Serum Susceptibility of *Haemophilus somnus* from Bovine Clinical Cases and Carriers†

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The serum susceptibility of 64 isolates of *Haemophilus somnus* from cattle was determined in a bactericidal assay with undiluted fresh or inactivated bovine serum with serial dilutions of bacterial suspension in RPMI 1640 medium. A total of 27 strains isolated from cattle with clinical disease (4 with thromboembolic meningencephalitis, 13 with pneumonia, and 10 with reproductive failure) were compared with 35 strains from asymptomatic carriers (11 from the vagina and 24 from the prepuce). Essentially, all clinical isolates were serum resistant, whereas approximately 25% of preputial isolates were serum susceptible, as judged after 1 h of incubation in serum; a majority of vaginal isolates showed delayed serum susceptibility. Lysozyme played no role in serum killing, and the alternative complement pathway played only a minor role. Iron saturation, however, appeared to impart greater serum resistance to serum-susceptible strains from the vagina and prepuce. Perhaps the serum-susceptible strains from carriers would be useful vaccine candidates, but resistant strains from carriers may be pathogenic.

*Haemophilus somnus* is associated with a spectrum of clinical conditions in cattle and, occasionally, sheep (8). The organism was initially isolated from cases of thromboembolic meningencephalitis (TEME) in feedlot cattle (8). Subsequently it has become clear that this pathogen is involved in the pathogenesis of septicemia, arthritis, pneumonia, calf diphtheria, abortion, and perhaps infertility (8, 16). The relationship with disease is not always clear, however, because *H. somnus* may inhabit the upper respiratory tract without causing disease (4, 5) and is very often part of the normal genital flora (7, 8, 29). In fact, in recent studies by us (29) and others (7), approximately 75% of bulls and 10 to 20% of cows were asymptomatic genital carriers. Observations that *H. somnus* is involved in a wide spectrum of syndromes indicate that strains of the organism associated with a particular syndrome or carrier state may have unique virulence attributes. One virulence factor often associated with the ability to cause septicemia and its sequelae is resistance to killing by antibody and complement (7, 21, 24, 25, 28). The importance of complement-mediated killing in resistance to *H. somnus* disease was shown by demonstration that more severe disease developed in cobra venom-treated cattle than in controls inoculated with *H. somnus* alone (17). Since most cattle have natural cross-reacting antibody to *H. somnus* (L. B. Corbeil, unpublished data) and since antibody-independent activation of complement also aids in defense against gram-negative infections (13, 21), it is likely that surface factors contributing to serum resistance are important in allowing *H. somnus* to invade. Conversely, the lack of invasion by organisms causing disease at the mucous surfaces only may be attributable to the lack of complement activity at those sites. For example, many isolates of *Neisseria gonorrhoeae* from gonorrhea are serum susceptible, whereas those from disseminated gonococcal infection are not (25). To investigate the association of serum resistance of *H. somnus* with disease, we collected isolates from cattle with different clinical syndromes and from asymptomatic carriers and tested their ability to resist killing by a pool of normal fresh bovine serum. Also, because cattle lack the bacteriolytic enzyme lysozyme (19) and low levels of serum iron are believed to contribute to resistance to infection (12), the roles of these two factors were evaluated as well.

**MATERIALS AND METHODS**

**Bacterial cultures.** Isolates of *H. somnus* were obtained from cattle with clinical cases of TEME, pneumonia, or reproductive failure (abortion or infertility) submitted to the Washington Animal Disease Diagnostic Laboratory and from P. Little, Ontario Veterinary College, University of Guelph, Ontario, Canada. Organisms were identified as *H. somnus* by standard methods (8). The following characteristics were considered to be positive identification: slow growth, flat tan colonies, capnophilic nature, yellow pigment, no growth on Mueller-Hinton agar plates, and gram-negative pleomorphic rods seen in smears. Isolates from asymptomatic carriers were cultured from preputial swabs of 33 bulls from two beef herds and vaginal swabs of young cows from the University of Idaho herd. Samples were cultured, and isolates were grown on Columbia blood agar (Difco Laboratories, Detroit, Mich.) plates containing 10% bovine blood at 37°C in 10% CO2 except in one experiment with broth cultures (10% bovine serum in brain heart infusion) as described in the text (see Fig. 4). Isolates were preserved after one to three subcultures by suspension from plate cultures in buffered glycerol (40% phosphate-buffered saline and 60% glycerol [vol/vol]) and freezing at −70°C. A rough strain of *Escherichia coli* (15) with no lipopolysaccharide side chains was used as a positive control in the bactericidal assays since it is extremely serum susceptible.

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TABLE 1. Serum susceptibility of *H. somnus* isolates from various sources

<table>
<thead>
<tr>
<th>Source of isolate</th>
<th>Total no. of isolates</th>
<th>No. of isolates susceptible to serum killing*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEME</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Reproductive failure</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Normal vagina</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Normal prepuce</td>
<td>24</td>
<td>6</td>
</tr>
</tbody>
</table>

* Serum-susceptible is defined as a greater than 1-log decrease in CFU after a 1-h incubation in fresh serum.

**Fresh bovine serum.** Blood was collected from two normal adult cattle, and serum was removed after 1 h at room temperature and 1 h at 4°C. This serum was pooled, divided into portions, and frozen at −70°C so that a fresh thawed portion from the same pool could be used in each test. The serum had a microagglutination titer of 1:024, which is the median titer for cattle in the northwestern United States as determined by the Washington Animal Disease Diagnostic Laboratory serology laboratory. For preparation of control serum with inactivated complement, portions were heated at 56°C for 30 min.

**Bactericidal assay.** A microtiter assay with undiluted serum in equal volumes of serial dilutions of bacterial suspension was used. This system was chosen because the homologous complement was present in in vivo proportions with antibody and other bactericidal or bacteriostatic factors in serum. Cultures of *H. somnus* (18 h, unless stated otherwise) were suspended in saline at 10⁶ CFU (estimated spectrophotometrically [75% T at 610 nm] and confirmed each time by plate counts). Serial 10-fold dilutions of bacteria were made in RPMI 1640 medium (RPMI) (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum (FCS). This medium was used because it supported the growth of *H. somnus* in preliminary studies better than other tissue culture media did. Tenfold dilutions (100 μl) of each isolate were incubated with fresh serum, heated serum, or RPMI-FCS in 96-well microtiter plates. The heated-serum controls (heated at 56°C for 30 min) and RPMI-FCS controls gave essentially equivalent counts, indicating that agglutination did not occur and therefore was not a factor in lowering colony counts. The microtiter plates were incubated at 37°C in 10% CO₂, and after 1 to 3 h, 2 drops (25 μl each) were removed and placed on slightly dried Columbia blood agar plates. The drops were allowed to soak into the agar, and the plates were incubated overnight at 37°C in 10% CO₂. Colony counts between 10 and 100 were used for the estimation of CFU per milliliter. Complement-mediated killing was determined by subtracting the log of the CFU per milliliter in fresh serum from the log of the CFU per milliliter in heated serum. In rare cases, when the heated-serum and RPMI-FCS controls were not equivalent, the results were discarded and the assays were repeated. Bactericidal activity was reported as logs of kill (i.e., the difference between CFU after incubation in heated serum and CFU after incubation in fresh serum), with greater than 1 log kill considered serum susceptible and less than 1 log kill considered serum resistant (3, 15).

Furthermore, since it has been shown with *E. coli* that bacteria are more susceptible to killing in the exponential phase than in the stationary phase of growth, we tested the susceptibility of representative *H. somnus* strains in these two phases of growth. Preliminary studies showed that *H. somnus* was in the exponential phase at 6 h and in the stationary phase at 18 h.

**Iron-saturated serum bactericidal assay.** The serum complement bactericidal assay previously described was altered to evaluate the effect of iron saturation on the bactericidal activity of serum. According to Kaneko (10), the total iron-binding capacity of adult bovine serum is 228 μg/dl with 97 μg of iron per dl normally present. Therefore, 131 μg of iron per dl was added to give 100% iron-saturated serum, and 359 μg/dl was added, to give 200% iron-saturated serum. Ferric ammonium citrate (Sigma Chemical Co., St. Louis, Mo.) was dissolved in RPMI plus 10% FCS to give the proper final concentration of iron when mixed with equal volumes of serum.

**Complement pathway evaluation.** The serum complement bactericidal assay was expanded to determine the pathway of complement killing of *H. somnus*. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma) and MgCl₂ were used to inhibit the classical pathway of complement but preserve the alternate pathway. EGTA

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TABLE 2. Comparisons of serum susceptibility of different clones from case 221V

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Log kill at 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>1</td>
<td>0.34</td>
</tr>
<tr>
<td>2</td>
<td>0.75</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
</tr>
<tr>
<td>4</td>
<td>0.08</td>
</tr>
</tbody>
</table>

FIG. 1. Kinetics of killing of clinical isolates of *H. somnus* by fresh bovine serum. Serum-susceptible strains were defined as those with more than 1 log₁₀ of killing (shaded line).
and MgCl₂ were dissolved in RPMI plus 10% FCS at a concentration of 86 mM for both. This solution (magnesium-EGTA) served as diluent in the bactericidal assay to give the same final concentration (43 mM) as that shown to be effective by MacDonald et al. (14).

**Evaluation of the role of lysozyme.** The effect of lysozyme was determined with lysozyme-deficient and lysozyme-positive rabbit serum (23). The presence or absence of lysozyme in the genetically deficient or positive rabbits (22, 23) was confirmed by testing against *Micrococcus luteus* (lyso-deikticus) in a diffusion assay (18).

**RESULTS**

Most isolates from cattle with TEME, pneumonia, and reproductive failure were serum resistant when the criterion of less than a 1-log drop in colony counts at 1 h was used (Table 1). Also, most isolates from vaginal carriers were serum resistant by the criterion of killing at 1 h, but 25% of the preputial isolates from normal bulls were serum susceptible. One pneumonia isolate (0004) was serum susceptible (1.33 logs killed at 1 h). This isolate was from an animal that also had large numbers of *Pasteurella multocida* isolated from the pneumonic lesion. One isolate from an animal with reproductive failure (1585-2-3) was also serum susceptible (1.84 logs killed). This isolate was derived from one colony on the original plate from animal 1585. A second colony from the same plate (1585-2-6) was very resistant to killing by serum (0.10 logs killed at 1 h). This observation prompted a study of different colonies from the same case. A vaginal isolate (221V) of marginal serum susceptibility was chosen.

Isolated colonies were cloned twice and tested for serum susceptibility on two different days. Of the four clones tested, three were serum resistant and one was relatively serum susceptible (Table 2). The reproducibility of the tests on days 1 and 2 was comparable to that found with other isolates.

Because it has been suggested that the kinetics of killing may be related to virulence differences among strains (23), the numbers of bacteria killed at 1, 2, and 3 h also were determined for selected isolates (see Fig. 1. 2, and 3). If a 1-log decrease in colony counts is taken as the criterion of serum susceptibility, all clinical isolates tested were classified as serum resistant after both 1 and 2 h of incubation, but delayed susceptibility to serum killing was detected at 3 h with 4 of 12 isolates (Fig. 1). Isolates from vaginal carriers had a pattern similar to that of clinical isolates at 1 and 2 h of incubation, but by 3 h, the majority (8 of 11) would be classified as serum susceptible (Fig. 2). Of the preputial isolates tested, both serum-susceptible and serum-resistant isolates were detected (Fig. 3).

In studies of the serum susceptibility of *H. somnus* in exponential and stationary phases of growth, no differences between 6-h broth cultures and 18-h plate cultures were detected (Fig. 4).

The mechanism of serum killing was investigated by determining the roles of iron, lysozyme, and the alternate complement pathway. Most clinical isolates were killed equally well in the presence of excess iron as in untreated serum (Fig. 5). The isolates from normal carriers, however, were usually more resistant to serum killing in the presence of excess iron. That is, three of four vaginal isolates and
three of four preputial isolates tested were more resistant to killing in serum with excess iron than in control serum. At both 100 and 200% iron saturation, sera with excess iron were statistically different from control sera in killing of these isolates ($P < 0.01$ with 100% iron saturation and $P < 0.02$ with 200% iron saturation, as determined by analysis of variance). Since cattle are deficient in lysozyme, we compared serum from normal rabbits with serum from a strain of rabbits deficient in lysozyme. The latter serum thus served as a bovine model. Both sera were equally efficient in killing *H. somnus* in the presence of bovine complement (Table 3). The results obtained with the alternate pathway of complement killing were variable (Fig. 6). With some isolates, the alternate pathway was involved in killing, whereas with other isolates, essentially all of the bactericidal effect was due to the classical complement pathway. In general, there was a larger proportion of alternate pathway killing of preputial isolates than of clinical or vaginal carrier isolates.

**DISCUSSION**

We have shown that nearly all isolates of *H. somnus* from cattle with clinical cases of TEME, pneumonia, and reproductive failure were classified as serum resistant after 1 h of incubation with fresh serum, as were nearly all isolates from vaginal carriers and greater than half of the isolates from preputial isolates. This is somewhat similar to the situation with *N. gonorrhoeae* infection in humans, in which isolates from disseminated gonococcal infections are serum resistant (25, 28) and those from locally invasive or uncomplicated disease have varying susceptibilities to complement-mediated killing (24). With the gonococcus, colony type has been associated with both virulence (11) and serum susceptibility (15). With that organism, serum susceptibility is controlled by several genetic loci (21, 26). It is likely that the differences in serum susceptibility between *H. somnus* isolates from various clinical diseases and clinically normal carriers is genetically determined also.

The data reported herein on *H. somnus* isolates from diseased animals suggest a strong correlation between virulence and serum resistance. The two serum-susceptible isolates from diseased animals may indicate that these variants were not the pathogenic organisms with major etiologic involvement. In the pneumonia case (0004), a serum-resistant *P. multocida* strain was isolated in large numbers along with *H. somnus*, so it is probable that *P. multocida* was the major pathogen. In reproductive failure case 1585, two colonies of *H. somnus* were tested; one was very susceptible and one was very resistant to complement-mediated killing. The resistant isolate 1585-2-6 was probably the major pathogen. The difference in serum susceptibility of several clones from one case were also demonstrated with isolate 221V. These examples should be taken as a warning that selecting one colony as representative of that pathogenic bacterium from a single clinical case may be misleading.

Although various clones differed in serum susceptibility, the stage of the growth cycle or type of culture media (broth or agar) did not seem to affect serum susceptibility.

To investigate the mechanisms of serum killing, magnesium-EGTA-treated serum (deficient in the classical pathway of complement) was compared with fresh, untreated serum for bactericidal effect on selected isolates. That the magnesium-EGTA-treated serum was able to kill some isolates with the same efficacy as the untreated serum indicated that the alternate pathway was involved in the killing of these strains. For other strains, the alternative pathway did not appear to be important. No pattern for the role of the alternate pathway in any particular syndrome or carrier state was shown.

Whether this alternate pathway killing was antibody dependent, as recently described for *Haemophilus influenzae* type b (27), could not be determined since essentially all cattle were shown to have antibodies to *H. somnus*.

The roles of lysozyme and serum iron were also considered, because lysozyme has been suggested as a factor in serum killing (28). Iron saturation of serum was studied because serum-resistant isolates of gonococci from disseminated gonococcal infection generally have superior abil-

![Graph](image-url)

**FIG. 4.** Kinetics of killing of cultures grown in broth for 6 h (exponential growth phase) and on agar plates for 18 h (stationary phase).

**TABLE 3. Role of lysozyme in killing of *H. somnus* strain 1P**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Bovine complement</th>
<th>Lysozyme</th>
<th>Complement + lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>3.6</td>
<td>0</td>
<td>3.7</td>
</tr>
<tr>
<td>2 h</td>
<td>4.2</td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>3 h</td>
<td>&gt;4.4</td>
<td>0</td>
<td>&gt;4.4</td>
</tr>
</tbody>
</table>

*Log$_{10}$ of plate count after incubation in heated serum (56°C for 30 min) minus $Log_{10}$ plate count after incubation in fresh serum. Bovine complement consisted of 1 volume of fresh bovine serum plus 1 volume of heated lysozyme-deficient rabbit serum. Lysozyme consisted of 1 volume of heated bovine serum plus 1 volume of heated normal rabbit serum. Complement plus lysozyme consisted of 1 volume of fresh bovine serum plus 1 volume of heated normal rabbit serum. (Lysozyme is not inactivated by heating.)*
FIG. 5. Killing of *H. somnus* isolates in the absence (□) or presence (▴) of 100% iron saturation or in the presence of 200% iron saturation (■).

FIG. 6. Total complement (■) and alternate (▴) pathway killing of *H. somnus* isolates.
ity to acquire iron than do serum-susceptible gonorrhea isolates (20), and iron added to serum abolishes the bacteriostatic effect of serum on Pasteurella septica (2). Bovine serum contains little free iron (10). In our studies, most of the isolates from respiratory, reproductive, or encephalitic disease were not more resistant to serum killing in the presence of excess iron, suggesting that they were able to acquire iron from iron-carrying proteins in bovine serum. Most isolates from the vaginal or preputial carrier state, however, were more resistant to killing in the presence of excess iron. This observation is similar to that of Kochran et al. (12), who found that iron supplementation of mice increased the mortality rates after inoculation with avirulent E. coli to mortality rates of untreated mice inoculated with virulent E. coli. These authors suggested that lipopolysaccharide in the cell surface and enterochelin in the cell wall are involved in iron acquisition and that iron deprivation may lead to permanent lesions in the bacterium cell surface. Since the structure of the bovine cell surface largely determines the serum susceptibility (9, 21, 26, 28), differences in cell surfaces of virulent and avirulent H. somnus isolates could be related to both serum susceptibility and iron acquisition.

Although lysozyme plays a role in the killing of some bacteria (28), no effect on H. somnus could be demonstrated in our studies. Our original hypothesis was that the lack of lysozyme in bovine serum and secretions may be partially responsible for the specificity of H. somnus for cattle. However, since serum killing was not enhanced by rabbit lysozyme, this does not appear to be the case.

The serum susceptibility of 25% of the preputial isolates tested in 1 h and the delayed susceptibility of most vaginal isolates suggest that these isolates would be unlikely to invade. They may be good candidates for live vaccines. The other 75% of the preputial isolates and 40% of the vaginal isolates should be considered as potential pathogens at this time, although other virulence factors are also likely to be important. If the serum-resistant isolates from vaginal and preputial carriers do have the other virulence attributes necessary to cause disease, then methods should be developed to terminate the carrier state. If, on the other hand, isolates from carriers are avirulent, the organisms present in the carrier state may benefit the host as resident autogenous vaccines.

LITERATURE CITED


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