 weeks.

The *in vitro* pathogenicity of *M. bovis* and *M. bovoculi* were examined in fetal bovine tracheal explant cultures (TEC). Both organisms replicated in TEC, but only *M. bovis* produced ciliostasis and only in serum-supplemented TEC medium. Generally, complete ciliostasis was evident within 62 ± 22 hours following inoculation of viable organisms; formalin-killed organisms did not affect TEC. Addition of catalase to the TEC did not inhibit ciliostasis. Organisms were observed by IF in association with ciliated epithelium in only 2 instances. Thin sections of infected explants revealed a transition from ciliated columnar epithelium to flat squamous epithelium suggesting that a toxin may have been associated with the ciliostasis. However, attempts to demonstrate a soluble toxin in cultures of *M. bovis* or sterile spent TEC medium were unsuccessful. Thus, it is concluded that in the presence of serum, viable *M. bovis* organisms produce a factor(s) which induces ciliostasis in fetal bovine TEC. This model should serve as means for further studies on the virulence mechanisms of *M. bovis* as they relate to pneumonia in calves.
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