Detection of Antibodies to the Biofilm Exopolysaccharide of *Histophilus somni* following Infection in Cattle by Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay (ELISA) was developed to detect bovine antibodies to *Histophilus somni* exopolysaccharide (EPS), which is created during biofilm formation. When an index value of 0.268 was used, the sensitivity of the assay for infected calves was 90.5% at 3 weeks postinfection, but the number of positive animals increased by week 4. The specificity of the assay for healthy calves was 92.5%. The EPS ELISA may aid in identifying calves with *H. somni* diseases.

*H*istophilus somni is associated with respiratory and systemic diseases in cattle and sheep. Although *H. somni* is commonly present in the genital and respiratory tracts, it is capable of causing diseases such as thrombotic meningoencephalitis, pneumonia, bacteremia, abortion, arthritis, myocarditis, mastitis, and infertility (1). Serum samples from healthy cattle may have positive titers to *H. somni* because this opportunistic pathogen may be part of the normal flora, because of previous subclinical or clinical infection, or because of cross-reactive antibodies with other bacteria. Cross-reactivity is a problem especially with agglutination tests, because cross-reactive IgM plays a major role in this assay (2, 3). Thus, the currently used serological assays are not adequately sensitive or specific for *H. somni* diagnosis (4, 5). Therefore, a definitive diagnosis of *H. somni* infection relies primarily on the isolation of the bacterium. Although multiplex PCR assays have been developed for diagnosing *H. somni* infection (6, 7), serological diagnosis would still be useful, as most laboratories can accommodate antibody detection formats that are rapid and inexpensive, such as enzyme-linked immunosorbent assay (ELISA).

Exopolysaccharide (EPS) is a major component of the *H. somni* biofilm matrix but is also produced under growth-restricting stress conditions (8), which are likely to occur during the disease process, as well as during colonization of the mucosal epithelia, such as in the vagina and prepuce. Furthermore, *H. somni* is known to form a biofilm and produce abundant EPS in cardiopulmonary tissue during the disease process (9). Whether biofilms are formed by *H. somni* in the carrier state on preputial, vaginal, or upper respiratory epithelia has not been determined. We have purified the EPS from *in vitro* biofilm growth, and the purified EPS can induce the production of antigen-specific antibodies (8). Therefore, we sought to identify the presence of antibodies to EPS in healthy cattle and in experimentally and naturally infected cattle and determine whether the EPS can be used in a direct ELISA for the serological differentiation of animals with *H. somni* disease from healthy animals.

H. somni pathogenic strains 738 and 7735 were grown overnight on brain heart infusion (BHI) agar with 5% sheep blood in 5% CO_2. The colonies were transferred to BHI broth supplemented with 1% yeast extract, 0.1% Trizma base, and 0.01% TMP (10). The bacteria were grown at 37°C with rapid shaking (200 rpm) to mid-log phase. To grow the cells as a biofilm, an aliquot of

bacteria at mid-log phase was transferred to a 1-liter bottle filled with Columbia broth supplemented with 1% glucose, 0.1% Trizma base, and 0.01% TMP, and it was grown for 5 days at 37°C with slow shaking (50 rpm) (11). EPS was purified from the 1-liter culture of *H. somni* strain 738 grown as a biofilm, as previously described (8). Briefly, the top ~900 ml of medium was removed; the EPS was extracted from the biofilm sediment with 45% phenol, dialyzed, and digested with DNase, RNase, and proteinase K; and any lipooligosaccharide (LOS) was removed by ultracentrifugation at $125,000 \times g$. The EPS was confirmed to be free of LOS by chemical analysis (8) and silver staining following electrophoresis (1). The EPS in the supernatant was precipitated with excess 95% ethanol containing 30 mM sodium acetate and incubated at −20°C overnight. Insoluble material was recovered by centrifugation, and the pellet was rinsed with 70% ethanol, suspended in distilled water, and lyophilized.

Blood samples were obtained from all healthy cattle available at the time ($n = 49$) with no history of respiratory disease. Additional cattle were experimentally ($n = 15$) and naturally ($n = 4$) infected with *H. somni*. The passive transfer status of the challenged calves was not determined, but all had been weaned ≥2 weeks prior to challenge. Most healthy cattle were ≥6 months of age. Five experimentally infected 8- to 12-week-old calves were infected intrabronchially with 10^7 CFU of *H. somni*, as previously described (12). Serum samples were obtained from these five challenged calves weekly until the bronchoalveolar lavage (BAL) fluid specimens were culture negative or until the experiment was terminated at 10 weeks postinfection. Random serum samples were also obtained from 10 additional 6- to 18-week-old male Holstein calves that were prechallenged with an attenuated strain of bovine herpesvirus 1 3 days before intranasal and intrabronchial chal-

lenge with 3×10^9 to 3×10^{10} CFU of *H. somni* strain 738 or 7735. Many of the calves were also treated with or (for those not given herpesviruses 1) only treated with 0.1 mg/kg of body weight/day of dexamethasone 4 days prior to challenge to further suppress innate immunity. The prechallenge with virus or dexamethasone was used to stress the animals and suppress the innate immune response. Without prior prechallenge, the animals become colonized but do not develop disease (13). These prechallenged stressed animals developed clinical symptoms of pneumonia. A postmortem examination revealed that the calves had various degrees of purulent bronchopneumonia with abscesses and multifocal purulent myocarditis. A more complete description of the pathology of each calf has been provided elsewhere (13). Normal respiratory tract bacteria, such as viridans group streptococci, were not isolated from the bronchoalveolar lavage fluid specimens, indicating that the *H. somni* isolates recovered from BAL fluid were not part of the normal upper respiratory tract flora. All procedures involving the experimental use of animals in this research at Virginia Tech were reviewed and approved by the University Animal Care and Use Committee to ensure humane care and treatment of the animals. The University Animal Welfare Assurance number on file with the Public Health Service Office of Laboratory Animal Welfare is A-3208-01, with an expiration date of 31 July 2017. Serum samples were also obtained from four naturally infected cattle from a local farm experiencing an outbreak of *H. somni* disease. The animals were clinically ill with pneumonia due to *H. somni*, which was confirmed by a culture of lung specimens from animals that had died. The serum prepared from the blood was stored at $-20°C$ until tested. The sources of all sera are shown in Table 1.

Microtiter plates (Immulon 4HBX-extra-high binding plates; Thermo Fisher Scientific, Inc., Waltham, MA) were coated overnight with 10 μg/ml purified EPS (0.1 ml per well) at room temperature in carbonate-bicarbonate buffer. The ELISA was modified from a previously described protocol (5). Briefly, the microtiter plates were washed three times with phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20 (washing buffer), blocked with 5% nonfat dry milk and 5% goat serum in PBS at 37°C for 1 h, and incubated overnight with the serum samples diluted 1:100 at 4°C. After the plates were washed 3 times with washing buffer, goat anti-bovine IgG, H+L chain (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), conjugated to horseradish peroxidase (diluted 1:1,000) was added and the plates were incubated at 37°C for 1 h. The color was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reagent (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), as described by the manufacturer. Color development was stopped by adding 100 μl of 2 N sulfuric acid, and the optical density (OD) at 450 nm (A_{450}) was determined from 3 replicates of each sample. The sera from cattle immunized with purified EPS were used as a positive control. The sera from the healthy calves with no evidence or history of disease due to *H. somni* were used as negative controls. All serum samples included a control lacking antigen to control for binding to nonspecific serum components, such as IgM, or to skim milk in the blocking buffer. A control lacking the enzyme conjugate was also included with the positive-control serum to EPS. The control background A_{450} values were subtracted from the test results and were generally ≤0.1.

The OD of all samples was transformed into a sample index by the formula (sample OD $- N$)/($P - N$), where N is the average of three optical density (OD) values for the normal calf sera (negative controls) and P is the average of three OD values of anti-strain 738 EPS calf serum (positive control). The optimal cutoff value was determined by the two-graph receiver operating characteristic (TG-ROC) curve analysis (14). Briefly, the sample indexes (ranging from -0.086 to 1.091, in increments of 0.07) were plotted against the OD from the sera of known positive animals (challenged) and negative animals (healthy; no history of *H. somni* disease) in a TG-ROC graph. The point at which the sensitivity and specificity curves crossed was considered the optimal cutoff value. Normal probability plots showed that the sample indexes followed an approximately normal distribution. Subsequently, a Student *t* test was used to compare the confirmed-positive samples with the confirmed-negative samples, irrespective of clinical group at 3 weeks follow-up. A P value of <0.05 was considered significant. The sensitivity of the assay at the sample index cutoff value was calculated as (number of true-positive samples/number of true-positive samples + number of false-negative samples) $\times$ 100, and specificity was calculated as (number of true-negative samples/number of true-negative samples + number of false-positive samples) $\times$ 100.

As shown in Fig. 1, the sera from the preinfected healthy calves (week 0) had low reactivity to EPS. After experimental challenge, the level of reactivity to *H. somni* EPS gradually increased during the first few weeks of infection but then decreased. However, the IgG antibody levels to whole cells remained elevated through ≥10 weeks postchallenge (data not shown). There was variation between the individual animals in the production of antibody to EPS. For calves E5 and E7, the infection was cleared by 6 weeks postchallenge, and the antibody levels to EPS were at their maximum during weeks 3 to 4. The infection persisted until 10 weeks postchallenge for calf 93, but calves 94 and 95 remained culture positive at 10 weeks, when the experiment was terminated. The anti-EPS titers of calves 93, 94, and 95 also peaked later than those for the 2 animals that cleared the infection earlier. The titer of antibody produced in response to the infection also varied. The antibody responses of calves E5, E7, and 95 were similar, except that the antibody titers peaked a few weeks later in calf 95 than in calves E5 and E7. Calf 93 responded with a stronger antibody response to EPS that peaked later than that of the other calves. The antibody response by calf 94 was lower than that of the other calves and was not clearly positive until 7 weeks postinfection. These differences are likely due to variation between individual animals and/or the course or severity of the infection. Although weekly serum samples were not obtained from the other 10 challenged animals in the study, the time-variable but consistent EPS responses of the individual animals shown in Fig. 1 appear to be representative of those of the other challenged animals. For each of these five calves, a peak index was determined as the highest index obtained during follow-up. The peak index was 0.495 at week 3 for E5, 0.363 at week 3 for E7, 0.748 at week 8 for calf 93, 0.289 at week 7 for calf 94, and 0.513 at week 6 for calf 95. The differences between the peak index values and those of the preinoculation sera also followed an approximately normal distribution and were significantly different from zero (paired *t* test, $P < 0.05$). The results indicated that antibodies to *H. somni* EPS increased after clinically symptomatic infection but remained at low levels in healthy animals.

To evaluate the ELISA as a diagnostic tool, additional serum samples from healthy animals and animals that were experimen-

TABLE 1 Antibody reactivities to *H. somni* EPS in sera from uninfected, naturally infected, and experimentally infected calves by ELISA compared to that in culture

Infection type and calf no.	Culture confirmation[a]	ELISA reactivity[b]	Sample index
Uninfected or prechallenged[c]			
09-14	−	−	0.159
118	−	+	0.286
617	−	−	0.008
6-24	−	−	0.073
112	−	−	0.115
10-21	−	−	0.200
113	−	−	0.098
09-11	−	−	0.048
05-1	−	−	0.257
08-2	−	−	0.174
106	−	−	0.198
100	−	−	0.040
08-22	−	−	0.202
105	−	−	0.020
01-03	−	−	−0.046
107	−	−	−0.035
09-23	−	−	0.183
117	−	−	0.199
598	−	−	0.152
957	−	−	0.031
674	−	−	0.253
627	−	−	0.201
293	−	+	0.673
4600	−	−	0.248
4645	−	−	0.119
680	−	−	0.025
623	−	−	0.186
629	−	−	0.160
921	−	−	0.064
4766	−	−	−0.027
494	−	−	0.282
736	−	−	0.052
4771	−	+	0.433
475	−	+	0.510
E5 R85/89 (0 wk)[d]	−	−	−0.023
E7 R85/89 (0 wk)	−	−	0.03
R86-93 (0 wk)	−	−	0.148
R86-94 (0 wk)	−	−	−0.086
R86-95 (0 wk)	−	−	−0.053
565 (0 wk)	−	−	0.003
670 (0 wk)	−	−	0.246
104 (0 wk)	−	−	0.091
13 (0 wk)	−	−	0.129
12 (0 wk)	−	−	0.268
494-1 (0 wk)	−	−	0.189
11 (0 wk)	−	−	0.178
161 (0 wk)	−	−	0.172
10 (0 wk)	−	−	0.028
167 (0 wk)	−	−	0.024
Experimentally infected[e]			
R86-94 (wk 3)	+	−	0.047
R86-94 (wk 4)	+	−	0.122
R86-94 (wk 7)	+	+	0.289
R86-93 (wk 3)	+	+	0.278
R86-93 (wk 4)	+	+	0.346
R86-93 (wk 7)	+	+	0.617
E7 R85/90 (wk 3)	+	+	0.363
E7 R85/90 (wk 4)	+	−	0.250

TABLE 1 (Continued)

Infection type and calf no.	Culture confirmation[a]	ELISA reactivity[b]	Sample index
E5 R85/89 (wk 3)	+	+	0.495
E5 R85/89 (wk 4)	+	+	0.482
R86-95 (wk 3)	+	−	0.242
R86-95 (wk 4)	+	+	0.317
R86-95 (wk 7)	+	+	0.469
565 (wk 3)	+	+	0.636
670 (wk 3)	+	+	0.828
104 (wk 3)	+	+	0.316
13 (wk 3)	+	+	1.031
12 (wk 3)	+	+	0.764
494-1 (wk 3)	+	+	0.980
11 (wk 3)	+	+	0.705
161 (wk 3)	+	+	0.780
10 (wk 3)	+	+	0.812
167 (wk 3)	+	+	0.846
Naturally infected			
580	+	+	0.734
155	+	+	0.586
563	+	+	1.091
588	+	+	0.748

[a] Confirmed diagnosis is based on clinical symptoms and isolation of *H. somni* from tissues.
[b] Result from this ELISA.
[c] Uninfected cattle were healthy, but asymptomatic carriage of *H. somni* was not determined. The prechallenged cattle were culture free of *H. somni* in the upper respiratory tract.
[d] Serum collected at 0 weeks was obtained just prior to experimental challenge.
[e] The number of weeks postinfection when the sera were collected is shown in parentheses.

tally infected or were naturally infected with *H. somni* were tested for all weeks sera were available. The sample index value determined by TG-ROC analysis to distinguish healthy from infected cattle was 0.268 at 3 weeks postinfection (Fig. 2 and Table 2). An infectious disease diagnosis can be made if a documented ≥4-fold rise in specific antibody titer is determined between the acute- and convalescent-phase serum samples. The time required for such a rise in titer is variable but usually occurs within 1 to 3 weeks (15). The serum samples obtained at clinical evaluation (acute) may actually be ≥1 week after the initial infection, and convalescent-phase serum samples obtained ≥2 weeks later would be ≥3 to 4 weeks postinfection. Therefore, the antibody response in the experimentally infected animals in this study at 3 to 4 weeks postinfection would be similar in timing to that of the naturally infected animals when convalescent-phase serum samples are taken ≥2 weeks after the initial evaluation. At 3 weeks postinfection (the only time for which all samples were available), the sensitivity and specificity of the assay were 90.5% and 92.5%, respectively. However, of the two calves that were ELISA negative at 3 weeks postchallenge, 1 became positive at 4 weeks postchallenge. The area under the ROC curve was 93.7% at 3 weeks postinfection, which indicates a high degree of diagnostic accuracy. The serum samples from the naturally infected clinically ill animals were collected about 2 weeks after culture confirmation (likely ≥3 to 4 weeks postinfection), and all were strongly positive.

Diagnosis by bacterial culture is time-consuming, and the identification of *H. somni* may be difficult due to overgrowth of contaminants, normal flora, or other pathogens. Real-time PCR,
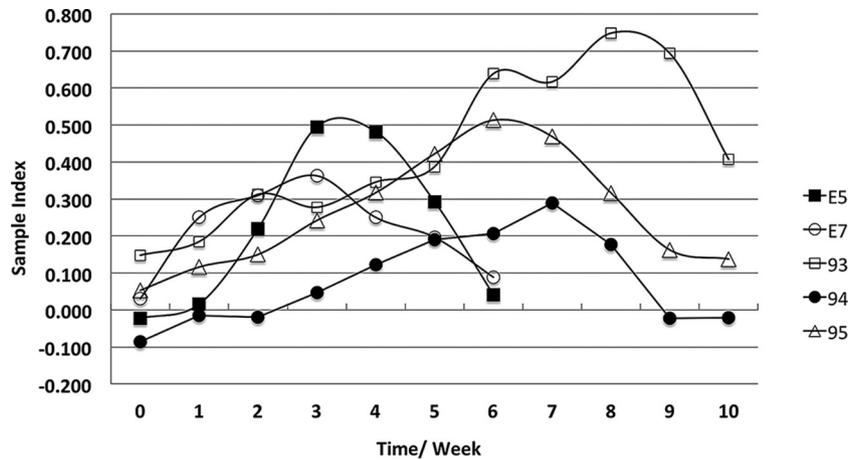
FIG 1 Antibody responses to EPS from 5 experimentally infected calves (E5, E7, 93, 94, and 95). The sera were initially collected at week 0, just prior to challenge. Blood samples were obtained weekly until bronchoalveolar lavage fluid cultures were negative or until the experiment was terminated (10 weeks postinfection). In calves E5 and E7, the infection was cleared by 6 weeks postchallenge, and therefore no additional serum samples were collected after 6 or 7 weeks, respectively. In calves 94 and 95, the animals were still culture positive at 10 weeks postchallenge, but calf 93 had cleared the infection at week 10 postchallenge. The IgG (H+L chain) response to EPS was measured.

though highly useful, may be unavailable in many diagnostic laboratories. In this study, *H. somni* EPS was used to develop an ELISA to differentiate *H. somni*-infected from healthy animals. The number of culture-confirmed samples available was limited, because infections due to *H. somni* occur predominately in feedlots, where environment, weaning, feed provided, stress, and other pathogens commonly predispose the animals to systemic *H. somni* infection (16–18). Feedlots are not present in the eastern United States, where this study was conducted. Nonetheless, *H. somni* commonly inhabits bovine mucosal sites. As a result, cattle may at some time develop cross-reactive antibodies to *H. somni* (4), making serology using whole cells as an antigen in serological
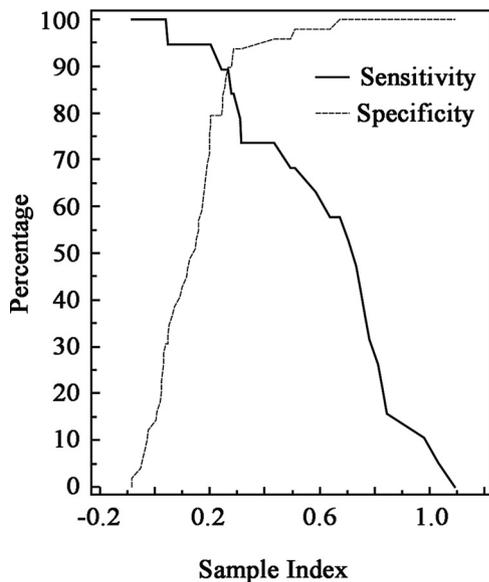

FIG 2 Two-graph receiver operating characteristic (TG-ROC) analysis of the ELISA. The sensitivity (solid line) and specificity (dashed line) at different cutoff sample indexes were plotted. At 3 weeks postinfection for the animals tested, the two curves intercross at a sample index of 0.268, which is the proposed optimal cutoff sample index value.

assays unreliable. Although agglutination assays predominately detect IgM antibodies, which are highly cross-reactive, several outer membrane proteins of *H. somni* have also been shown to cross-react with antigens of other Gram-negative bovine pathogens (19, 20). Therefore, an assay that measures the antibody response to an antigen that may be expressed predominantly during active disease may enhance serological sensitivity and specificity. Much earlier studies showed that detection of IgG2 antibodies to the large molecular size (approximately 270 K) fibrillar immunoglobulin binding proteins of *H. somni*, now called IbpA, by immunoblotting with peroxidase-conjugated protein A can also differentiate most animals with *H. somni* disease from asymptomatic carriers and culture-negative animals at 5 to 6 weeks postchallenge (earlier times were not tested) (21). More recent ELISA studies with the same 5 calves used in this study (E5, E7, 93, 94, and 95) showed that a strong IgG antibody response was made to the A5 recombinant subunit of IbpA by week 2, which further increased until week 5 and then persisted through the 6 or 10 weeks of the study (22). However, an advantage to the use of EPS as an antigen is that it is relatively easy to purify, and it is produced only during biofilm growth or under stress conditions; therefore, healthy animals and animals vaccinated with a killed *H. somni* vaccine would remain serologically negative. A disadvantage is that polysaccharide antigens are not strong immunogens, and a substantial antibody response to EPS may be weaker than that to a protein. This is reflected by the fact that the sera were used at a 1:100 dilution for the EPS ELISA. Therefore, if infection due to *H. somni* is suspected, the EPS ELISA is negative at the initial visit, and culture or real-time PCR is inconclusive or unavailable, testing of a repeat serum sample by EPS ELISA 2 weeks later may support the diagnosis.

H. somni produces an EPS *in vitro* when the bacteria form a biofilm, and the chemical structure of the EPS has been determined (8). Previous research from our lab has established that *H. somni* also forms a biofilm in the cardiopulmonary tissue of infected calves (9). A comparison of biofilm formation between a pathogenic and a serum-sensitive commensal strain of *H. somni*, which also lacks IbpA, indicated that the pathogenic strain pro-

TABLE 2 Sensitivity, specificity, and TG-ROC analysis results

ELISA diagnosis[a]	Confirmed diagnosis		Youden index		Area under the ROC curve (%)	Sensitivity 95% CI (%)	Specificity 95% CI (%)
	Positive	Negative	Youden index J (%)	Optimal cutoff			
Positive	16	4	88.61	>0.268	93.75	66.9–98.7	77.8–96.6
Negative	3	45					

[a] Data of the positive cases included in Table 1 are from 3 weeks postinfection.

duced more biofilm and EPS than the commensal strain. In addition to being abundantly produced under conditions that favor biofilm formation, EPS is also produced by *H. somni* under stress conditions, including anaerobiosis, stationary phase, and high salt concentration (8). Although biofilms and their matrix components (including EPS, and likely IbpA, other surface proteins, DNA, and lipids) may be produced at mucosal sites during colonization, a strong antibody response may not be made to some of these antigens except during pulmonary or systemic infection. Phagocytosis by neutrophils, macrophages, dendritic cells, etc., and presentation to T and B cells may be required to induce an adequate antibody response. Nonetheless, an association between an antibody response to EPS and bovine disease was apparent, and we propose that antibodies to EPS have the potential for use in assays to identify disease due to *H. somni* in cattle.

ACKNOWLEDGMENTS

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