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Cross-Reactivity of *Haemophilus somnus* Antibody in Agglutination and Complement Fixation Tests and in the Enzyme-Linked Immunosorbent Assay

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The specificity and sensitivity of agglutination, complement fixation, and enzyme-linked immunosorbent assay (ELISA) procedures in the detection of antibodies to *Haemophilus somnus* was investigated. *H. somnus* rabbit immune sera were found to agglutinate *Pasteurella multocida, Staphylococcus aureus*, and *Haemophilus agni* and, in some instances, also *Pasteurella haemolytica, Salmonella dublin, Streptococcus agalactiae*, and *Corynebacterium pyogenes*. In complement fixation tests with saline extracts as antigens, only *H. agni* reacted with *H. somnus* antisera to any significant degree. In ELISA tests with sonicated or heat-extracted antigens, cross-reactions were seen with the two *Pasteurella* spp. and with *H. agni*. When whole cells and saline extracts were used as antigens in ELISAs, only *H. agni* showed any cross-reactivity. The greatest specificity in distinguishing homologous from heterologous reactions was achieved by ELISA with saline extracts as antigens. *Escherichia coli* and *Brucella abortus* antigens failed to react with *H. somnus* antibody in any of the tests. A rabbit serum containing antibody to bovine type isolates of *P. multocida, P. haemolytica, S. aureus, S. agalactiae, S. dublin, C. pyogenes*, and *E. coli* gave no positive reaction in ELISA tests with saline extract of *H. somnus* as antigen. It is concluded that such saline extract, which appears to consist largely of *H. somnus* common antigen, has the potential of being a useful diagnostic reagent in the study by ELISA of antibody response to *H. somnus*.

Interest in the antigenic characteristics and serological behavior of the bacterium commonly referred to as *Haemophilus somnus* dates from the time of its first isolation and recognition as a significant pathogen of cattle (5). Serological approaches have been utilized in studies on the epidemiology of, and immunity to, *H. somnus* infections (1, 3–5, 16, 18). Several investigations utilizing diverse serological techniques addressed the question of antigenic relationships of *H. somnus* to other bacterial species. A variety of cross-reactivity patterns have been described, most consistently with so-called *H. agni* (3, 5, 12, 14) but also, with lesser regularity, with *Brucella abortus* and *Bordetella bronchiseptica* (14), various *Haemophilus* spp., *Pasteurella multocida* and *P. haemolytica* (3), *Listeria monocytogenes, Streptococcus agalactiae, Actinobacillus lignieresii*, and *Campylobacter fetus* (12). In some instances, reactions were reciprocal. In others, *H. somnus* antigen reacted with heterologous antisera, or antigens prepared from a number of other bacteria gave positive results with *H. somnus* antisera in one-way reactions. These observations raise vexing questions concerning the interpretations of tests which employ one of these antigens in screening sera of cattle with uncertain histories of exposure. The present investigation is directed at one of these aspects, namely, the behavior of *H. somnus* antibody toward various antigen preparations derived from common bovine commensal and pathogenic bacteria and used in several testing procedures, including agglutination, complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA). The most promising modification of the latter procedure was then used to study the reactivity with *H. somnus* antigen of heterologous antibodies likely to be encountered in cattle sera.

MATERIALS AND METHODS

Bacterial cultures. The strains selected were: four *H. somnus* strains; representatives of species previously reported to react with *H. somnus* antibody (*H. agni, S. agalactiae*); and species not reported so to react (*P.*

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TABLE 1. Designation and origin of antigen strains

Species	Strain no.	Source		
		Host species	Organ/condition	Geographic location
Haemophilus somnus	M734	Bovine	Pneumonic lung	Colorado (A. B. Hoerlein)
Haemophilus somnus	2106	Bovine	Pneumonic lung	California (VMTH)[a]
Haemophilus somnus	139/77	Ovine	Pneumonic lung	Switzerland (J. Nicolet)
Haemophilus somnus	927/75	Bovine	Pneumonic lung	Switzerland (J. Nicolet)
Haemophilus agni	902	Ovine	Septicemia	California (VMTH)
Pasteurella multocida	70-483-3B	Bovine	Pneumonic lung	California (VMTH)
Pasteurella haemolytica A1	H19	Ovine	Pneumonia	California (VMTH)
Corynebacterium pyogenes	493-11	Bovine	Abscess?	California (VMTH)
Staphylococcus aureus		Bovine	Mastitis	California (VMTH)
Streptococcus agalactiae		Bovine	Mastitis	California (VMTH)
Salmonella dublin	SA22	Bovine	Ileum	California (VMTH)
Escherichia coli	80-144-10A	Bovine	Lung and pericardial fluid	California (VMTH)

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multocida, P. haemolytica, Salmonella dublin, Staphylococcus aureus) but common in the bovine environment. Also included were *Corynebacterium pyogenes* and *Escherichia coli*, organisms associated with cattle but not previously studied. All strains were of bovine origin except for *H. agni, P. haemolytica*, and one *H. somnus* culture, all of which had been recovered from sheep. The *P. haemolytica* culture represented the biotype A and serotype 1 prevalent in cattle. The four *H. somnus* cultures included two representatives of each of two serological subpopulations of the species previously described by us, i.e. the Swiss (S) and American (A) (2). Sources of antigen strains are shown in Table 1.

The identity of all cultures except *H. somnus* and *H. agni* was established by standard methods (8) at the Microbiology Service, Veterinary Medical Teaching Hospital, University of California, Davis. That of the *H. somnus* cultures had been confirmed by cultural tests in an earlier study (2). The strain of *H. agni* was one of the isolates from an outbreak of lamb septicemia, during the course of which the species was first recognized and described (6).

H. somnus and *H. agni* were propagated on chocolate agar in candle jars. All other cultures were grown on blood agar in air. Incubation was at 37°C for 48 h.

Immune sera. The preparation of *H. somnus* antisera has been described elsewhere (2). The antisera used in this study were raised in rabbits with the four *H. somnus* cultures listed in Table 1.

A composite immune serum intended to reflect the antibody content of the serum of cattle exposed to common commensal and pathogenic agents was also prepared. Cells of *P. multocida, P. haemolytica, C. pyogenes, S. aureus, S. agalactiae, E. coli*, and *S. dublin* were grown on blood agar plates at 37°C for 48 h. The growth was harvested and washed three times in physiological saline. Each strain was suspended to produce an absorbency of 0.39 at 540 nm in a spectrophotometer (Spectronic 20; Bausch & Lomb, Inc., Rochester, N.Y.), and equal volumes were combined in the immunizing antigen. The rest of the immunization protocol was identical to that previously described (2).

Agglutination test. All aspects of the agglutination test procedure have been described previously (2). In brief, cells were propagated on chocolate agar, harvested and washed with physiological saline solution (pH 8), heated at 60°C for 90 min as a precaution against autoagglutination, and suspended in 0.5% phenolized saline solution (pH 8) to an optical density of 0.39 at 550 nm in a Spectronic 20 spectrophotometer. Serum dilutions tested ranged from 1:10 to 1:2,000. The tests were read after incubation at 37°C for 24 h.

CF test. The technique of the CF test and its preliminary titrations were those described by Lennette and Schmidt (9). Bacteria for antigen preparation were propagated as for the agglutination test. Cells were harvested with sterile physiological saline, pH 7.4. The cell concentration was adjusted to correspond to an optical density of 4.0 at 540 nm (Spectronic 20 spectrophotometer). This suspension was incubated at 4°C for 24 h and then sedimented at 8,000 × *g* in a refrigerated centrifuge (RC 1; Ivan Sorvall, Inc., Norwalk, Conn.) for 15 min. The supernatant constituted the antigen. It was titrated by block titration with positive and negative rabbit sera and stored until the next day, when the actual test was performed. The complement (C. F. Meyerdick Rabbitry, Vacaville, Calif.) in the titration was used at a dilution of 1:30. Hemolysin (California State Department of Public Health, Berkeley, Calif.) was titrated with sheep erythrocytes obtained from Animal Resources Service, University of California, Davis.

The test was performed in microtiter trays (Flow Laboratories, Englewood, Calif.). Twofold dilutions of inactivated serum, ranging from 1:8 to 1:1,024, were placed into successive wells in 0.025-ml amounts. Identical amounts of antigen (at the determined dilution) and of complement (2 exact units) were added to each well. After overnight incubation at 4°C, the trays were warmed for 10 min at 37°C, and 0.05 ml of sensitized sheep erythrocytes was added to each well. The trays were reincubated for 30 min at 37°C and read by inspection. Reactions in wells showing less than 50% hemolysis were considered positive. The usual anticomplementary and hemolytic controls for sera, antigen, and saline were included.

ELISA. The protocol for the ELISA was an adaptation of the technique described by Saunders (13).

Preparation of antigens. Four types of antigen were

used. "Cellular" antigen was prepared as for the agglutination test. "Sonicated" antigen was cellular antigen subjected to sonic oscillation (Sonic 300 Dismembrator; Artex Systems Corp., Farmingdale, N.Y.) for 20 min at high intensity, followed by centrifugation at 8,000 × *g* for 15 min. The supernatant was stored at 4°C until the next day, when the test was performed. "Heat-extracted" antigen was cellular antigen boiled for 1 h, centrifuged, and separated and stored like the sonicated antigen. "Soluble" antigen was identical to the CF antigen.

Titration of test components. Block titrations were used to determine optimal dilutions of the antigens for coating the solid phase. The antigens were diluted in cold (4°C) carbon-bicarbonate buffer, pH 9.6 (17). Fifty microliters of diluted antigens was pipetted into each well of microtiter trays (substrate plates; Dynatech Corp., Alexandria, Va.), which were then incubated at 37°C for 2 h of adsorption of the solid phase. Serum was diluted with Tris buffer, pH 7.4 (10). Optimal dilutions varied according to the antigen preparation used from 1:50 (soluble) to 1:100 (cellular, sonicated, heat extracted), as determined by block titration. The appropriate dilution of enzyme-labeled antibody conjugate (goat anti-rabbit immunoglobulin G serum, peroxidase-conjugated; Northeast Biomedical Laboratory, South Windham, Maine) was also obtained by block titration. Tris buffer was the diluent. The substrate mixture consisted of a solution of 0.05 ml of 40 mM 2,2-azino-di-(3-ethylbenzthiazoline sulfonic acid) (Sigma Chemical Co., St. Louis, Mo.) and 0.4 ml of 0.5 M hydrogen peroxide in 10 ml of citrate buffer, pH 4 (15). The reaction was stopped with a solution of 0.1 M hydrofluoric acid (pH 3.3) containing 0.38 mg of EDTA tetrasodium per ml (M. Bartlett, personal communication).

Test procedure. (i) Step 1. Previously coated plates were washed twice with buffer wash (17), inverted, and tapped on absorbent material for removal of residual fluid.

(ii) Step 2. Fifty microliters of each diluted serum was dispensed into the appropriate wells. Duplicate wells were used for each antigen-serum combination. This procedure allowed six antigens per plate to be tested in duplicate. A high-precision micropipettor (eight-channel pipettor, 0.050 to 0.200 ml; Dynatech) was used for dispensing of all reagents.

(iii) Step 3. Plates were covered and incubated for 15 min at 37°C and then washed three times and drained as described in step 1.

(iv) Step 4. Fifty microliters of enzyme-labeled antibody conjugate dilution was added to each well.

(v) Step 5. Plates were covered and incubated for 15 min at 37°C and washed twice as in step 1.

(vi) Step 6. One hundred microliters of substrate mixture was added to each well.

(vii) Step 7. Plates were covered and incubated for 20 min at 37°C for appropriate color development.

(viii) Step 8. Two hundred microliters of stop solution was pipetted into each well.

(ix) Step 9. Reactions were read on a Multiskan microplate reader (Flow Laboratories) at 415 nm. The reader was "blanked" with a new empty plate before each use.

For interpretation of the readings, means and standard deviations were determined for the absorbency values obtained with *H. somnus* antigens and with non-*H. somnus* antigens. Confidence intervals were adopted as extending to 2 standard deviations of the respective means. Absorbency values falling within one of these intervals were interpreted as positive or negative for reactivity with *H. somnus* antigen. Those values falling in the zone where the confidence intervals of *H. somnus* and non-*H. somnus* reactivities overlapped were read as doubtful (see Fig. 1).

RESULTS

The cross-agglutination titers of the four hyperimmune sera are shown in Table 2. It is apparent that all sera agglutinated all *H. somnus* antigens (referred to hereafter as homologous antigens), although to various titers. *P. multocida*, *S. aureus*, and *H. agni* antigens were agglutinated by all sera, whereas *P. haemolytica* was agglutinated by three, and *S. dublin*, *S. agalactiae*, and *C. pyogenes* by two, of the four sera. None of the sera reacted with *E. coli* or *B. abortus*. (All antigens other than those derived from *H. somnus* strains are referred to as heterologous in text and figures).

Of 36 possible reactions with heterologous antigen, 21 were positive (58.3%). Of the 29 titers of 1:≥100, 11 (37.9%) were with heterologous antigens, i.e., "false positives." None of the preimmunization sera agglutinated any of the antigens.

In the CF tests, impressive cross-reactions, comparable in titer to those involving homologous antigens, occurred between *H. agni* and

TABLE 2. Agglutination reactions between four *H. somnus* antisera and homologous and heterologous bacterial antigens

Antiserum to:	Antigen[a]												
	H. somnus				*P. multocida*	*P. haemolytica*	*S. dublin*	*E. coli*	*S. agalactiae*	*S. aureus*	*C. pyogenes*	*B. abortus*	*H. agni*
	M734	2106	927/75	139/77									
M734	1,000	400	400	400	100	400	50	<20	100	200	20	<20	200
2106	200	1,000	100	400	50	100	<20	<20	<20	200	<20	<20	100
927/75	400	1,000	1,000	400	50	50	20	<20	<20	200	<20	<20	100
139/77	200	100	200	1,000	200	<20	<20	<20	50	100	20	<20	100

[a] Values are reciprocals of highest dilution of serum causing 2+ agglutination. 1:20 was the lowest dilution tested.

TABLE 3. Complement fixation reactions between four *H. somnus* antisera and homologous and heterologous bacterial antigens

Antiserum to:	Antigens[a]												
	H. somnus				*P. multocida*	*P. haemolytica*	*S. dublin*	*E. coli*	*S. agalactiae*	*S. aureus*	*C. pyogenes*	*B. abortus*	*H. agni*
	M734	2106	927/75	139-77									
M734	256	128	128	128	8	8	<8	<8	<8	<8	<8	<8	128
2106	128	64	32	32	<8	<8	<8	<8	<8	<8	<8	<8	32
927/75	64	64	128	64	8	<8	<8	<8	<8	<8	<8	<8	32
139/77	64	64	64	<8	<8	<8	<8	<8	<8	<8	<8	<8	32
Preimmunization serum	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8

[a] Values are reciprocals of highest titer.

the *H. somnus* antisera (Table 3). Low cross-reacting titers with the two pasteurella antigens (1:8) were observed with only one of the antisera.

In the ELISAs, a diphasic distribution of reactivities was evident with three of the four antigen preparations: cellular, heat extracted, and sonicated. At the top of the absorbency scale (i.e., most positive) was found a cluster headed by *H. somnus* antigens, followed by *H. agni*, and eventually the two *Pasteurella* spp. Separated from this and occupying the lower end of the scale (i.e., most negative) were the absorbencies produced with the remaining six antigens, in a cluster so tight that consistent distinctions between reactivities of the several antigens could not be detected. This pattern is illustrated in Fig. 1. It will be noted that the confidence intervals of the means of homologous and heterologous reactivities overlapped, creating a doubtful zone which covers absorbencies produced by both homologous and heterologous reactions. This situation obtained with the three test series with, respectively, cellular, heat-extracted, and sonicated antigens. The unequivocally positive range never covered any but homologous reactions. It should be added that the three homologous reactions producing the lowest absorbency readings and falling into the doubtful zone with all three antigen

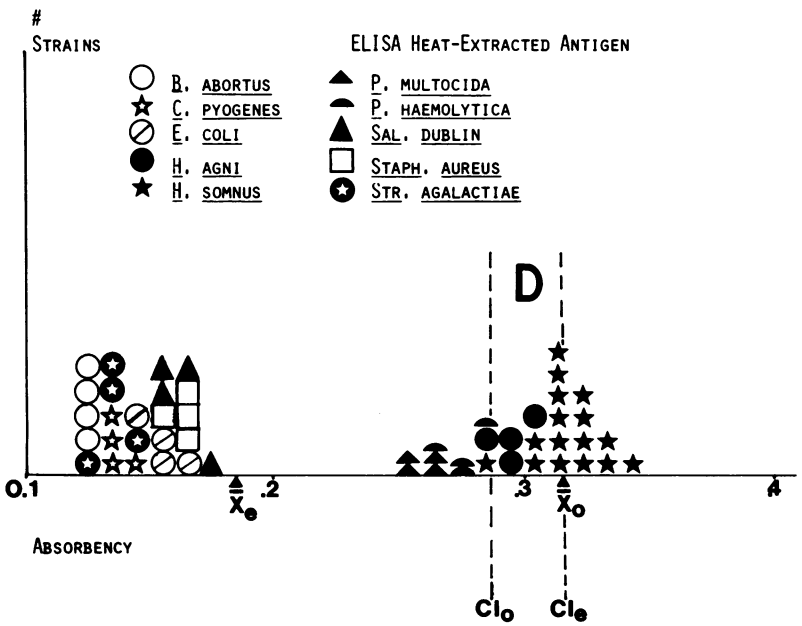

FIG. 1. ELISA reactions between four *H. somnus* antisera and heat-extracted antigens of homologous and heterologous bacterial strains. Abscissa, Absorbency at 415 nm. Ordinate, Strains reacting. $\bar{X}$, Mean; CI_o, homologous confidence interval; CI_e, heterologous confidence interval; D, doubtful zone.

preparations were either between an S antigen (strain 139/77 or 927/75) and an antiserum to an A *H. somnus* culture (strain 2106), or between an A antigen and an S antigen-specific serum (strain 139/77).

A different pattern was observed with the soluble antigen (as defined above; Fig. 2). A diphasic distribution was not as obvious as with the other three series. If such a distribution can be read into these data at all, the *Pasteurella* readings would have to be included into the cluster at the lower end of the scale, i.e., among the heterologous reactions. Most significantly, however, the confidence intervals of the means of homologous and heterologous reading did not overlap, thereby eliminating the doubtful zone. Only two of four *H. agni* reactions fell into the positive range, which otherwise comprised only and all homologous readings.

Since *H. somnus* antibodies exhibited the highest degree of specificity via-à-vis the soluble antigen, further tests were performed to determine its likely suitability as a diagnostic reagent. These involved the use of a rabbit antiserum to a mixture of bacteria which are common in the bovine environment and some of which had been reported to cross-react with *H. somnus* (3, 12). Figure 3 shows the reactions of this serum in an ELISA system, using soluble antigens prepared from the immunizing strains and from *H. somnus* 927/75. Positive reactions were observed only with the antigens used to raise this serum. *H. somnus* antigen, although reacting to somewhat higher titer after, than before, immunization, tested unequivocally negative in the immune serum.

DISCUSSION

Our observations have shown that the cross-reactivity of *H. somnus* antibodies depended greatly on the type of serological procedure and method of antigen preparation employed. Therefore, our results are not readily comparable to those of previous investigations into the cross-reactivity of *H. somnus*. Thus, Dierks et al. (3) in their studies with CF used whole cells as the antigen and observed some positive reactions with antiserum to *P. haemolytica* and *P. multocida*. Miller et al. (12) used indirect hemagglutination in some of their tests. Only the whole cell agglutination tests of Shigidi and Hoerlein (14) and Miller et al. (12) suggest comparability with part of our studies. The former observed no agglutination of *P. haemolytica* in either of two antisera to A strains but did report agglutination of *B. abortus* at low titer by both. Miller et al. observed agglutination of *S. agalactiae* at low titer, but none for *S. dublin*. These differences may well be due to minor antigenic variations among *H. somnus* strains, since considerable diversity in cross-reactivity occurred among *H. somnus* antisera in the several studies, including ours. The one constant feature was the agglutination of *H. agni* by all *H. somnus* sera.

Cross-reactivity was broadest in agglutination tests with whole cell antigens and lowest in ELISA procedures with a soluble extract obtained by the mildest extraction technique. The high degree of specificity attained with this antigen was also approximated by CF tests with the same type of extract and by ELISA tests with whole cell antigens. In the latter instance, it is likely that the soluble antigen is the chief constituent of whatever solid phase is adsorbed onto the tray and subsequently reacts with the sera. Of the four types of antigens tested in the ELISA procedure, the one prepared by sonic disruption and, to a lesser extent, the one obtained by heat extraction gave potentially false positive readings with unrelated antigens. *H. agni* represents a special case to be discussed below.

The diphasic distribution of reactivities, which would be expected in tests of sera with

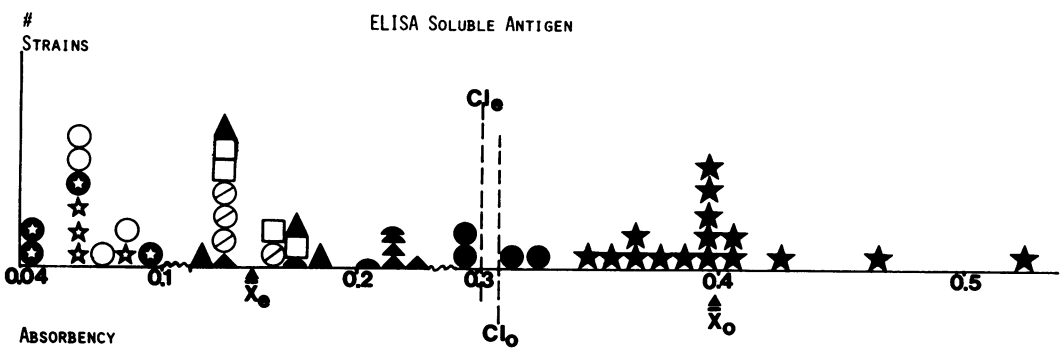

FIG. 2. ELISA reactions between four *H. somnus* antisera and soluble antigens of homologous and heterologous strains. For explanation of symbols and abbreviations, see Fig. 1 and its legend.

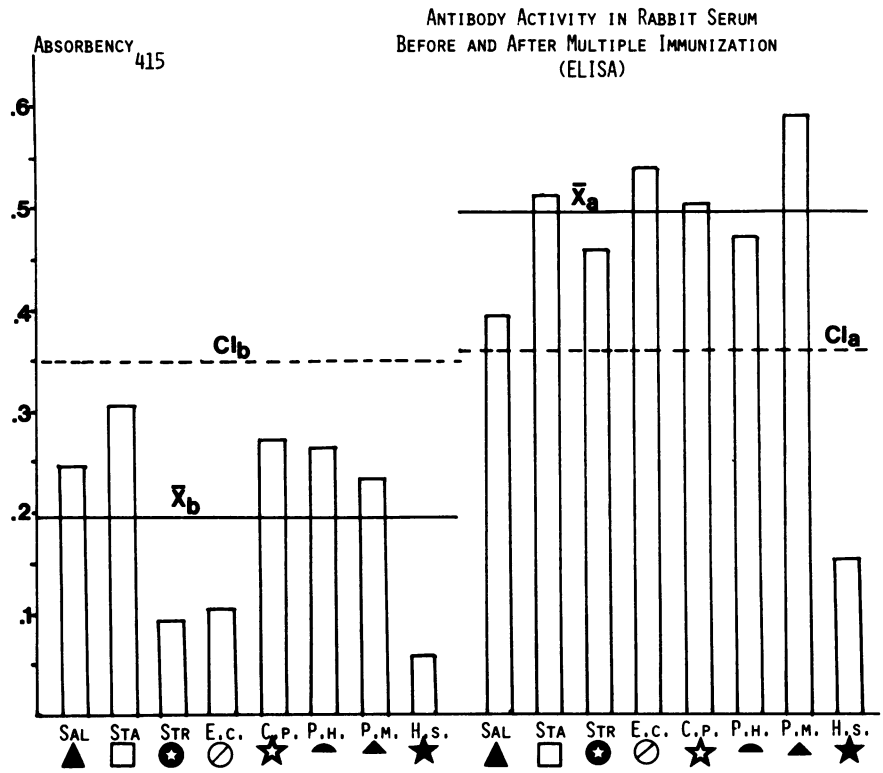

FIG. 3. Antibody activity in rabbit serum before and after multiple immunization. CI, Confidence interval; $\bar{X}$, mean; b, before immunization; a, after immunization. For other symbols, see Fig. 1.

homologous and heterologous antigens, had some noteworthy features in the case of the ELISAs: most of the heterologous reactions were clustered at the low end of absorbency values. With three of the four types of antigen preparations used, however, the upper end of the absorbency scale was occupied not only by the values representing homologous reactions but also, continuous with them in the lower end of the cluster, by those produced with *H. agni* and *Pasteurella* antigens. This phenomenon was most obvious in the series of tests done with heat-extracted antigens (Fig. 1) but was clearly discernible in those with whole cells and sonic extracts as well. This pattern suggests a certain relatedness between the pasteurellae on the one hand and *H. agni* and *H. somnus* on the other that agrees with recent observations of other similarities and possible taxonomic ties among this group of bacteria (11). Only by the soluble antigen were the pasteurellae clearly separable from the *H. somnus-H. agni* range of reactivities (Fig. 2).

The close resemblance of *H. agni* and *H. somnus* was recognized at the time the latter organism was first described, and serological cross-reactivity was reported as part of that resemblance (5). The question of whether the two agents deserve separate specific recognition is probably legitimate. On the basis of the results of the ELISA tests, despite consistent cross-reactivity, they do appear to be serologically distinct ($P < 0.01$; with soluble antigen, $P < 0.002$).

The superior specificity and sensitivity shown by soluble antigens in ELISA with homologous antibody was confirmed in a model simulating field conditions under which a serological test for *H. somnus* might be used. A serum raised against a number of agents (other than *H. somnus*) common in cattle and shown to contain antibody to such agents failed to react positively with *H. somnus* soluble antigen. Previous investigators had reported cross-reactivity of several of these antibodies with *H. somnus* (3, 12).

We reported previously (2), on the basis of agglutinin cross-adsorption studies, on the existence of several sets of surface antigenic determinants in *H. somnus*: a common (C), an A, and an S factor, which can occur in various combinations. Of the four *H. somnus* strains used in the present study, all had been shown previously to possess the C, two the A, and two the S factor (2). Since all antigens prepared from them reacted with all of the sera, the presence of C factor in all the preparations tested, including particularly

the soluble ones, may be assumed. The participation and significance of the A and S factors cannot be unequivocally established from our data. Although in the ELISA series the lowest readings with *H. somnus*-derived antigen were always obtained when S antigen reacted with A serum and vice versa, the difference between such heterologous reactions and the homologous reactions was not statistically significant ($P > 0.05$, Wilcoxon's sum of ranks test; 7). Antibodies to C factor are apparently not responsible for the various cross-reactions observed with other microbial species except possibly *H. agni*, since none of the antigens from these other species reacted significantly with *H. somnus* antiserum in the ELISA series with soluble antigen. The results of ELISA tests with soluble antigen suggest a highly sensitive and specific approach to serological studies of *H. somnus* infections.

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