Isolation of Pathogenic Strains of *Haemophilus somnus* from the Female Bovine Reproductive Tract

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ABSTRACT

The prevalence of *Haemophilus somnus* in the genital tract of slaughtered and live cows in southern Ontario was investigated. The vagina and uterus of slaughtered cows were swabbed separately. Live cows were examined and sampled in two field surveys: Centre A and Centre B. In the former, aspirated mucus secretions and in the latter, specimens obtained by guarded swabbing were examined bacteriologically. *Haemophilus somnus* was isolated from 28 genital tracts of 461 slaughtered (6.1%), and seven of 199 live (3.5%) cows during the centre B survey. The isolates were recovered from both normal and diseased reproductive tracts. Fourteen strains isolated from genital organs were examined for pathogenicity *in vivo* to test the occurrence of pathogenic isolates. In the initial stage of the *in vivo* study on pathogenicity, each of the fourteen isolates was examined on one calf using an intracisternal inoculation. Subsequently, one pathogenic and one nonpathogenic strain were inoculated into five calves each to statistically confirm their pathogenic potential. Of 14 genital isolates of *H. somnus* examined in an intracisternal calf assay, six (43%) caused a fatal peracute neurological disease, while eight were nonpathogenic. A comparative pathological study of pathogenic and nonpathogenic isolates showed that the former caused a severe fatal suppurative meningoencephalitis whereas the latter caused no lesions whatsoever or a mild leukocytic leptomeningitis. The salient data obtained in this study indicate that there are pathogenic strains of *H. somnus* in the genital tract of apparently normal cows as well as of those with inflammatory disease.

RÉSUMÉ

On a étudié la prévalence d'*Haemophilus somnus* dans le tractus génital de vaches abattues et de vaches vivantes. Chez les vaches abattues, le vagin et l'utérus furent écouvillonnés séparément. L'examen et l'écouvillon chez les vaches vivantes furent menés au cours de deux études cliniques: étude A et étude B. Dans le premier cas, des sécrétions et du mucus étaient aspirés; dans le second cas, les spécimens étaient obtenus au moyen d'un écouvillon protégé avant d'être évalués au plan bactériologique.

***Haemophilus somnus* fut isolé de 28 tractus génitaux de 461 spécimens d'abattoir (6.1%) et de sept des 199 spécimens provenant d'animaux vivants (3.5%) dans l'étude B. Les isolats furent obtenus de tractus génitaux normaux et anormaux. La pathogénicité *in vivo* de 14 souches isolées des organes génitaux fut examinée pour évaluer la fréquence des isolats pathogènes.**

Au début de l'étude *in vivo* sur la pathogénicité, chacun des 14 isolats fut examiné chez un veau après une inoculation intracisternale. Par la suite, une souche pathogène et une souche non pathogène furent inoculées chez cinq veaux pour confirmer la pathogénicité.

Des 14 isolats génitaux d'*H. somnus* étudiés par inoculation dans la citerne cérébello-médullaire chez le veau, six (43%) provoquèrent une maladie neurologique suraiguë fatale tandis que huit n'étaient pas pathogènes.

Une étude pathologique comparative des isolats pathogènes et nonpathogènes a démontré que les premiers causent une méningo-encéphalite suppurative fatale tandis que les nonpathogènes ne provoquent aucune lésion ou provoquent une légère leptoméningite leucocytaire.

Les résultats qui ressortent de cette étude indiquent qu'il y a des souches pathogènes d'*H. somnus* dans le tractus génital de vaches apparemment normales aussi bien que chez celles présentant une maladie inflammatoire.
(Traduit par Dr Patrick Guay)

INTRODUCTION

Haemophilus somnus is an important pathogen in cattle causing peracute fatal septicemia and thrombotic meningoencephalitis (TME) (1), pneumonia (2,3), arthritis (2), myocardial abscessation (4), mastitis (5–7), and abortion (3,8). The frequent isolation of *H. somnus* from the genital tract (9) and semen (10,11–13) of bulls is not accompanied by overt disease (9). Cows harbor this organism in the genital tract at a lower frequency (10–27%) but prevalence may vary depending on the geographical area surveyed (3,14–17). *Haemophilus somnus* may cause inflammatory female genital disease (8,17) or persist in the genital tract for a considerable time as a commensal (3,17). No work has been carried out to determine the pathogenicity of isolates from the genital tract of the cow. In limited

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in vivo studies, seminal and preputial strains were determined to be nonpathogenic in an intracisternal (I/C) calf assay (18,19), a method which is regarded as the reliable way to differentiate pathogenic from nonpathogenic strains of *H. somnus*. Fatal peracute meningitis, however, occurred in young calves inoculated I/C with TME and pneumonic isolates (18,19) which correlates with their ability to cause septicemia and lesions typical of TME after intravenous inoculation (20,21). This paper addresses the important epidemiological issue of the pathogenicity of strains of *H. somnus* isolated from normal and diseased female bovine genital tracts.

MATERIALS AND METHODS

ABATTOIR SURVEY

Reproductive tracts of dairy and beef cows were opportunistically sampled at a local abattoir (J.M. Schneider's Inc., Kitchener, Ontario). The vagina was cut away from the rectum, and together with the cervix and uterus examined for gross mucosal lesions. A sterile swab (6 inch absorbent buds, Johnson and Johnson Ltd., Toronto, Ontario) was inserted to sample the mucosa of the anterior vagina and posterior cervix. The serosa of the uterus was rinsed with 70% alcohol, the wall incised, and a separate swab inserted into the exposed uterine cavity. The swabs were placed separately into sterile tubes containing 1 mL of phosphate buffered saline (PBS), pH 7.2, with 0.1% porcine gelatin (Sigma Chemical Company, St. Louis, Missouri) as a transport medium.

The specimens, maintained at ambient temperature, were plated on solid media within 3 h. The vaginal and uterine swabs were cultured on two kinds of media: (I) a Columbia agar selective medium, developed for the isolation of *H. somnus* (22) and adapted in our laboratory through substitution of Columbia agar (Oxoid) with Columbia agar (Difco) (23), and (II) a brain heart infusion agar (BHI-agar) used as a nonselective medium (18). Plates were incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. These conditions of incubation were used for all bacterial cultures in this work. Colonies of *H. somnus* were identified morphologically (24,25), and the isolation confirmed by an indirect fluorescent antibody test (IFAT) and immunodiffusion test (IDT).

In the IFAT the colonies were suspended in PBS, mounted on a glass microscope slide, air-dried and fixed with acetone for 10 min. Anti-*H. somnus* bovine hyperimmune serum 704 against reference strain 43826 (20,26) was applied for 20 min and incubated at room temperature in a humidified chamber. After washing with PBS, a rabbit anti-704 immune serum conjugated with fluorescein isothiocyanate was applied for 20 min and then the slide washed with PBS. The reference strain 43826 of *H. somnus* was prepared in the same manner and used as a positive control. The slides were examined using a Leitz Wetzlar® Orthoplan fluorescent microscope (Walter A. Carveth Ltd., Toronto, Ontario) and were considered positive when there was an "apple" green fluorescence associated with the bacterial cell wall. The IDT was performed using *H. somnus* reference strain 43826 and the antigens tested were prepared by sonication of bacteria (18).

FIELD SURVEY

Numerous dairy herds in southern Ontario were visited with theriogenologists from centre A (United Breeders Inc., Guelph, Ontario) and centre B (Western Ontario Breeders Inc., Woodstock, Ontario). The cows were selected for sampling according to the breeding history which indicated a reproductive problem. A genital examination per rectum was performed by the theriogenologist during the visit.

Cows in survey A (Centre A) were sampled by introduction of a regular insemination catheter into the uterus and/or cervix and aspiration of some mucus or mucopurulent material into the catheter. One to three mL of the aspirate was immediately placed into sterile tubes containing 1 mL of the transport medium. Cows examined in survey B (Centre B) were sampled using guarded sterile swabs (Kalayjian Industries Inc., Long Beach, California), which were exposed in the uterus or the posterior cervix and immediately placed into the transport medium.

The mucus specimens collected in survey A were transported at ambient temperature to the laboratory within 12 h and cultured on the media used in the abattoir survey and also on McConkey agar to aid in further bacteriological evaluation of the specimens.

The swab specimens from survey B were cultured on BHI-agar, McConkey agar, and a modified Columbia agar as a selective medium. The medium differed from the selective medium used in the abattoir survey and in survey A in that it was devoid of neomycin and the concentration of sodium azide was lowered to 2 μg/mL (from 50 μg/mL) (23). The isolation of *H. somnus* was performed using methods described for the abattoir survey. Organisms other than *H. somnus* were identified using standard methods in the Clinical Microbiology Laboratory, Veterinary Teaching Hospital, Ontario Veterinary College.

Nine strains of *H. somnus* isolated from the slaughtered cows and five strains isolated from live cows were cultivated in the yolk sac of five to seven day old embryonating eggs and stored at −70°C (20). Seven of those strains were recovered from inflammatory uterine exudate, one from the cervix of a live cow with vulvitis and the remaining six isolates were recovered from grossly normal cervix or vagina.

STUDY OF STRAIN PATHOGENICITY

Fourteen genital strains of *H. somnus* were selected on the basis of whether the genital tract had inflammatory lesions. The isolates were tested for pathogenicity by intracisternal inoculation into young Holstein bull calves (#1–14), whereby each strain of *H. somnus* was examined separately in one animal. Two strains, 87121531 and 88159, were later inoculated into five calves (#15–24) to confirm their pathogenicity.

A total of 24 Holstein bull calves three to eight weeks old were kept in isolation facilities, fed with antibiotic free-rations and examined clinically for one week prior to the challenge. The Animal Care Committee guidelines were followed during all experiments. The inoculum of each strain examined *in vivo* was prepared as described previously (20). The prepared *H. somnus*

suspension was estimated to contain 10^5 colony-forming units (CFU)/mL and 1 mL of this suspension was used as an intracisternal inoculum within 1 h after preparation. The actual number of bacterial CFU inoculated was counted in residual inoculum immediately after the animal inoculation (20).

The calves were sedated with an intramuscular dose of xylazine (Rompun®, Bayvet Division, Chemagro Ltd., Etobicoke, Ontario) 0.25–0.5 mg/kg body weight and then by an intravenous dose of chloral hydrate-$MgSO_4$ solution until profound narcosis occurred (20,21). The inoculation technique into the cisterna magna has been described (20). Approximately 1 mL of cerebrospinal fluid (CSF) was withdrawn and 1 mL of the inoculum containing *H. somnus* was injected.

All calves were observed during recovery from anesthesia and then repeatedly examined clinically until euthanasia was performed. The calves with a severe acute neurological disease were euthanized when in lateral recumbency near extremis, while transiently mildly affected animals were killed three to four days after the challenge. Withdrawal of some CSF from the cisterna magna through the spinal needle was attempted immediately after euthanasia. A routine total postmortem examination was performed. The brain and other tissues were removed for histopathology and a CSF sample and sections of the brain, lung, liver, kidney and spleen were taken for bacteriological examination and cultured on BHI-agar. Tissues for histopathology were fixed by immersion in 10% buffered formalin, and the sections of basal ganglia, parietal cortex, thalamus, midbrain, cerebellum and rostral medulla, and caudal medulla, as well as sections of the other organs were processed routinely for histological examination. The examination of the microscopic slides was performed with no knowledge of the severity of the clinical disease and findings on the postmortem examination.

Two genital isolates, 88159 and 87121531, were selected from the group of 14 to confirm *in vivo* the reproducibility of their pathogenic potential. Both strains were recovered from the normal vagina of slaughtered cows. Ten calves were kept in isolation and handled as described previously and examined for presence of homologous IgG_2 anti-*H. somnus* antibodies prior to the challenge. The titers were measured using a dot-blot immunoassay described previously (27).

The first group of five animals (#15–19) were inoculated with strain 88159 shown in the preliminary trial to be pathogenic and the second group of five (#20–24) with strain 87121531 which was considered nonpathogenic.

Histological lesions observed in two sections, the thalamus and the cerebellum/rostral medulla of the calves, were assessed using 13 criteria in order to compare the same type of lesions among the examined calves. The 13 histological criteria were as follows: in meninges, infiltration by intact leukocytes (i) and infiltration by necrotic leukocytes (ii); in choroid plexus, severity of the interstitial inflammation (iii); in pyencephalus, numbers of intact leukocytes (iv) and numbers of necrotic leukocytes (v); vasculitis in the brain parenchyma, perivascular leukocytosis (vi), fibrinoid vascular necrosis (vii), and perivascular protein-rich fluid exudation or hemorrhage (viii). The remaining criteria included; vasculitis in the meninges, perivascular leukocytosis (ix), fibrinoid vascular necrosis (x), and perivascular protein-rich fluid exudation or hemorrhage (xi); neutrophilia in the neuropil (xii), and microglial reaction in the neuropil (xiii). The lesions in each category were scored according to severity: 0, 1, 2 and 3. The total scores for the section of cerebellum/medulla and thalamus from five calves in the first group were compared with the scores for both these sections for the second group. An analysis of variance (ANOVA) procedure from SAS Inc. (28) was used to determine statistically significant differences.

RESULTS

ABATTOIR SURVEY

A total of 461 reproductive tracts from 405 dairy and 56 beef cows were examined. *Haemophilus somnus* was isolated from 28 cows (6.1%) comprising 24 isolates from dairy (5.9%) and four from beef (7.1%) cows. Twenty-four (85.7%) strains were isolated from grossly healthy genital organs and four strains (14.3%) from diseased organs of two cows with pyometra and two with endometritis (Table I). *Haemophilus somnus* colonies cultured on Columbia agar (22) were small, nonhemolyzing and grey. Numbers of colonies were markedly higher on BHI-agar than on Columbia agar. Twenty-one specimens were overgrown by contaminants including *Escherichia coli* in 12 plates, *Proteus* sp. in seven, *Actinomyces pyogenes* in one, and *Pasteurella multocida* in one. Of 28 isolated strains of *H. somnus*, 27 were positively identified on the IFAT and 12 on the IDT.

FIELD SURVEY

Survey A

A total of 154 dairy cows were examined and sampled in 30 herds. One strain of *H. somnus* was isolated in a mixed culture with other organisms from an asymptomatic repeat breeder. Of the examined animals four cows had a purulent metritis, 60 were repeat breeders, 30 were recently calved and had a prolonged mucopurulent postparturient discharge requiring antimicrobial treatment. Five cows aborted within a few weeks prior to the sampling and required treatment, and 55 cows which were apparently normal were examined and sampled in the postparturient interval. In total, ten bacterial species, including *H. somnus*, were identified from the 126 cows. A large number of specimens con-

TABLE I. Isolation site of *H. somnus* from the genital tract of slaughtered cows

	Condition of the genital tract		
Isolation site	Normal	Diseased[a]	Total (%)
Vagina only	14	0	14 (50)
Uterus only	5	1	6 (21.4)
Both sites	5	3	8 (28.6)
Total (%)	24 (85.7)	4 (14.3)	28 (100)

[a]Inflammatory lesions were associated in two animals with endometritis and two with pyometra as diagnosed on gross examination

tained contaminants. The most commonly isolated bacteria were *E. coli*, 69 times, α-streptococci, 57, *Bacillus* sp., 30 and *A. pyogenes*, (22).

Survey B

A total of 197 dairy and two beef cows were examined and sampled in 85 different herds. Seven (3.52%) strains of *H. somnus* were isolated from seven dairy cows of which four were repeat breeders, two had a prolonged postparturient discharge and one had aborted four days prior to the sampling. All strains of *H. somnus* were isolated either in a mixed or pure culture on BHI-agar and four strains on the KD-Columbia agar, when it was employed. Colonies of *H. somnus* cultured on the KD-Columbia agar were similar in size and morphology to colonies grown in parallel on BHI-agar.

Overall, 13 cows were diagnosed as having metritis, 141 were repeat breeders, 31 had a prolonged inflammatory postparturient discharge which required treatment, and 14 cows had aborted in the past few weeks. From 99 (49.75%) cows which were positive on bacterial culture, α-streptococci were isolated most frequently, at 39 times, and were followed in number by *E. coli*, 24, *A. pyogenes*, 22, *Bacillis* sp., 19, diphtheroids, 9, nonhemolyzing staphylococci, 8, and *H. somnus*, 7. Bacterial contamination of the specimens was not a problem in this part of the field study.

The presence of moderate to high numbers of *E. coli* and α-streptococci and the presence of *Bacillus* sp. colonies on the nonselective medium was considered bacterial contamination. The proportion of samples containing potential contaminants to the total number of samples for the repeat breeder category was calculated in both field surveys and the values compared using a Chi square test (29). The repeat breeder cows sampled in survey A had a significantly higher incidence of bacterial contamination in mucus samples than those sampled using guarded swabbing in survey B ($p < 0.05$). Although the difference in the proportion of contamination between the total number of cows in both field studies appeared obvious, it was not found to be significant statistically.

PATHOGENICITY STUDIES

The number of bacterial CFU in the inocula of the 14 I/C studies varied from $0.11\text{–}15 \times 10^5$. This moderate variation of numbers of bacteria used in the challenge did not appear to influence the mortality of calves. All challenged animals recovered well from anesthesia within 30–60 min after the inoculation.

Clinical signs

Six calves (#1–6) developed severe neurological disease, became anorexic, progressively depressed, ataxic and recumbent within 5 h to 10 h after the challenge and developed progressively severe opisthotonus and rigidity of the limbs. These signs were followed by hypothermia and lateral recumbency with severe tonic-clonic convulsions of the limbs prior to euthanasia, 16–29 h after the challenge. The six genital isolates of *H. somnus* inoculated I/C in these six calves were designated pathogenic strains.

In the remaining group of eight animals (#7–14) the intracisternal inoculation of *H. somnus* had only mild effects, characterized by mild to moderate anorexia, hyperthermia, depression, mild ataxia and variable opisthotonus all of which were transient. All eight calves were healthy when euthanized three to four days after the I/C challenge. The genital strains of *H. somnus* inoculated I/C in these eight calves were designated nonpathogenic strains.

Necropsy

The six calves with prominent neurological disease had severe lesions in the central nervous system (CNS). All of these animals had a moderate amount of fibrinopurulent exudate in the atlantooccipital joint and a large volume of cloudy CSF containing strands of fibrin. Cloudy yellow colored meninges covered the flattened cerebral gyri and there was a cone shaped posterior cerebellar herniation into the foramen magnum. There was extensive fibrin deposition in the meninges of the lower brain stem and rostral cervical spinal cord. Both lateral and third ventricles were mildly to moderately distended with cloudy CSF. All six animals had marked pulmonary congestion, edema and petechial and ecchymotic hemorrhages which were variably scattered on the serosal surfaces of organs of the thoracic and abdominal cavity.

Of the eight calves which did not develop fatal neurological disease after the I/C challenge, six had no gross pathological lesions. One animal (#8) had a minor amount of slightly cloudy CSF and cloudy yellow basal leptomeninges, whereas the other (#9) had a mild fibrinous exudate in the meninges over the dorsal aspect of the medulla oblongata.

Bacteriology

On bacteriological examination of the CSF, large numbers of *H. somnus* were found in all calves with fatal neurological disease. Basilar sections of the rostral brain collected from these calves yielded large to moderate numbers of *H. somnus* from four calves (#1,3,5,6) and no bacterial growth from two calves (#2,4). *Haemophilus somnus* colonies were always isolated from the CNS in pure culture. There was no significant bacterial growth from the remaining organs cultured.

Of the eight nonfatally affected animals, the CSF and brain tissue from all were negative on bacterial culture, except for low numbers of *H. somnus* colonies in the CSF of one calf (#14).

Histopathology

On histological examination five severely affected calves (#1–5) had pronounced fibrinopurulent meningitis composed of a massive infiltration of intact and necrotic neutrophils and mononuclear cells sometimes accompanied by a localized leukocytic infiltration of the subjacent brain parenchyma (Fig. 1). Large bacterial colonies were frequent in the leptomeninges of the brain stem. Meningeal and parenchymal blood vessels adjacent to the meninges and ependyma had fibrinoid necrosis of the tunica adventitia and tunica media, accompanied by intravascular leukocytosis and swollen or exfoliated endothelium (Fig. 2). Perivascular exudation of a protein rich fluid and hemorrhage surrounded some damaged meningeal and parenchymal blood vessels. In all cerebral ventricles, there was severe purulent inflammation of the choroid plexus and large amounts of fibrinous pyencephalus containing scattered

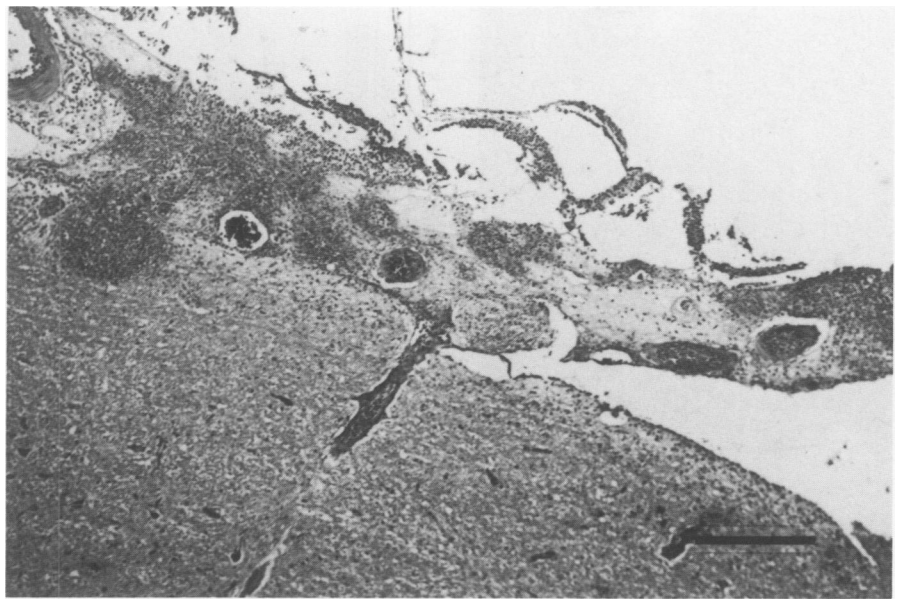

Fig. 1. Section of the rostral medulla oblongata from calf #2 inoculated I/C with a pathogenic strain of *H. somnus* 8711337 and euthanized 21 h after challenge, showing severe fibrinopurulent meningitis and localized invasion of the adjacent brain parenchyma by inflammatory cellular exudate. There is leukocytic infiltration of the Virchow-Robin space of the adjacent parenchymal blood vessels. H&E. Bar 300 μm.

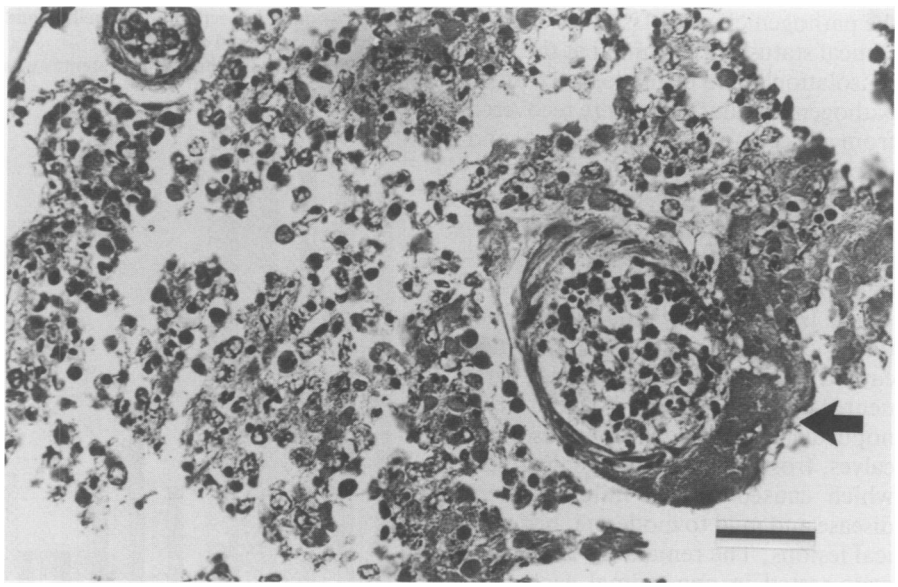

Fig. 2. Midbrain section from calf #1 inoculated I/C with a pathogenic strain of *H. somnus* 87102721 and euthanized 16 h after challenge, showing severe fibrinopurulent meningitis. A large bacterial colony (arrow) is adjacent to the wall of a blood vessel with mural fibrinoid necrosis, loss of endothelium and intravascular leukocytosis with fibrin deposition. Also, massive mixed leukocytic infiltration of the perivascular space is composed of neutrophils and mononuclear cells, large numbers of which are necrotic. H&E. Bar 40 μm.

bacterial colonies and much leukocytic necrosis. A pituitary adenitis was due to neutrophilic infiltration of the pars nervosa and intermedia and adjacent meningitis. The remaining fatally affected calf (#6) had similar CNS lesions, but the inflammatory infiltrate was predominantly composed of intact leukocytes. The vascular lesions were also less severe in this animal than in the remaining five severely affected calves.

Of eight calves with mild and non-fatal disease, two animals, (#11,12) had no histological lesions. Two calves (#13,14) had a mild diffuse leptomeningitis (Fig. 3) and calf #9 additionally had mild leukocytic cuffing of some of the parenchymal blood vessels and mild inflammation of the choroid plexus. This animal had a severe leptomeningitis adjacent to the pituitary with visible bacterial colonies. Animal #7 had a moderate leptomeningitis of the brain stem and moderate vasculitis in the meninges, adjacent parenchyma and subependymal neuropil. Inflammation of the choroid plexus and pyencephalus was composed of intact leukocytes occasionally surrounding small bacterial colonies. This animal also had a fibrinopurulent pituitary adenitis.

The thalamic section of calf #13 and the cerebellomedullary section of calf #14 were not examined microscopically. Total histological lesion scores in the thalamic and cerebellomedullary sections of the 14 calves are presented graphically in Fig. 4.

Statistical study

Ten calves with no significant IgG_2 anti-*H. somnus* antibody titers were inoculated with two strains, five animals per strain. Group 1 (#15–19) were each inoculated with 2.5×10^5 CFU of strain 88159, which had been shown to be pathogenic. These all recovered well from the general anesthesia but subsequently all succumbed to a severe neurological disease and were euthanized when in lateral recumbency, and near death within 12 to 21 h after the challenge. All had a severe fibrinopurulent meningitis and the cultured CSF samples yielded heavy pure growth of *H. somnus*. The brain sections from these animals yielded large to low numbers of *H. somnus*, while the remaining organs sampled were bacteriologically negative. Histologically severe fibrinopurulent meningitis with a large proportion of necrotic leukocytes was associated with scattered bacterial colonies. The lesions of vasculitis, inflammation of the choroid plexus and pituitary adenitis were similar to those in severely affected calves examined previously.

Group 2 (#20–24) were inoculated with 1.2×10^6 CFU of *H. somnus* strain 87121531 each and showed only transient clinical signs of anorexia, apathy and elevation in body temperature. No animal in this group had any abnormal findings on gross necropsy examination when killed three days postinoculation. Numbers of *H. somnus* colonies ranging from three to 20 were

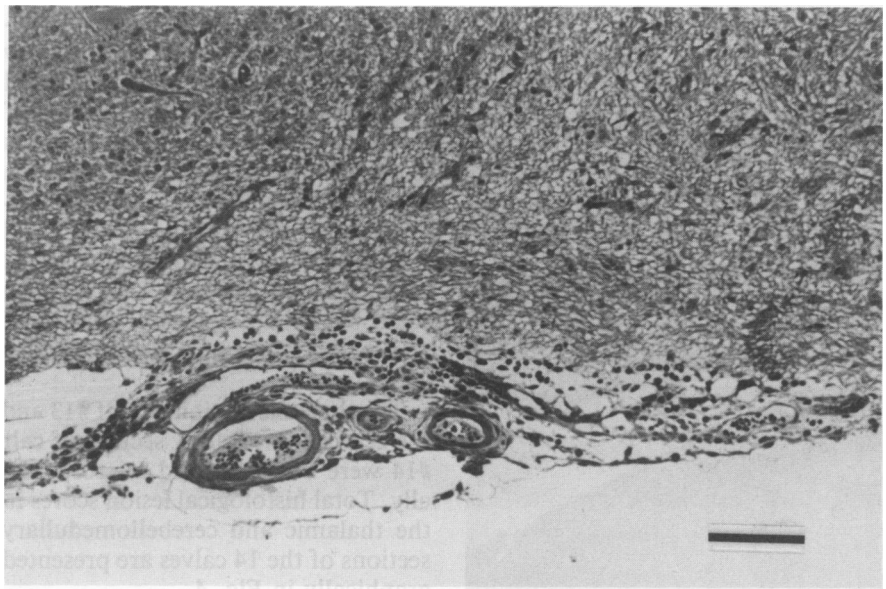

Fig. 3. Section of caudal medulla oblongata from calf #7 inoculated I/C with a nonpathogenic strain of *H. somnus* 87121531 and euthanized 95 h after the challenge, showing mild subacute meningitis with low numbers of mononuclear leukocytes scattered in the leptomeninges. H&E. Bar 100 μm.

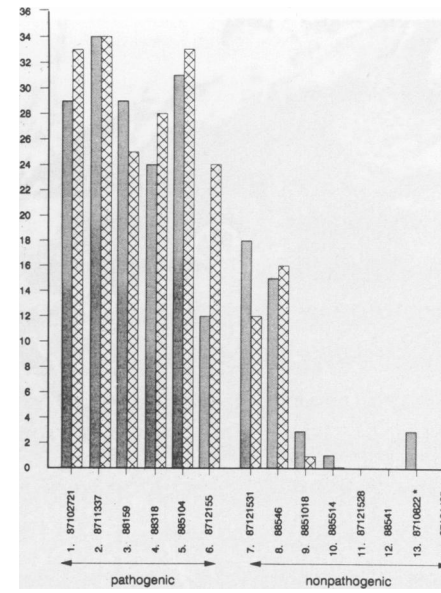

Fig. 4. Scoring of severity of histological lesions observed in the section of cerebellum and medulla and the section of thalamus following intracisternal inoculation of 14 genital strains of *H. somnus* into 14 calves.
solid bar — cerebellum and medulla, crossed bar — thalamus,
8710622* — only the section of cerebellum and medulla were examined and scored,
87101435† — only the section of thalamus was examined and scored.

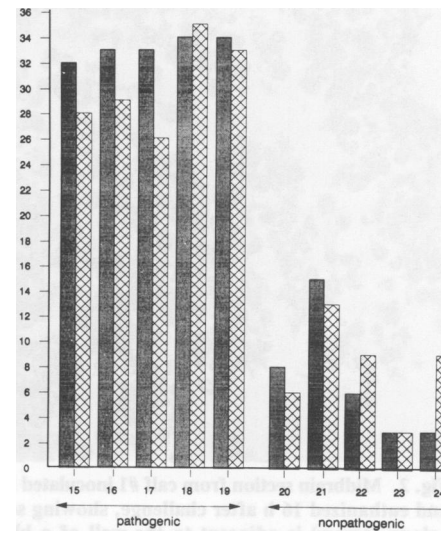

Fig. 5. Scoring of histological lesions in the thalamic region and in the region of the cerebellum and medulla of ten calves, following intracisternal inoculation of a pathogenic, 88159 or nonpathogenic, 87121531 strain of *H. somnus*. The scores for calves inoculated with the pathogenic strain were significantly higher than those for calves inoculated with the nonpathogenic strain, $p < 0.0001$.
Solid bar — cerebellum and medulla, crossed bar — thalamus.

isolated from the CSF of three animals (#20,21 and 24), whereas the CSF from the two remaining calves, (#22 and 23), and the remaining organs of all animals were negative on culture. Histopathological examination of the CNS revealed mild generalized leukocytic leptomeningitis and mild leukocytic cuffing of the parenchymal vasculature adjacent to the meninges or ependyma. There was mild inflammation of the choroid plexus and calf #24 had a fibropurulent coagulum in the fourth ventricle with intact and necrotic leukocytes. All calves of this group had severe suppurative pituitary adenitis and adjacent meningitis, and calf #22 had scattered bacterial colonies in the meningeal exudate.

The scores of histological lesions for the section of cerebellum/medulla and the section of thalamus from five calves from the first group were significantly higher than the scores for the corresponding sections from five calves from the second group ($p < 0.0001$ for both sections). Total scores of the histological lesions in both sections from both groups of calves are presented graphically in Fig. 5.

DISCUSSION

This study demonstrated the prevalence of pathogenic strains of *H. somnus* in the female bovine reproductive tract. No association between the pathogenicity of 14 isolates and the clinical status of the animal at the time of isolation could be made, since of six pathogenic strains three were recovered from grossly normal genital tracts and three from diseased genital tracts. Similarly, of eight nonpathogenic strains, four were recovered from normal and four from diseased reproductive tracts.

The I/C calf assay reliably differentiated one pathogenic strain, which caused a rapidly progressing fatal neurological disease and severe fibrinopurulent meningoencephalitis in six calves, from one nonpathogenic isolate which caused only a mild transient disease and mild to moderate, histological lesions. The remaining 12 isolates were tested in one animal each, and therefore can only presumptively be called "pathogenic" (five strains) or "nonpathogenic" (seven strains). Histological lesions produced in the CNS by these 14 strains indicated the absence of an all or none phenomenon regarding pathogenicity of genital isolates of *H. somnus*. In addition to a well defined group of isolates producing mild or no damage, and a group of strains producing severe lesions, there were also strains which fell into an intermediate group. One pathogenic and two nonpathogenic isolates produced lesions in the CNS of a similar moderate degree. Although histological lesions indicated a substantial inflammatory response to both

"nonpathogens", the mild course of clinical disease caused by these isolates showed this term to be useful.

Differences among individual animals used for the I/C testing of isolates in the initial stage of the study might have had an influence on their susceptibility to infection. *Haemophilus somnus* antibody titers were not examined in those 14 calves, but all of them were procured from one herd with no history of *H. somnus*-related disease and were raised in isolation prior to the experiments. Calves used in the statistically valid test originated from the same herd and had no significant IgG_2 anti-*H. somnus* antibody titers. Measurement of the IgG_2 class of antibody has been proposed as the most reliable method to assess serologically the status of prior infection with *H. somnus* in cattle (30).

The prevalence data obtained in live cows examined in field survey B (3.52%), was lower than that obtained in the abattoir ($p < 0.1$). Comparison of both data cannot be made in this study however, because in contrast to the centre B study, the origin of slaughtered cows was not determined.

The bacteriological results from both field surveys indicated that survey B provided reliable data on the prevalence of *H. somnus* in live cows. A guarded swabbing technique combined with KD-Columbia agar as a selective medium (survey B), was found to be superior to the technique of aspiration of genital mucus (survey A) for successful isolation of *H. somnus*. Guarded swabs had little contamination and the KD-Columbia agar was not suppressive for *H. somnus* but inhibited gram-positive organisms, particularly α-streptococci, the most important contaminant in survey B. The sampling of the anterior vagina and cervix was elected in the abattoir and survey B to avoid fecal contamination. Although the rate of isolation of *H. somnus* can be higher in the vestibular glands than in the anterior vagina and cervix (14,16), sampling of vestibular glands in live animals is difficult and prone to fecal contamination.

The I/C calf assay has been used previously by Humphrey (18) and Groom (19) to test virulence of isolates of *H. somnus* including those recovered from the male genital tract. Both authors were able to reliably and reproducibly differentiate pathogenic from nonpathogenic isolates. The pathogenicity of an *H. somnus* isolate in the I/C test corresponds to its potential to cause septicemia and lesions of TME after intravenous inoculation of calves as shown by Stephens *et al* (20,21). In the present study the ability of genital isolates of *H. somnus* to cause disease syndromes other than meningoencephalitis, i.e. arthritis, myocardial abscessation, pneumonia and inflammation of the genital tract, was not investigated. The other *in vivo* models used for testing the pathogenicity of strains of *H. somnus*, i.e. a calf subcutaneous chamber inoculation (19) and a pulmonary assay (31) have been used to a limited extent. Also testing of a limited number of strains has been done by the infusion of the bacterial suspension into the nongravid uterus or cervix, and infection of the pregnant uterus (reviewed in 32). Pathogenic strains tested in the intravenous septicemic challenge produced disease in 60–70% of animals and needed large numbers of calves for validation (20,21). The I/C calf assay was used in the present study because of its confirmed clearcut reliability to determine pathogenicity of *H. somnus* isolates and the relatively simple inoculation and examination procedure, contrary to most of the other *in vivo* assays.

Since pathogenic strains of *H. somnus* were found among genital isolates from the cow this provides an important link in understanding the epidemiology of the TME syndrome. The female bovine reproductive tract is a source of not only nonpathogenic but also pathogenic strains of *H. somnus*. As suggested before, orovaginal contact between cattle may lead to transient infection of the nasal passages and upper respiratory tract (25) and in suitable circumstances be followed by pneumonia and/or bacteremia with subsequent septicemia and TME. This work supports observations made by other workers using the I/C calf assay (18,19) that pathogenicity of *H. somnus* for the CNS is determined by individual strains.

The comparison of the prevalence of *H. somnus* in cows with genital disease and in healthy cows indicates, similarly to other studies (32), that *H. somnus* is a commensal well adapted to the genital mucosa, rarely causing a local inflammatory response.

It should now be determined whether there are pathogenic strains of *H. somnus* in the genital tract of the bull and in semen samples. Humphrey (18) and Groom (19) tested a limited number of male genital isolates in the I/C calf assay and found them to be nonpathogenic, but the testing of a large number of strains is required to clarify this issue. The development of an *in vitro* model for the reliable testing of large numbers of genital strains of *H. somnus* is an important requirement to replace the I/C calf assay which is costly and raises serious humane concerns. Such a putative *in vitro* test would be an important step for further studies on the epidemiology of the *H. somnus* disease complex and the screening of exported live cattle and semen samples for pathogenic isolates. The identification of important factors of bacterial virulence may also be accelerated by the use of such a test, and the findings could lead to the development of more effective vaccines.

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