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Characterization of a 78-Kilodalton Outer Membrane Protein of *Haemophilus somnus*

STEPHEN A. KANIA,[1,2]† RONALD P. GOGOLEWSKI,[2]‡ AND LYNETTE B. CORBEIL[1]*

Department of Pathology H811F, University of California San Diego Medical Center, 225 Dickinson Street, San Diego, California 92103,[1] and Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040[2]

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A 78-kilodalton (kDa) outer membrane protein (OMP) of *Haemophilus somnus* was one of the two antigens most consistently and most intensely immunoreactive in Western immunoblots of whole cells of *H. somnus* reacted with convalescent-phase serum obtained from cattle with experimental *H. somnus* pneumonia. This antigen was isolated by gel filtration chromatography of sodium dodecyl sulfate-solubilized OMP. Reactions of Western blots with bovine monospecific antiserum prepared against the 78-kDa antigen indicated that this 78-kDa OMP was present in each of 22 isolates of *H. somnus* obtained from cattle with pneumonia, thromboembolic meningoencephalitis, and abortion as well as from vaginal or preputial carriers. The 78-kDa OMP was also present in each isolate obtained weekly throughout the course of experimental *H. somnus* pneumonia in a calf. Monospecific antiserum to the 78-kDa OMP also reacted with proteins from closely related bacterial species in the family *Pasteurellaceae* but not with bacteria of 13 other genera. The 78-kDa OMP of *H. somnus* is of interest because it is surface accessible, highly conserved, immunogenic, cross-reactive with other members of the family *Pasteurellaceae*, and reactive with convalescent-phase serum which is passively protective against *H. somnus* pneumonia.

Haemophilus somnus is a gram-negative bacterium that causes several diseases in cattle, including pneumonia (1, 19, 25), abortion (11, 35, 47), septicemia (37), arthritis (37), and thromboembolic meningoencephalitis (TEME) (2, 25, 28). It also can be present in a nasal, preputial, or vaginal carrier state (24, 49). No biochemical or morphological differences have been identified to distinguish carrier strains from pathogenic strains (25), except that some carrier strains are serum sensitive and Fc receptor negative (53). Although the host-parasite relationship in *H. somnus* disease and in the asymptomatic carrier state must be different, little is understood about host defense against *H. somnus*. However, antibodies to *H. somnus* are likely to be important, because convalescent-phase serum from animals recovering from experimental *H. somnus* pneumonia passively protects calves against *H. somnus* challenge (17).

Diagnosis of *H. somnus* disease is commonly made by isolation of the organism (30) or by serology (27). Diagnosis by bacteriologic culture may be negative because it is not often possible to isolate *H. somnus* from cattle treated with antibiotics (25). Furthermore, the presence of *H. somnus* is not necessarily indicative of disease, because carriers are asymptomatic (24, 49). Therefore, interpretation of cultural results is difficult. Presently used serological assays such as the microagglutination test (16) appear not to be very specific for *H. somnus*. The specificity of an *H. somnus* serological assay might be improved by the use of a single antigen that is highly conserved among strains of *H. somnus* and that is not cross-reactive with the normal flora. Since many epidemiological studies are based on serological assays (38, 39), a more specific test should improve our knowledge of the incidence of *H. somnus* diseases as well as providing a more accurate means of diagnosis.

Currently, protection against *H. somnus* infection involves the use of killed whole-cell vaccines. However, epidemiological studies indicate that the ability of these vaccines to prevent pneumonia is questionable (33). The relative lack of protection attributed to killed whole-cell vaccines may be due to a poor immune response against the most protective antigens. A vaccine composed of only antigens which confer protection would eliminate irrelevant proteins and focus the immune response on antigens that are important in conferring protective immunity. Therefore, an appropriate *H. somnus* antigen may solve either or both of the problems of serological specificity in immunodiagnostic tests and less than optimal protection with killed whole-cell vaccines.

We chose to investigate a 78-kilodalton (kDa) outer membrane protein (OMP) of *H. somnus* for these applications because we had previously shown it to be in the outer membrane and accessible to antibody (17, 18). Also, convalescent-phase serum, which passively protects calves against experimental *H. somnus* pneumonia (17), reacted strongly with the 78-kDa OMP in Western immunoblots (17). In this communication we report a method for isolation of the 78-kDa antigen of *H. somnus*, production of monospecific bovine antiserum for antigen characterization, conservation of this antigen among isolates of *H. somnus*, and its absence in most other gram-negative species.

MATERIALS AND METHODS

Bacteria. Isolates of *H. somnus* described in Table 1 were frozen at −70°C in phosphate-buffered saline–glycerol (40:60) within two subcultures of original isolation, with the exception of strains received from other investigators. These included strains 42836, 91-1, and B83-145 (P. Little, Ontario Veterinary College, Guelph, Ontario, Canada) and strain 8025 (Washington Animal Disease Diagnostic Laboratory)

* Corresponding author.
† Present address: College of Veterinary Medicine, University of Florida, Gainesville, FL 32610.
‡ Present address: Central Veterinary Laboratory, Glenfield, New South Wales 2167, Australia.

TABLE 1. Characteristics of *H. somnus* isolates used for the study of the conservation of the 78-kDa antigen

Strain[a]	Disease	Serum killing[b]	Source	Sex[c]	Age	Region
43826	TEME	0.76	Brain	M/C	NA	Ontario, Canada
0289	TEME	0.15	Brain	NA[d]	10 wk	Washington
8025	TEME	0.79	NA	NA	NA	NA
91-1	TEME	0.08	NA	NA	NA	Ontario, Canada
109B	Pneumonia/TEME	0.04	Lung	M/C	9 mo	Washington
1542	Pneumonia	0.26	Lung	M	4 mo	Washington
2336	Pneumonia	0.47	Lung	M	5 wk	Washington
3581	Pneumonia	1.06	Lung	F	2 mo	Idaho
3415-2	Pneumonia	0.86	Lung	M/C	1 yr	Idaho
1297	Pneumonia	0.62	Lung	M/C	1 yr	California
41VC	Carrier	0.63	Vagina	F	Mature	Washington
202V	Carrier	0.64	Vagina	F	Mature	Idaho
208V	Carrier	0.51	Vagina	F	Mature	Idaho
221V	Carrier	1.16	Vagina	F	Mature	Idaho
1P	Carrier	1.93	Prepuce	M	Mature	Washington
24P	Carrier	3.21	Prepuce	M	Mature	Washington
127P	Carrier	2.14	Prepuce	M	Mature	Washington
129Pt	Carrier	2.11	Prepuce	M	Mature	Washington
1030	Abortion	0.24	Uterus	F	Mature	Washington
2069	Abortion	0.23	Cervix	F	2 yr	Idaho
570	Abortion	0.15	Cervix	F	3 yr	Washington
B83-145	Abortion	0.0	NA	F	Mature	Alberta, Canada

[a] References for strains: strain 43826 (44, 45); strain 8025 (7).
[b] Serum killing is expressed as the $\log_{10}$ reduction in colony counts after a 1-h incubation in fresh normal bovine serum as compared with heated serum (4).
[c] Abbreviations: M/C, castrated male; M, male; F, female.
[d] NA, Not available.

(originally from L. N. Brown [7]). *H. somnus* was plated from frozen stock onto Columbia blood agar plates (Difco Laboratories, Detroit, Mich.) containing 10% bovine blood (CBA) and incubated at 37°C in 10% CO_2. Broth cultures were prepared in brain heart infusion broth containing 0.1% Tris and 0.001% thiamine monophosphate (BHI-TT) as described by Inzana and Corbeil (26).

Other species of bacteria are described in Table 2. *Campylobacter fetus* subsp. *fetus* was plated on CBA, incubated in a microaerophilic environment (Campylobacter pack; Oxoid Ltd., Basingstoke, England), and then transferred to BHI containing 0.1% Tris (BHI-T). *Haemophilus influenzae* type b was grown on BHI agar with X and V factors in 10% CO_2 and transferred to BHI-T with X and V factors. All other bacteria were plated on CBA in 10% CO_2 and then grown in BHI-T broth. Serum susceptibility was determined as described previously (4), by using undiluted fresh normal bovine serum with autologous complement.

Preparation of *H. somnus* OMP. *H. somnus* 2336 was used because its virulence in *H. somnus* pneumonia has been established (19). It was recovered from a calf with *H. somnus* pneumonia and has been used to induce experimental pneumonia in calves (17–20). The first subculture of a single colony of *H. somnus* 2336 was used to inoculate BHI-TT. After 4 h in a 37°C shaking water bath, 25 ml of culture was inoculated into 1.5 liters of BHI-TT and incubated at 37°C for 16 h with shaking. Bacteria were centrifuged for 15 min at 12,000 × *g*, washed twice in 50 mM Tris (pH 7.8), and suspended at 100 mg (wet weight) per ml of 50 mM Tris–2 mM $MgCl_2$. Bacteria were disrupted in a French press at 15,000 lb/in^2 and then centrifuged for 15 min at 12,000 × *g*. The supernatant was centrifuged at 4°C for 70 min at 230,000 × *g* in a Ti 50.2 rotor. Membrane-associated proteins from each gram of bacteria were suspended in 10 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) containing 2% (wt/vol) *N*-lauroylsarcosine (Sigma Chemical Co., St. Louis, Mo.) and incubated for 20 min at room temperature. The samples were then centrifuged for 110 min at 125,000 × *g* at 4°C. The pellet was suspended in HEPES buffer, and total protein was determined as described by Bradford (5) (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, Calif.).

Solubilization of OMP. Preliminary experiments showed that 2% sodium dodecyl sulfate (SDS) completely solubilized

TABLE 2. Cross-reactivity of antibody to the *H. somnus* 78-kDa antigen in Western blots of related and unrelated gram-negative bacteria

Species	Isolate[a]	Cross-reacting antigen	
		Intensity[b]	Mol mass (kDa)
Moraxella bovis	ATCC 10900	0	–
Bordetella bronchiseptica	83-7931	0	–
Acinetobacter calcoaceticus	2273-140b	0	–
Haemophilus agni	R17	++	74
Campylobacter fetus subsp. *fetus*	ATCC 27374	0	–
Haemophilus influenzae type b	Eagan	+/−	76
Enterobacter cloacae	LRS	+/−	78
Citrobacter freundii	LRS	0	–
Proteus mirabilis	LRS	0	–
Leptospira interrogans	LRS	0	–
Pseudomonas aeruginosa	LRS	0	–
Brucella abortus	2308	0	–
Salmonella typhimurium	ATCC 14028	0	–
Klebsiella pneumoniae	ATCC 13883	0	–
Escherichia coli	JL9	0	–
Pasteurella multocida	0361	++	80
Pasteurella haemolytica	0156	++	84
Actinobacillus lignieresii	131	++	85
Actinobacillus equuli	1723-6	++	86

[a] Strain designations or LRS (laboratory reference strain).
[b] Relative staining of bands in Western blots with antiserum to the *H. somnus* 78-kDa OMP (1:4,000 dilution). Key: +/−; barely detectable reaction, +; faint reaction, ++ distinct reaction, +++ very intense staining of bands.

all detectable *H. somnus* OMP as determined in Western blots, whereas other detergents (Zwittergent [Calbiochem-Behring, La Jolla, Calif.], 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], and octylglucoside, all at 1% and at 0.1%) did not completely solubilize all of the OMP. Therefore, 5 mg of OMP was suspended in 7.5 ml of the solubilization buffer described by Hindennach and Henning (23) (2% SDS, 10 mM Tris [pH 7.5], 5 mM EDTA). The sample was incubated at 37°C for 30 min and centrifuged for 110 min at 125,000 × *g* at 18°C to remove insoluble material.

Column chromatography. Solubilized OMP in 2% SDS were applied to a column (75 by 1.6 cm) containing Sepharose 4B (Pharmacia, Uppsala, Sweden) in 1% SDS–10 mM Tris (pH 7.5)–5 mM EDTA. The column was operated with a hydrostatic pressure of 80 cm, and the protein elution profile was determined with a UV monitor at 280 nm. From each column fraction with detectable protein, a 250-μl sample was concentrated by lyophilization and suspended in 50 μl of water for electrophoresis. Relative molecular masses of eluted proteins were determined by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoreactivity of each protein by Western blotting against convalescent-phase serum as described below.

Preparation of bovine antiserum to the 78-kDa protein. Chromatographically enriched 78-kDa protein was extensively dialyzed against water. An 11-week-old calf (with no antibody to *H. somnus* detectable by Western blots) was immunized intramuscularly with approximately 200 μg of 78-kDa *H. somnus* antigen emulsified in incomplete Freund adjuvant. Identical inoculations were given at weeks 4 and 7. Serum obtained 7 days after the final inoculation was designated anti-78-kDa serum.

Sera and isolates from animals with experimental *H. somnus* disease. The preparation of convalescent-phase sera from animals with experimentally induced *H. somnus* pneumonia was described previously (17). In this experiment, *H. somnus* 2336 was doubly cloned by streaking one colony for isolation on two successive CBA plates to ensure that a genetically homogeneous population of organisms was used for infection. In each of five 12-week-old calves, 10^7 CFU of *H. somnus* 2336 was inoculated intrabronchially into a caudal lobe. Mild chronic pneumonia resulted. Bronchial lavage samples were culture negative for *H. somnus* before inoculation, and weekly lavage samples were culture positive for 6 to 10 weeks. *H. somnus* isolates from weekly lavage samples were stored at −70°C as described above. Convalescent-phase serum used in this study was collected at week 6 after inoculation.

Serum P4 was collected from a cow which had been inoculated intrabronchially with 10^{10} live *H. somnus* 649 organisms in an experimental abortion study (55). During the convalescent phase, her immune response was boosted by three biweekly immunizations with subcutaneous injections of 10^{10} formalinized *H. somnus* 649 organisms in Freund incomplete adjuvant (55).

SDS-PAGE. Samples for electrophoresis consisted of Sepharose 4B column fractions, bacteria, OMP, or the soluble fraction of culture supernatant. Bacteria grown in broth culture to 40% light transmittance at 610 nm were washed three times in phosphate-buffered saline and used at approximately 10^8 bacteria per lane. All samples were boiled in SDS sample buffer (31) for 2 min prior to SDS-PAGE. Samples were electrophoresed in slab gels with a 5% stacking gel and a 7.5 to 17.5% gradient or 10% resolving gel, using the discontinuous buffer system of Laemmli (31). Proteins were stained with Coomassie blue or transferred to nitrocellulose for Western blots.

Western blots. For Western blots, proteins were transferred electrophoretically to nitrocellulose paper in a Trans-Blot cell (Bio-Rad) at 30 V for 16 h followed by 70 V for 1 h by using the transfer buffer system of Towbin et al. (46). Convalescent-phase bovine serum collected 6 weeks after inoculation (as described above) was diluted in Tris-buffered saline (20 mM Tris, 500 mM NaCl; pH 7.5) containing 0.05% Tween 20 and incubated with immobilized antigen for 2 h. The blots were washed, and peroxidase-conjugated anti-bovine immunoglobulin G (IgG) (heavy and light chains) (ICN Immunobiologicals, Lisle, Ill.) diluted 1:2,000 in Tris-buffered saline–0.05% Tween 20 was added for 1 h. After washing, antigen-antibody complexes were stained with 4-chloro-1-naphthol and H_2O_2 (17). Nonimmunogenic proteins were detected by overstaining selected blots with 0.1% amido black.

Anti-78-kDa serum was used to probe a panel of *H. somnus* isolates from cattle with TEME, abortion, or pneumonia and from asymptomatic preputial or vaginal carriers, as well as isolates obtained from weekly bronchial lavage samples from a calf with experimental pneumonia. Other gram-negative bacteria, including bovine pathogens and normal flora, were also tested for reactivity with antibody to *H. somnus* 78-kDa antigen. Western blots were incubated with monospecific antisera diluted 1:4,000, unless otherwise stated, and processed as above.

RESULTS

Isolation of the 78-kDa protein. SDS solubilized all of the major OMP of *H. somnus*, as determined by Western blot analysis of pellet and supernatant fractions following ultracentrifugation. A 78-kDa protein was the first of the SDS-solubilized OMP to elute from the sizing column. Coomassie stained SDS-polyacrylamide gels of several fractions showed a single band with an apparent molecular mass of 78 kDa (data not shown). Western blot analysis showed that the pooled column fractions of the 78-kDa protein contained only the 78-kDa antigen as detected by reaction with convalescent-phase serum and amido black overstain (Fig. 1). In the lanes containing whole solubilized *H. somnus* and OMP, the 78-kDa protein is not the most abundant protein as detected by amido black overstain, even though this 78-kDa protein is one of the two OMP staining most intensely in immunoblots of whole *H. somnus* or OMP with convalescent-phase serum. In lanes containing the purified 78-kDa antigen, no other proteins were detected by amido black staining.

The 78-kDa antigen detected by sera from five animals convalescent from *H. somnus* pneumonia is different from the 76-kDa antigen which we previously demonstrated to be a predominant antigen recognized by sera from 18 animals convalescent from *H. somnus* abortion (13). Both the 76- and 78-kDa antigens can be detected in Western blots of whole solubilized *H. somnus* 2336 or 649 cells (Fig. 2). The 78-kDa antigen reacted strongly with convalescent-phase serum obtained from animals with pneumonia (calves E7 and E5), whereas only faint reactivity with the 76-kDa antigen was detected. However, the 76-kDa antigen and not the 78-kDa antigen reacted with convalescent-phase immune serum obtained from an animal (calf P4) in an experimental abortion study. Serum from animals with pneumonia (calves E5 and E7) detected only the 78-kDa antigen in pooled column fractions of 78-kDa antigen (Fig. 2). Serum (from cow P4)

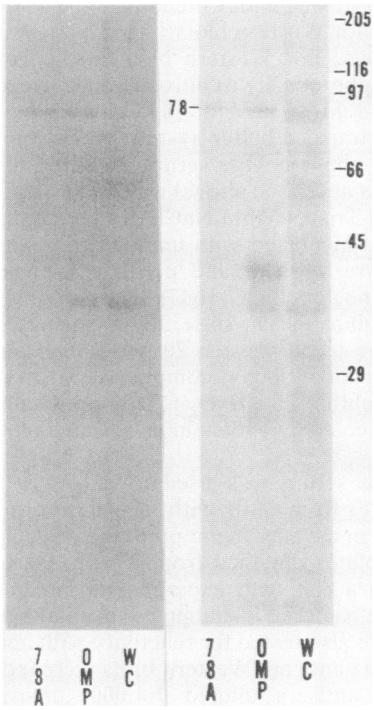

FIG. 1. Western blot of convalescent-phase serum (from calf E5, at 1:1,500) against the 78-kDa antigen (78A), *H. somnus* outer membrane-enriched fractions (OMP), and whole *H. somnus* cells (WC) solubilized in SDS sample buffer. The panel on the left is stained with convalescent-phase serum plus peroxidase-labeled antibody and substrate, whereas the panel on the right is also overstained with amido black to detect proteins not reactive with antibody.

which recognized the 76-kDa antigen but not the 78-kDa antigen in blots of whole solubilized *H. somnus* cells also did not react with the purified 78-kDa antigen (Fig. 2). Controls incubated with preimmunization serum or conjugate alone were negative.

Specificity of bovine antiserum to isolated 78-kDa antigen. To determine whether bovine antisera to 78-kDa antigen reacted with other *H. somnus* antigenic determinants, we solubilized whole *H. somnus* bacteria by boiling them in SDS sample buffer and separated antigens by SDS-PAGE. Serum from a calf immunized with pooled fractions of 78-kDa antigen was compared with convalescent-phase serum obtained from calf E5 in Western blots (Fig. 3). Although convalescent-phase serum reacted with many antigens in the whole bacterial preparation, the antiserum to the 78-kDa antigen reacted essentially only with the 78-kDa antigen. Serum obtained prior to immunization with the 78-kDa antigen did not react with 78-kDa antigen even at a serum dilution of 1:1,000 (the lowest dilution tested). However, after the second immunization, activity with the 78-kDa antigen was readily detectable with serum diluted at 1:4,000. After the third immunization, serum reactivity in Western blots was still readily detected at 1:8,000. At a 1:2,000 dilution, serum collected after the third immunization reacted more intensely with the 78-kDa antigen than did convalescent-phase serum at a 1:1,500 dilution (Fig. 3).

Conservation of the 78-kDa protein. The monospecific anti-78-kDa serum reacted with approximately equal intensity against the 78-kDa antigen in Western blots of all isolates obtained weekly during the course of experimental pneumonia, as well as in Western blots of the doubly cloned inoculum strain. Western blots of 22 whole solubilized *H. somnus* isolates, representing five disease or carrier groups (Table 1), showed one protein in each preparation which

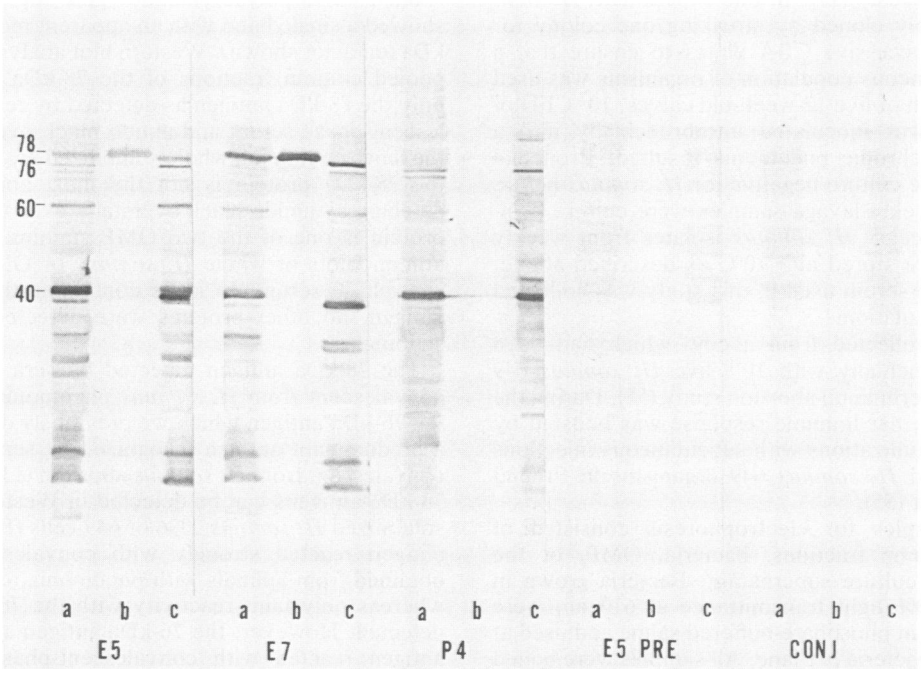

FIG. 2. Western blot with immune serum (from animals E5, E7, and P4) or controls (preimmunization serum from animal E5 [E5 pre] or conjugate alone [CONJ]) reacted against whole solubilized cells of *H. somnus* 2336 (lanes a), purified 78-kDa OMP from *H. somnus* 2336 (lanes b), or whole solubilized cells of *H. somnus* 649 (lanes c). Serum was diluted 1:1,500. Apparent molecular masses in kilodaltons are on the left.

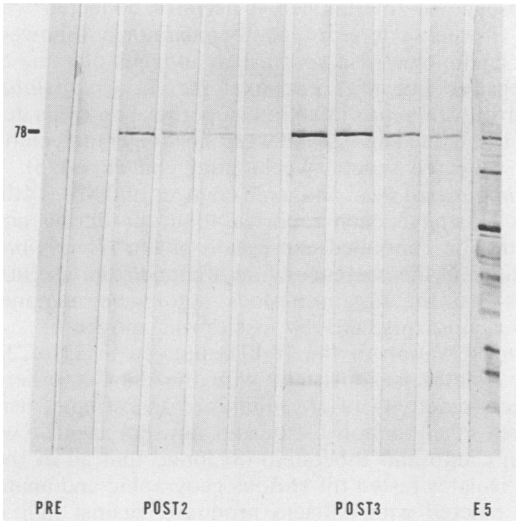

FIG. 3. Western blot of solubilized whole cells of *H. somnus* 2336 reacted with serum from a calf immunized with purified 78-kDa antigen in incomplete Freund adjuvant or with convalescent-phase serum (lane E5). Lanes: PRE, preimmunization serum at a 1:1,000 dilution; POST2, serum collected after the second immunization at dilutions of 1:1,000, 1:2,000, 1:4,000, and 1:8,000 (from left to right); POST 3, serum collected after the third immunization at dilutions of 1:1,000, 1:2,000, 1:4,000, and 1:8,000 (from left to right); E5, convalescent-phase serum from calf E5, at a dilution of 1:1,500.

reacted with anti-78-kDa serum with similar intensity (Fig. 4). Three of the four vaginal isolates appear to have immunoreactive proteins with slightly higher apparent molecular masses than those of the other 19 isolates, which had apparent molecular masses of 78 kDa (Fig. 4). No differences were detected between serum-sensitive and serum-resistant isolates.

Cross-reactivity with other bacterial species. Serum from a calf convalescent from *H. somnus* pneumonia reacted in Western blots with many antigens of each of the members of the family *Pasteurellaceae* tested (data not shown). Monospecific anti-78-kDa serum reacted not only with *H. somnus* but also with single proteins of *Pasteurella multocida*, *Pasteurella haemolytica*, *Haemophilus agni*, *Actinobacillus lignieresii*, and *Actinobacillus equuli*. A much weaker reaction occurred against single proteins of *Enterobacter cloacae* and *Haemophilus influenzae* type b. These immunoreactive proteins ranged in molecular mass from approximately 74 to 86 kDa (Table 2). Like *H. somnus*, *P. multocida* and *P. haemolytica* cause pneumonia (10) and, occasionally, reproductive failure (48) in cattle. Therefore, several isolates of these species from cattle with pneumonia or reproductive failure and from bovine normal flora were examined (Table 3). These included both serum-sensitive and serum-resistant isolates (Table 3). All 11 isolates of *P. haemolytica* tested had a cross-reactive protein of approximately 84 kDa, and all 10 isolates of *P. multocida* tested had a cross-reactive protein of approximately 80 kDa. Of the other 11 gram-negative species and 3 gram-positive species tested, no proteins reacted with the antisera at intensities above background (Table 2).

DISCUSSION

In this study, we isolated the 78-kDa antigen of *H. somnus* by gel filtration chromatography of SDS-solubilized, outer membrane-enriched fractions of *H. somnus* 2336 (17–19). This protein appeared as a single band with an apparent molecular mass of 78 kDa in Coomassie blue-stained SDS-polyacrylamide gels and in Western blots with convalescent-phase serum. Serum from a calf immunized with purified 78-kDa antigen reacted with a single band in Western blots

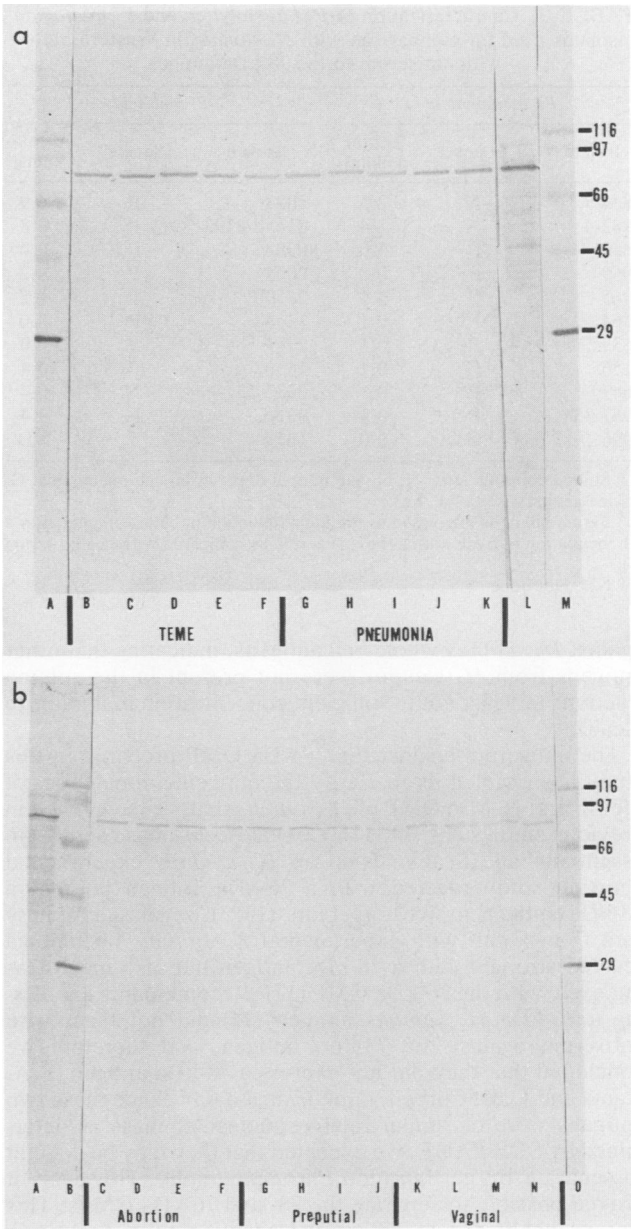

FIG. 4. Western blots of disease and carrier isolates of *H. somnus* with bovine anti-78-kDa serum at a dilution of 1:4,000. (a) TEME and pneumonia isolates. Lanes: A and M, protein standards; B, isolate 43826; C, isolate 0289; D, isolate 8025; E, isolate 109B; F, isolate 91-1; G, isolate 1542; H, isolate 2336; I, isolate 3581; J, isolate 3415-2; K, isolate 1297; L, isolate 2336 (control). Lanes A, L, and M were overstained with 0.1% amido black. (b) Abortion, preputial, and vaginal isolates. Lanes: A, isolate 2336 (control); B and O, protein standards; C, isolate 41VC; D, isolate 202V; E, isolate 208V; F, isolate 221V; G, isolate IP; H, isolate 24P; I, isolate 127P; J, isolate 129Pt; K, isolate 1030; L, isolate 2069; M, isolate 0570; N, isolate 83-145. Lanes A, B, and O were overstained with 0.1% amido black.

TABLE 3. Characterization of *P. haemolytica* and *P. multocida* isolates used for comparison with *H. somnus* in Western blots with antiserum to the 78-kDa antigen

P. haemolytica			*P. multocida*		
Isolate	Source[a]	Serum killing[b]	Isolate	Source[a]	Serum killing[b]
0834-7	N	2.5	2881	P	0.9
0982-4	N	2.4	0145b 3192	P	0.8
1846	P	3.0	0982	P	0.4
0069	P	0.2	0004	P	0.2
2065	P	0.1	2850b	P	0.2
1564	P	0.1	1308	P	0.0
1639	P	0.1	2743	P	0.0
0156	G	0.1	1630	P	0.4
1647	P	0.1	0982-2	N	0.4
1967-334-1	P	0.1	93NC	N	3.4
1096	G	0.0	1059	T	ND[c]

[a] Source abbreviations: N, bovine normal flora; P, bovine pneumonia; G, bovine genital disease; T, turkey.

[b] Serum killing is expressed as the $\log_{10}$ reduction in colony counts after a 1-h incubation in fresh normal bovine serum as compared with heated serum (4).

[c] ND, Not done.

against *H. somnus* whole-cell antigens, indicating that other antigens from *H. somnus* were not present in the column fractions (at least not in sufficient concentration to immunize a calf).

The antiserum against the 78-kDa OMP prepared in this study has enabled us to clarify the antigenic nonidentity of the 78- and 76-kDa OMP of *H. somnus* that we have noted in previous studies (13, 14, 17). Convalescent-phase sera from essentially all 18 animals in an *H. somnus* experimental abortion study reacted with a 76-kDa antigen but not a 78-kDa antigen in Western blots (13). Convalescent-phase sera from a calf with experimental *H. somnus* pneumonia reacted strongly with a 78-kDa antigen but also much less intensely with the 76-kDa OMP (17). Recombinants expressing a 76-kDa *H. somnus* antigen (14) did not react with antiserum against the 78-kDa antigen, and therefore we concluded that they did not express a 78-kDa antigen (S.A. Kania and L. B. Corbeil, unpublished data). Since these two antigens were of similar relative molecular mass as determined by SDS-PAGE, we expected that they may be difficult to separate by gel filtration chromatography. However, it proved possible to separate the 78- and 76-kDa OMPs. This was probably due to differential partitioning of these two antigens in broth cultures. The 76-kDa antigen is found predominantly in the soluble culture supernatant, and the 78-kDa antigen is found in the sarcosyl-insoluble outer membrane fraction of *H. somnus* (Kania and Corbeil, unpublished). Interestingly, in our studies, reactivity to the 76-kDa protein was detected primarily in serum from animals convalescent from experimental abortion (13) and reactivity to the 78-kDa antigen was seen primarily in serum from animals with experimental pneumonia (17). Although the reason for this difference is not known, the fact that experimental abortion was preceded by a septicemic phase (13), whereas blood cultures were not positive in calves with experimental pneumonia (19), suggests that differences in the ability of *H. somnus* to invade may be related to the differences in humoral immune responses to these two antigens.

The 78-kDa OMP was conserved in *H. somnus* isolates collected throughout a 10-week course of infection as well as in 22 of 22 *H. somnus* isolates obtained from many sources. The presence of this antigen in isolates collected weekly over a 10-week course of acute pneumonia followed by chronic pneumonia is in contrast to antigens of some other gram-negative bacteria. For example, in *Campylobacter fetus* subsp. *venerealis* infection, superficial antigens detectable in the cloned inoculum were no longer detectable in isolates collected several weeks after challenge (15). With *Neisseria gonorrhoeae*, antigenic changes of OMP II (40, 56) and pili (22) have been reported to occur during natural infection. The continued expression of the *H. somnus* 78-kDa antigen in the presence of an immune response in vivo suggests that the organism does not evade immune responses against this OMP by loss of this antigen.

The conservation of the 78-kDa antigen in 22 of 22 *H. somnus* isolates is consistent with the data of others on serological reactivity of *H. somnus*. For example, using a whole-cell agglutination assay and antisera against whole bacteria, Canto and Biberstein (8) found that all of the *H. somnus* isolates tested (of various geographic and anatomic origins) reacted with antisera produced against individual isolates. Analysis of antibody reactivity after adsorption of the antisera with heterologous isolates demonstrated antigenic heterogeneity among *H. somnus* isolates. Agglutination of every *H. somnus* strain by each antiserum indicates that there are likely to be antigens common to all strains of *H. somnus* in addition to heterogeneous antigens. However, agglutination and adsorption techniques do not allow the identification of specific antigens that are common and/or heterogeneous among isolates. This problem is solved in our Western blot analysis, since we show that the 78-kDa antigen is highly conserved among *H. somnus* isolates from carriers or animals with *H. somnus* disease. The presence of the 78-kDa antigen in isolates from diverse regions (including two Canadian provinces and several locations in the western United States) shows that it is conserved in isolates from a wide geographic area as well as in isolates from different syndromes. The lack of association of the 78-kDa antigen with susceptibility to killing by fresh normal bovine serum contrasts with our with our studies with a 270-kDa OMP of *H. somnus* (53). However, since this normal bovine serum did not contain specific antibodies to the 78-kDa OMP, our studies do not imply that antibody plus complement would not kill the organism.

The cross-reactivity of the 78-kDa antigen with antigens of other members of the family *Pasteurellaceae* is consistent with data showing the close relationship among these bacteria. Canto et al. demonstrated that antiserum produced against *H. somnus* cross-reacted with other bacterial species in whole-cell serologic assays (9). Also, *H. somnus* has been shown to be genotypically (21) and phenotypically (42) similar to other members of the family *Pasteurellaceae*. Although a leukotoxin is an important virulence factor and protective immunogen in disease caused by *P. haemolytica* (41), somatic antigens are also important in protection (12, 41). Therefore, the somatic antigen which is cross-reactive between *H. somnus* and the pasteurellas may be worth studying for cross-protection.

Since the 78-kDa OMP is a conserved antigen in *H. somnus*, is surface exposed, and is recognized by protective convalescent-phase serum (17), its protective capacity against *H. somnus* infection may merit investigation. OMP antigens of some other gram-negative bacteria (3, 6, 29, 32) are protective. Furthermore, Stephens et al. (44, 45) elicited protection against bovine TEME with membrane extracts of *H. somnus*. Although this preparation contained several antigens, the studies provided evidence that *H. somnus*

membranes can protect against disease. Furthermore, *H. somnus* OMP may be more relevant in protection than outer membranes of some other gram-negative bacteria, because *H. somnus* does not appear to have a capsule or pili (24, 43). In a parallel study (18), we tested protection by the antiserum to the 78-kDa OMP used to characterize the isolates in the present study. This antiserum did not protect calves against experimental pneumonia (18). However, the antiserum tested contained predominantly IgG1 antibodies (18). Others have shown that IgG2 is important in protection against pyogenic infections in cattle (36, 50–52), and our previous experiments with *H. somnus* infection indicate that IgG2 antibodies are likely to be important in protection against bovine infection with *H. somnus* also (17, 18, 54, 55). This may be because bovine IgG2 is more opsonic than bovine IgG1 (34). In a separate study of chronic pneumonia (20), we showed that *H. somnus* remains in the bronchoalveolar fluid as long as IgG1 and IgA antibody titers predominate but that the bacteria are cleared as these isotypic antibody titers decrease and IgG2 titers increase. These studies suggest that it is important to investigate protection against *H. somnus* pneumonia by IgG2 antibodies to the 78-kDa antigen.

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LITERATURE CITED

1. **Andrews, J. J., T. D. Anderson, L. N. Slife, and G. W. Stevenson.** 1985. Microscopic lesions associated with the isolation of *Haemophilus somnus* from pneumonia lungs. Vet. Pathol. **22**:131–136.
2. **Bailie, W. E., H. D. Anthony, and K. D. Weide.** 1966. Infectious thromboembolic meningoencephalitis (sleeper syndrome) in feedlot cattle. J. Am. Vet. Med. Assoc. **148**:162–166.
3. **Barenkamp, S. J.** 1986. Protection by serum antibodies in experimental nontypable *Haemophilus influenzae* otitis media. Infect. Immun. **52**:572–578.
4. **Blau, K. A., A. C. S. Ward, D. J. Prieur, and L. B. Corbeil.** 1987. Serum susceptibility of bovine Pasteurella. Can. J. Vet. Res. **51**:157–161.
5. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Brodeur, B. R., Y. Larose, P. Tsang, J. Hamel, F. Ashton, and A. Ryan.** 1985. Protection against infection with *Neisseria meningitidis* group B serotype 2b by passive immunization with serotype-specific monoclonal antibody. Infect. Immun. **50**:510–516.
7. **Brown, L. N., C. Reggiardo, M. W. Khan, P. G. Eness, and H. L. Self.** 1972. Application of the complement fixation test to the study of *Haemophilus somnus* infections of cattle, p. 502–508. *In* Proceedings of the 76th Annual Meeting of the U.S. Animal Health Association.
8. **Canto, G. J., and E. L. Biberstein.** 1982. Serological diversity in *Haemophilus somnus*. J. Clin. Microbiol. **15**:1009–1015.
9. **Canto, J., E. L. Biberstein, T. A. Schulte, and D. Behymer.** 1983. Cross-reactivity of *Haemophilus somnus* antibody in agglutination and complement fixation tests and in the enzyme-linked immunosorbent assay. J. Clin. Microbiol. **17**:500–506.
10. **Carter, G. R.** 1967. Pasteurellosis: *Pasteurella multocida* and *Pasteurella hemolytica*. Adv. Vet. Sci. **11**:321–379.
11. **Chladek, D. W.** 1975. Bovine abortion associated with *Haemophilus somnus*. Am. J. Vet. Res. **36**:1041.
12. **Confer, A. W., R. J. Panciera, M. J. Gentry, and R. W. Fulton.** 1987. Immunologic response to *Pasteurella haemolytica* and resistance against experimental bovine pneumonic pasteurellosis, induced by bacterins in oil adjuvants. Am. J. Vet. Res. **48**:163–168.
13. **Corbeil, L. B., J. E. Arthur, P. R. Widders, J. W. Smith, and A. F. Barbet.** 1987. Antigenic specificity of convalescent serum from cattle with *Haemophilus somnus* induced experimental abortion. Infect. Immun. **55**:1381–1386.
14. **Corbeil, L. B., G. Chikami, M. Yarnall, J. Smith, and D. G. Guiney.** 1988. Cloning and expression of genes encoding *Haemophilus somnus* antigens. Infect. Immun. **56**:2736–2742.
15. **Corbeil, L. B., G. G. D. Schurig, P. J. Bier, and A. J. Winter.** 1975. Bovine venereal vibriosis: antigenic variation of the bacterium during infection. Infect. Immun. **11**:240–244.
16. **Corbeil, L. B., P. R. Widders, R. Gogolewski, J. Arthur, T. J. Inzana, and A. C. S. Ward.** 1986. *Haemophilus somnus*: bovine reproductive and respiratory disease. Can. Vet. J. **27**:90–93.
17. **Gogolewski, R. P., S. A. Kania, T. J. Inzana, P. R. Widders, J. D. Liggitt, and L. B. Corbeil.** 1987. Protective ability and specificity of convalescent serum from calves with *Haemophilus somnus* pneumonia. Infect. Immun. **55**:1403–1411.
18. **Gogolewski, R. P., S. A. Kania, H. D. Liggitt, and L. B. Corbeil.** 1988. Protective ability of antibodies against 78- and 40-kilodalton outer membrane antigens of *Haemophilus somnus*. Infect. Immun. **56**:2307–2316.
19. **Gogolewski, R. P., C. W. Leathers, H. D. Liggitt, and L. B. Corbeil.** 1987. Experimental *Haemophilus somnus* pneumonia in calves and immunoperoxidase localization of bacteria. Vet. Pathol. **24**:250–256.
20. **Gogolewski, R. P., D. C. Schaefer, S. K. Wasson, R. R. Corbeil, and L. B. Corbeil.** 1989. Pulmonary persistence of *Haemophilus somnus* in the presence of specific antibody. Infect. Immun. **27**:1767–1774.
21. **Gonzales, H. F., and D. P. Bingham.** 1983. Genetic relatedness of *Haemophilus somnus* to select genera of bacteria. Am. J. Vet. Res. **44**:193–195.
22. **Hagblom, P., E. Segal, E. Billyard, and M. So.** 1985. Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. Nature (London) **315**:156–158.
23. **Hindennach, I., and U. Henning.** 1975. The major proteins of the *Escherichia coli* outer cell envelope membrane. Eur. J. Biochem. **59**:207–213.
24. **Humphrey, J. D., P. B. Little, L. R. Stephens, D. A. Barnum, P. A. Doig, and J. Thorsen.** 1982. Prevalence and distribution of *Haemophilus somnus* in the male bovine reproduction tract. Am. J. Vet. Res. **43**:791–794.
25. **Humphrey, J. D., and L. R. Stephens.** 1983. *Haemophilus somnus*: a review. Vet. Bull. **53**:987–1004.
26. **Inzana, T. J., and L. B. Corbeil.** 1987. Development of a defined medium for *Haemophilus somnus* isolated from cattle. Am. J. Vet. Res. **48**:366–369.
27. **Keister, D. M.** 1981. *Haemophilus somnus* infections in cattle. Comp. Cont. Educ. **3**:S260–S264.
28. **Kennedy, F. L., E. L. Biberstein, J. A. Howarth, L. M. Frazier, and D. L. Dungworth.** 1960. Infectious meningoencephalitis in cattle, caused by a haemophilus-like organism. Am. J. Vet. Res. **21**:403–409.
29. **Kimura, A., P. A. Gulig, G. H. McCracken, Jr., T. A. Loftus, and E. J. Hansen.** 1985. A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. Infect. Immun. **47**:253–259.
30. **Krogh, H. V., K. B. Pedersen, and E. Blom.** 1983. *Haemophilus somnus* in semen from Danish bulls. Vet. Rec. **112**:460.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
32. **Loeb, M. R.** 1987. Protection of infant rats from *Haemophilus influenzae* type b infection by antiserum to purified outer membrane protein a. Infect. Immun. **55**:2612–2618.
33. **Martin, W., S. Acres, R. Janzen, P. Willson, and B. Allen.** 1984. A field trial of preshipment vaccination of calves. Can. Vet. J. **25**:145–147.
34. **McGuire, T. C., and A. J. Musoke.** 1981. Biologic activities of bovine IgG subclasses. Adv. Exp. Med. Biol. **137**:359–366.
35. **Miller, R. B., D. H. Lein, K. E. McEntee, C. E. Hall, and S.**

Shin. 1983. *Haemophilus somnus* infection of the reproductive tract of cattle: a review. J. Am. Vet. Med. Assoc. **182**:1390–1392.

36. **Nansen, P.** 1972. Selective immunoglobulin deficiency in cattle and susceptibility to infection. Acta Pathol. Microbiol. Scand. Sect. B **80**:49–54.
37. **Pritchard, D. G., J. Shreeve, and R. Bradley.** 1979. The experimental infection of calves with a British strain of *Haemophilus somnus*. Res. Vet. Sci. **26**:7–11.
38. **Sanfacon, D., and R. Higgins.** 1983. Epidemiology of *Haemophilus somnus* infection in dairy cattle in Quebec. Can. J. Comp. Med. **47**:456–459.
39. **Sanfacon, D., R. Higgins, K. R. Mittal, and G. L'Archeuque.** 1983. *Haemophilus somnus*: a comparison among three serological tests and a serological survey in beef and dairy cattle. Can. J. Comp. Med. **47**:304–308.
40. **Schwalbe, R. S., P. F. Sparling, and J. G. Cannon.** 1985. Variation of *Neisseria gonorrhoeae* protein II among isolates for an outbreak caused by a single gonococcal strain. Infect. Immun. **49**:250–252.
41. **Shewen, P. E., and B. N. Wilkie.** 1988. Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. Can. J. Vet. Res. **52**:30–36.
42. **Stephens, L. R., J. D. Humphrey, P. B. Little, and D. A. Barnum.** 1983. Morphological, biochemical, antigenic, and cytochemical relationships among *Haemophilus somnus*, *Haemophilus haemoglobinophilus*, *Histophilus ovis*, and *Actinobacillus seminis*. J. Clin. Microbiol. **17**:728–737.
43. **Stephens, L. R., and P. B. Little.** 1981. Ultrastructure of *Haemophilus somnus*, causative agent of bovine infectious thromboembolic meningoencephalitis. Am. J. Vet. Res. **42**:1638–1640.
44. **Stephens, L. R., P. B. Little, B. N. Wilkie, and D. A. Barnum.** 1981. Humoral immunity in experimental thromboembolic meningoencephalitis in cattle caused by *Haemophilus somnus*. Am. J. Vet. Res. **42**:468–474.
45. **Stephens, L. R., P. B. Little, B. N. Wilkie, and D. A. Barnum.** 1984. Isolation of *Haemophilus somnus* antigens and their use as vaccines for prevention of bovine thromboembolic meningoencephalitis. Am. J. Vet. Res. **45**:234–239.
46. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
47. **van Dreumel, A. A., and M. Kierstead.** 1975. Abortion associated with *Haemophilus somnus* infection in a bovine fetus. Can. Vet. J. **16**:367–370.
48. **Ward, A. C. S., L. B. Corbeil, and J. F. Everman.** 1985. Pasteurellosis—a fatal link in bovine abortions? Vet. Med. **80**:118–122.
49. **Ward, A. C. S., L. B. Corbeil, W. D. Mickelsen, and V. F. Sweet.** 1983. A selective medium for Gram-negative pathogens from bovine respiratory and reproductive tracts. Proc. Am. Assoc. Vet. Lab. Diagn. **26**:103–112.
50. **Watson, D. L.** 1981. Immunologically-specific resistance to infection with particular reference to staphylococcal mastitis. Adv. Exp. Biol. Med. **137**:579–590.
51. **Watson, D. L.** 1984. Evaluation of attenuated, live staphylococcal mastitis vaccine in lactating heifers. J. Dairy Sci. **67**:2608–2613.
52. **Watson, D. L.** 1987. Serological responses of sheep to live and killed *Staphylococcus aureus* vaccines. Vaccine **5**:275–278.
53. **Widders, P. R., L. Dorrance, M. Yarnall, and L. B. Corbeil.** 1989. Immunoglobulin binding activity among pathogenic and carrier isolates of *Haemophilus somnus*. Infect. Immun. **57**:639–642.
54. **Widders, P. R., S. C. Dowling, R. P. Gogolewski, J. W. Smith, and L. B. Corbeil.** 1989. Isotypic antibody responses in cattle infected with *Haemophilus somnus*. Res. Vet. Sci. **46**:212–217.
55. **Widders, P. R., L. G. Paisley, R. P. Gogolewski, J. F. Evermann, J. W. Smith, and L. B. Corbeil.** 1986. Experimental abortion and the systemic immune response to "*Haemophilus somnus*" in cattle. Infect. Immun. **54**:555–560.
56. **Zak, K., J. L. Diaz, D. Jackson, and J. E. Heckels.** 1984. Antigenic variation during infection with *Neisseria gonorrhoeae*: detection of antibodies to surface proteins in sera of patients with gonorrhea. J. Infect. Dis. **149**:166–173.