Virulence Differences Among Three Strains of *Haemophilus somnus* Following Intratracheal Inoculation of Calves

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ABSTRACT

Pneumonia was induced in four month old Holstein calves by intratracheal inoculation of 1 x 10^9 colony forming units of *Haemophilus somnus*. Twenty calves were divided into four groups of five and challenged with a pneumonic strain (Group 1), an encephalitic strain (Group 2), a preputial strain (Group 3), or a placebo (Group 4). The clinical score, neutrophil count, respiratory rate, and temperature were significantly increased in group 1 by day 1 postinoculation ($P < 0.05$) and maintained until day 6 postinoculation ($P < 0.05$). The macroscopic pathological changes were significantly greater in group 1 ($P < 0.05$). *Haemophilus somnus* was consistently isolated from pneumonic tissue of group 1 only. Groups 2 and 3 had mild transient increases in all parameters measured and macroscopically only small focal lesions were present. It is concluded that virulence differences exist between *H. somnus* strains following intratracheal challenge of bovine lungs.

RÉSUMÉ

Cette expérience visait à provoquer une pneumonie, chez des veaux Holstein âgés de quatre mois, en leur introduisant dans la trachée 1 x 10^9 unités formatrices de colonies d'*Haemophilus somnus*. Les auteurs en utilisèrent à cette fin quatre groupes qui comptaient chacun cinq sujets. Ceux du groupe #1 reçurent une souche isolée d'un cas de bronchopneumonie purulente; ceux du groupe #2, une souche isolée d'un cas de méningo-encéphalite thromboembolique; ceux du groupe #3, une souche isolée du prépuce d'un taureau reproducteur; ceux du groupe #4, un placebo. Dès le premier jour après leur infection, les veaux du groupe #1 manifestèrent des signes cliniques, une neutrophilie, un rythme respiratoire et une hyperthermie significatifs ($P < 0,05$) qui persistèrent durant six jours ($P < 0,05$); ils développèrent aussi des lésions macroscopiques significativement plus marquées ($P < 0,05$) et on n'isola constamment *H. somnus* que de leurs lésions pulmonaires. Les veaux des groupes #2 et #3 ne manifestèrent par ailleurs qu'une intensification transitoire des paramètres précités et ne développèrent que de petites lésions focales. Il semble par conséquent exister des différences relatives à la virulence de diverses souches d'*H. somnus*, suite à leur introduction expérimentale dans la trachée des bovins.

INTRODUCTION

Haemophilus somnus is the cause of thromboembolic meningoencephalitis (1,2). The organism has also been associated with cranioventral suppurative bronchopneumonia in 6 to 12 month old beef and dairy cattle (3-9). Necrosis of the bronchiolar epithelium with infiltration of neutrophils is a characteristic histological change (4,8,9). Early reports emphasized fibrinous bronchopneumonia rather than suppurative bronchopneumonia (10,11). *Haemophilus somnus* may be present in pure culture (3,8,9,12) or mixed with a variety of organisms such as *Pasteurella haemolytica*, *Pasteurella multocida* (3,8-14), *Mycoplasma* and *Ureaplasma* spp. (3,9,12,13).

Pneumonia has been reproduced by a variety of routes. Intratracheal challenge has been used more successfully (15-19) than nasal instillation (16,20,21), aerosol exposure (22) or intravenous injection (15,16). Descriptions of *H. somnus* experimental pneumonia suggest that the lesions are similar to those seen in field cases (18,19). The ability of *H. somnus* strains other than pneumonic isolates to induce pneumonia has not been fully investigated (16).

Strain virulence differences have been described following intracisternal and intravenous inoculation (16,23). Also, differences in chick lethality (24), cytotoxicity (23), serum resistance (23,25,26), and adherence to turbinate cells *in vitro* (27) have been reported. Well defined virulence differences have not been demonstrated following intratracheal inoculation.

The purpose of the present study was to examine the ability of a pneumonic, encephalitic, and preputial isolate of *H. somnus* to induce pneumonia in calves following intratracheal inoculation.

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MATERIALS AND METHODS

ANIMALS

Twenty four month old Holstein Friesian calves were obtained from a commercial herd. The animals were held in single pens for ten weeks and thereafter two calves were held in each pen. All experiments followed the guidelines of the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

BACTERIA

Three strains identified as *H. somnus* were used (28). Strain 70986 was isolated from a natural case of suppurative bronchopneumonia, strain 43826 was from a natural case of thromboembolic meningoencephalitis and strain 26-16 from the prepuce of a breeding bull. Strains 70986 and 43826 were virulent in four month old calves inoculated intracisternally whereas strain 26-16 was not (23,29). The strains were stored at −70°C after being passaged once on brain heart infusion agar with 5% bovine blood and 0.5% yeast extract (BHIBYE) (30). To prepare the challenge suspension, each strain was cultured on BHIBYE and suspended in phosphate buffered saline pH 7.2 with 0.5% gelatin (PBS-gel) (Sigma Chemical Company, St. Louis, Missouri). The suspensions were adjusted to give an optical density of 0.2 at 440 nm wavelength, corresponding to 1×10^8 colony-forming units (CFU)/mL. The challenge suspensions were used immediately after preparation.

INOCULATION

The calves were divided into four groups of five animals. Group 1 was inoculated with strain 70986, group 2 with strain 43826, group 3 with strain 26-16 and group 3 was given PBS-gel. The calves were tranquilized with Rompun 0.1 mg/kg (Haver Lockhart, Rexdale, Ontario) and injected percutaneously into the trachea with 10 mL of challenge suspension.

DATA COLLECTION

The calves were examined daily for clinical signs of demeanor, anorexia, coughing, increased respiratory rate, nasal discharge, increased rectal temperature and abnormal sounds based on thoracic auscultation. Attempts were made to recover *H. somnus* from nasal mucus and blood before inoculation and on days 1,3,5 and 7 after inoculation (PI). At necropsy, tracheal mucus, lung tissue from the cranioventral lobes and functional areas between normal and consolidated tissue were cultured for mycoplasmas and bacteria. The number of *H. somnus* per gram of lung tissue was calculated by counting colonies growing from serial tenfold dilutions of 1.0 g tissue suspended in 1.0 mL PBS-gel. The presence of *H. somnus* in the lungs at necropsy was also ascertained by direct immunofluorescence microscopy, using a fluorescein isothiocyanate conjugated rabbit immunoglobulin against strain 43826 (19).

Blood samples stabilized in ethylenediaminetetracetate (EDTA- Vacutainer Brand, Becton-Dickinson, Rutherford, New Jersey) were collected from all animals before inoculation and at days 1,3,5 and 7 PI and analyzed for total erythrocyte counts, total white blood cell (WBC) counts and differential counts.

Serum was removed from clotted blood samples taken on the same days and titrated for antibodies against *H. somnus* strain 70986 by the bacterial agglutination test (30).

All animals were euthanized seven days PI and necropsied. A visual estimate was made of the proportion of the lung volume that had developed lesions using a previously described method (31). The estimated lung lesion surface areas were outlined on lung diagrams.

Specimens were taken for histological examination, fixed in 10% formalin, sectioned at 6 μm, and stained with hematoxylin and eosin. Selected lung sections were stained with Brown and Brenn, Warathin Starry, and Victoria blue stains. A detailed blind histological examination was made and tabulated as previously described (32).

STATISTICAL ANALYSIS

The ratios of lung lesion area to total lung area were calculated and transformed using arcsin tables (33). Hematological counts, bacterial counts, clinical indices, and gross indices are expressed as means ± standard deviation. Bacterial counts were transformed to $\log_{10}$ for calculations of means ± standard deviations. Data means were compared using analysis of variance, t-test and Duncan's range test where applicable. A probability of 0.05 or less was accepted as significant. All analyses were carried out using the APL Statistical Package (APL StatLib, University of Guelph, Guelph, Ontario).

RESULTS

CLINICAL RESPONSE

All calves were free of clinical respiratory disease before inoculation. All animals were febrile one day PI. Clinical response varied between individual calves and groups of calves. Spontaneous coughing and tachypnea were associated with lung sounds identified as dry or moist rales. All animals had significantly increased respiratory rates one and two days PI, but the calves in group 1 had significantly higher rates ($p < 0.001$) that were significantly greater than in calves in any other group ($p < 0.05$). No significant increase in rectal temperature was observed. All group 1 animals had more severe clinical signs than any of the animals in other groups. All five calves in group 1 had increased lung sounds and animals 4 and 5 were dull, depressed and

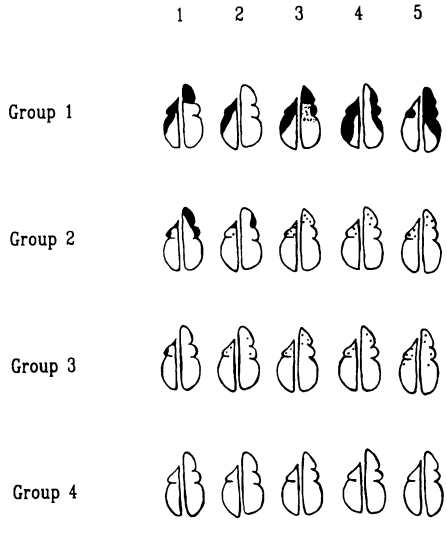

Fig. 1. Illustration of the distribution, extent and frequency of lung lesions of four groups of calves inoculated with *H. somnus*. Black areas represent the areas of consolidation evident viewing the dorsal aspect of the lungs.

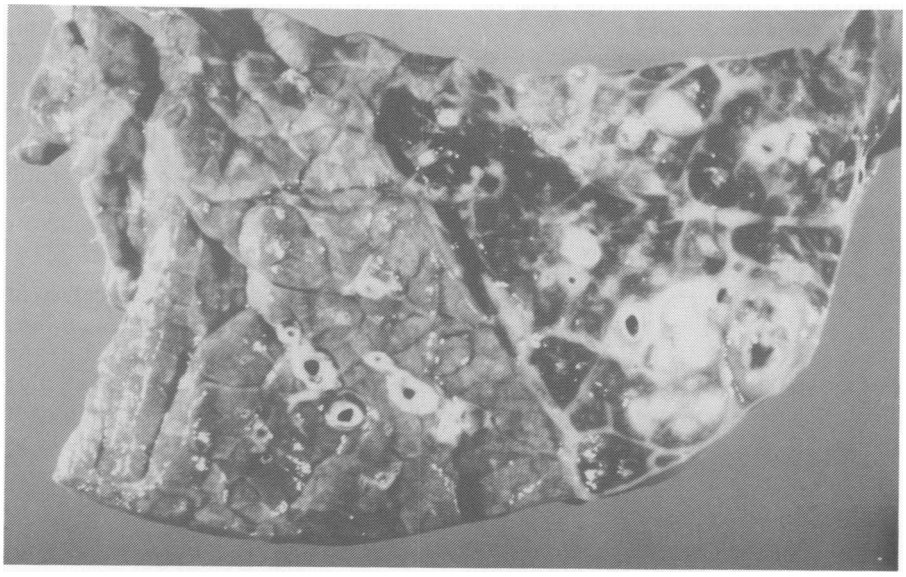

Fig. 2. Portion of lung from a calf in group 1, inoculated with a virulent pneumonic strain of *H. somnus*. Cut surface through area of consolidation. Notice dilated bronchioles filled with necrotic debris, abscessation, sequestration, and dilated interlobular septa.

inappetant. Clinical signs referable to the respiratory tract were obvious on day 7 PI. Animals in groups 2, 3 and 4 appeared normal by day 4 PI.

HEMATOLOGY

There was a significant increase in total WBC count in all groups at day 1 PI but these counts were normal by two days PI. Band neutrophils were significantly increased in all groups at day 1 PI, but were normal at day 2 PI except in group 1 in which the counts remained significantly elevated. Lymphocyte, monocyte and eosinophil levels reflected a stress response in the groups and were not evaluated statistically.

MACROSCOPIC CHANGES

The distribution, frequency, and extent of pulmonary lesions are summarized in Fig. 1. Macroscopic lesions were present in all groups except group 4 (controls) and were most severe in group 1, as indicated by the consolidated areas of lung (Fig. 1).

Group 1 — Lungs were enlarged with firm cranioventral purple consolidation of the left lungs and emphysema of the caudal lobes (Fig. 2). Approximately 32.0% ± 8.7 of the lungs were consolidated significantly greater than groups 2 and 3 ($p < 0.01$) and group 4 ($p < 0.001$). Grey foci, 1 to 2 cm in diameter, were visible through the pleura, and interlobular septae were distended. Purulent material was present in dilated bronchi and numerous abscesses, necrotic foci and sequestra were scattered throughout the parenchyma.

Groups 2 and 3 — Macroscopic lesions were mild with multifocal, 1 to 3 cm diameter areas of atelectasis (Fig. 3) coalescing to form firm consolidation of the left intermediate and right cranial lobes with mucopurulent material in the bronchioles. The areas of consolidation, 9.2% ± 7.6 and 10.7% ± 3.4 in groups 2 and 3 respectively were significantly greater ($p < 0.01$) than in control group 4 animals.

Group 4 — The lungs had no macroscopic abnormalities. All test groups had a significantly greater surface area of consolidation than did group 4.

BACTERIOLOGY

Haemophilus somnus was not isolated from the blood of any calf during the experiment. Group 1 had *H. somnus* in 80% (four) of the lung lesions and all other groups had a prevalence of 20% (one) (Table I). *Mycoplasma* spp. and other bacteria were isolated in moderate numbers and only in those cases from which *H. somnus* was recovered (Table I). *Haemophilus somnus* was recovered in pure culture from the junctional area of all six cases and mixed with *E. coli* and *Pasteurella* spp. in the cranioventral portion. The number of *H. somnus* per g tissue was not significantly different between treated groups. *Haemophilus somnus* was not cultured from the nares prior to inoculation in any animal. Nasal isolation PI was highly variable and

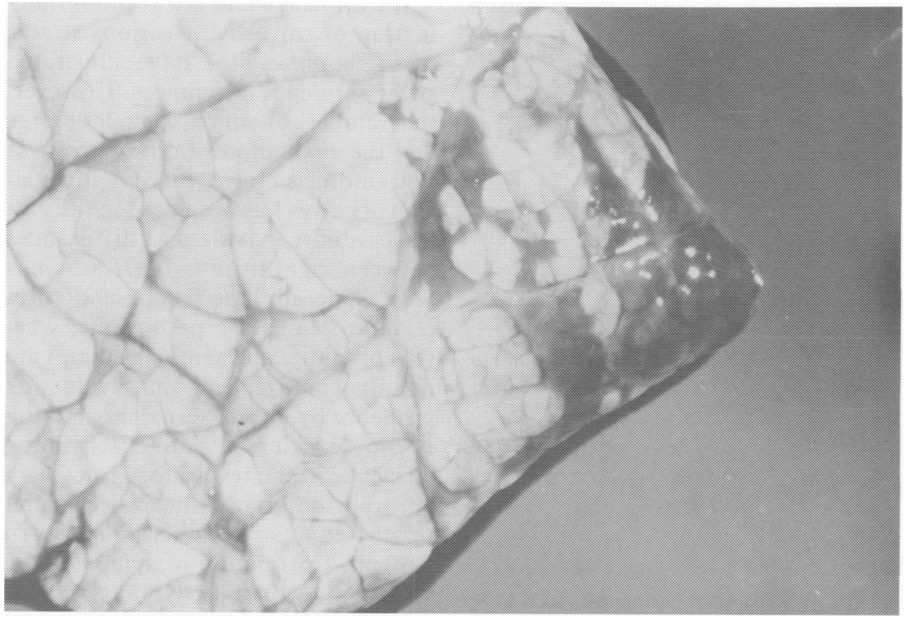

Fig. 3. Portion of lung from a calf in group 2, inoculated with an avirulent preputial strain of *H. somnus*. Mild atelectasis at tips of intermediate lobe.

TABLE I. Isolation of *H. somnus*, Other Bacteria (OB) and *Mycoplasma* from the Nasal and Tracheal Mucus and Lungs of Four Groups of Calves Inoculated with *H. somnus*

Group[a]	*H. somnus* Lung	OB Lung	*Mycoplasma* Lung	*H. somnus* Nasal Mucus	*H. somnus* Tracheal Mucus
1	4/5	4/5	4/5	2/5	3/5
2	1/5	1/5	1/5	3/5	1/5
3	1/5	1/5	1/5	0/5	1/5
4	0/5	0/5	0/5	0/5	0/5

[a]Group 1, pneumonic Strain 70986; Group 2, encephalitic Strain 43826; Group 3, preputial Strain 26-16; Group 4, control

did not reflect accurately isolation from the lung (Table I). Tracheal isolation at necropsy was made only in animals which had *H. somnus* in the lung (Table I).

HISTOPATHOLOGY

All groups had lesions which had exudative and proliferative components and were most severe in bronchioles and alveoli. Changes were similar in all groups with little variation among individuals of the same group.

Group 1 — Fibrin frequently distended interlobular septa and alveolar lumens and large areas of coagulation necrosis were centered over bronchioles (Fig. 4). The inflammatory infiltrate was predominantly neutrophilic with mild macrophage accumulation and the occasional giant cell. Bacterial aggregates were occasionally seen.

Groups 2 and 3 — Both groups had similar lesions. Bronchioles had a mild mononuclear infiltrate in the lamina propria with mild to moderate peribronchiolar fibrosis and lymphoid hyperplasia (Fig. 5) and a mild neutrophil infiltrate in the lumens. Neutrophils, macrophages and an occasional giant cell were present in alveolar lumens.

Group 4 — All control animals had mild focal atelectasis with mild neutrophil accumulation in bronchiolar and alveolar lumens.

IMMUNOFLUORESCENCE

Fluorescence was demonstrated only if *H. somnus* had been isolated from tissues where macroscopic and microscopic evidence of consolidation was present. Fluorescence was most intense in terminal bronchioles in the areas of necrosis associated with sloughed bronchiolar and alveolar epithelium and in luminal necrotic debris.

SEROLOGY

Serum antibody titers of calves at the time of challenge with *H. somnus* could not be correlated with the subsequent response to challenge (Table II). There was no significant increase in *H. somnus* titers PI day 7 as a result of challenge.

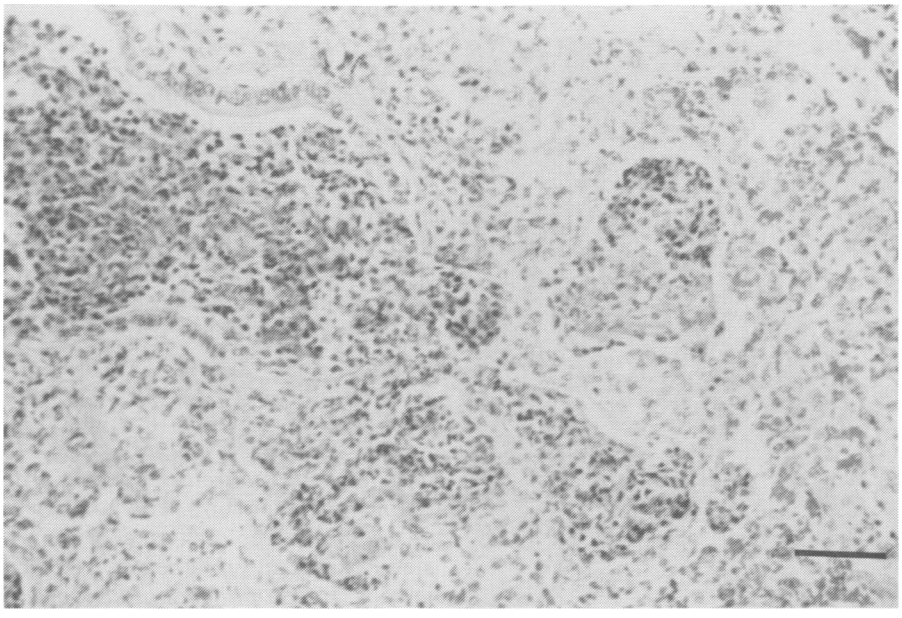

Fig. 4. Section of lung from a calf in group 1, inoculated with a virulent pneumonic strain of *H. somnus*. Bronchioles filled with inflammatory infiltrate, fibrin and necrotic debris extending into the alveoli. Notice bronchiolar epithelial necrosis and sloughing. H & E. Bar equals 150 μm.

DISCUSSION

Experimental pneumonia was consistently induced by pneumonic Strain 70986 but encephalitic and preputial strains failed to induce significant pneumonia. Assessment of clinical indices, hematology and gross indices clearly demonstrated differences between strains of *H. somnus* and their ability to induce bovine pneumonia. The pneumonic strain was virulent following intracisternal (23,29) and intratracheal challenge while the encephalitic strain was virulent following intracisternal challenge (23,29) but not intratracheal challenge. The preputial isolate was consistently avirulent (23,29).

Intratracheal and intravenous inoculation of calves with various strains of *H. somnus* (16) have illustrated strain virulence differences. However, the numbers of animals were not large and the evaluation of differences was not outlined. Intracisternal inoculation of calves has been used in the past to evaluate virulence differences between eight strains of *H. somnus* (23). Generally pneumonic and encephalitic strains are pathogenic, whereas preputial and seminal isolates are not. It would appear that evaluating virulence by intracisternal inoculation is not sufficient for determining virulence in the respiratory tract.

Strain differences have been demonstrated using several *in vitro* systems. Correlation of demonstrable virulence in these systems to *in vivo* virulence has not been made. Inoculation of embryonated eggs (24) indi-

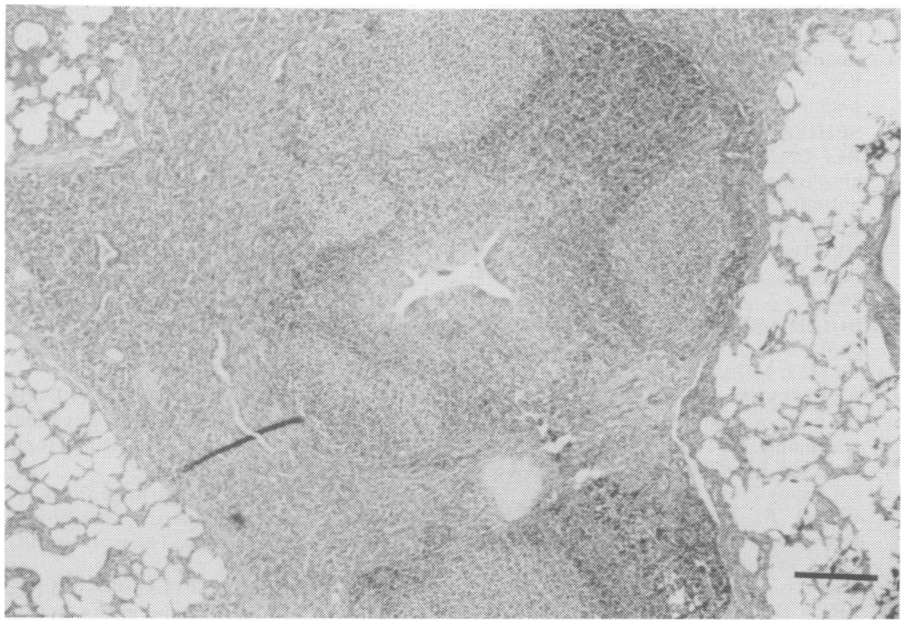

Fig. 5. Section of lung from a calf in group 2, inoculated with an avirulent preputial strain of *H. somnus*. Atelectasis with mild alveolar inflammatory infiltrate. H & E. Bar equals 600 μm.

cated strain virulence differences. Differences have also been shown between adhesion to cultured bovine turbinate cells and correlated to colonial morphology of these strains (27). However, cytotoxicity differences were not demonstrated on a variety of cell lines (23). Some investigators have reported that serum susceptibility varies between strains with encephalitic isolates being resistant and preputial isolates being susceptible while others have failed to demonstrate these differences (23,25,26). The biochemical components responsible for these differences are being investigated but outer membrane protein polyacrylamide gel electrophoretic analyses have failed to show significant differences between strains (34).

The gross appearance of the induced pneumonias are similar to those described for field cases of *H. somnus*-associated pneumonia (18,19). The lesions differ somewhat from some early reports of *H. somnus*-associated pneumonia (10,11) which described more fibrin and spreading coagulation necrosis in the lesions. All animals in this study were euthanized seven days PI and lesions at 48 to 96 h would undoubtedly have contained more fibrin. Sequential studies are necessary to determine the full spectrum of lesions possible with *H. somnus* pneumonia. Histologically the lesions reflected what was seen grossly and were also similar to described field cases (8,9).

None of the animals in this study developed bacteremia. Systemic infection has been infrequently induced previously following intratracheal inoculation (15,16). In our study organisms were usually localized to the necrotic debris in the alveolar and bronchiolar lumens as demonstrated by immunofluorescence and were seldom seen in the interstitium. Similar findings have been reported using immunoperoxidase staining of the bacteria (18). The findings in this experiment would suggest that invasion and systemic spread from the lungs is uncommon. The pneumonic strain was readily isolated in moderate numbers from the lung while encephalitic and preputial strains were infrequently isolated suggesting more efficient clearance of these last two strains from the lung parenchyma. It is possible that this ability of the pneumonic strain to persist in the lung is a mechanism for increased virulence in the lung. Neutrophils and macrophages were prominent in the lung lesions of those calves inoculated with the pneumonic strain but not in the lungs with less severe lesions. It is possible that avirulent strains are efficiently removed from the lung but virulent strains are able to persist and induce an inflammatory response of greater severity. The animals inoculated with the pneumonic strain had a significant peripheral neutrophilia with an increased circulating band neutrophil population. It is known that *H. somnus* has the ability to inhibit neutrophils *in vitro* (35-37). The role of neutrophil inhibition *in vivo* requires further study. *Haemophilus somnus* may also persist in pulmonary parenchyma as a facultative intracellular organism in alveolar macrophages (38). The role of specific antibody in the removal of *H. somnus* is unknown. There was no correlation of gross lesion severity to serum antibody level as measured by the bacterial agglutination test. One would then question the importance of circulating antibodies in the clearance of virulent organisms from the lung in light of these findings. Other researchers have not been able to correlate susceptibility to experimental systemic challenge and serum antibody levels (30).

Future work investigating experimentally induced pneumonia should recognize the differences between strains in their ability to induce pneumonia. Although a general statement on strain virulence differences related to anatomical site of isolation cannot be made it would appear from our work and that of others (16,23) that pathogenic differences do indeed exist. The bacterial factors responsible for these differen-

TABLE II. Means and ± Standard Deviation of Serum Bacterial Agglutination Titers from Four Groups of Calves Inoculated with *H. somnus*

	Group 1	Group 2	Group 3	Group 4
Day 0	5.6 ± 2.1	7.2 ± 5.2	13.6 ± 11.2	7.2 ± 5.2
Day 1	10.4 ± 5.3	17.6 ± 14.2	18.4 ± 13.1	8.0 ± 4.9
Day 4	8.8 ± 4.3	13.6 ± 11.2	14.4 ± 11.5	9.6 ± 6.1
Day 7	8.8 ± 4.4	14.4 ± 11.5	10.4 ± 5.4	8.0 ± 4.9

Group 1, pneumonic Strain 70986; Group 2, encephalitic Strain 43826; Group 3, preputial Strain 26-16; Group 4, control

ces warrant further investigation as does the role of the host in determining the outcome of *H. somnus* infection, specifically the contribution of antibody and phagocytic function in bacterial removal and their involvement in the genesis of inflammatory lesions.

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