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Serological Differentiation Between Infected and Vaccinated Cattle by Using Purified Soluble Antigens from *Brucella abortus* in a Hemagglutination System

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Serological tests used in current brucellosis eradication schemes, such as bacterial tube agglutination, do not readily distinguish between infected animals and those immunized with strain 19 or 45/20 *Brucella abortus* vaccines. In this study, sera from naturally infected cattle were used to identify serologically important antigens in extracts of virulent *B. abortus* by gel diffusion techniques. Antisera from rabbits hyperimmunized with selected precipitation lines were used for purification by affinity chromatography of two precipitating and one nonprecipitating antigen from crude bacterial extracts. A passive hemagglutination test using these antigens was developed. A number of characterized bovine sera were screened by passive hemagglutination and conventional bacterial tube agglutination tests. A considerable improvement in discrimination between sera from infected and vaccinated cattle was obtained with the hemagglutination test compared with bacterial tube agglutination.

Current schemes for brucellosis eradication rely on herd vaccination, with identification and slaughter of infected animals (2). Difficulties in differentiating infected animals from those immunized with strain 19 or 45/20 *Brucella abortus* vaccines can result in unnecessary slaughter of animals (1, 6, 7). This economic factor has hindered eradication schemes, and as a consequence several investigators have looked for serological differences between these two groups but have been unsuccessful in achieving good discrimination (5, 9, 14, 20, 21, 26).

In this investigation, sera from naturally infected cattle were used to identify serologically important antigens in extracts of virulent *B. abortus* by gel diffusion techniques. Rabbits were immunized with selected precipitation lines, and after removal of antibovine activity, the antisera were used for purification of antigens from crude bacterial extracts by affinity chromatography. Purified antigens and crude extracts were coupled to fixed erythrocytes and used in a passive hemagglutination (HA) system to screen serum samples from infected and vaccinated cattle.

MATERIALS AND METHODS

Serum samples. Eighty-eight sequential serum samples taken from a total of six cows (numbers 1 through 6) were used. Details of the history of each cow are summarized: numbers 1 and 2 were vaccinated with Duphavac (N. V. Philips-Duphar, Amsterdam) (dead rough strain 45/20) on day 1; number 3 was vaccinated with strain 19 (smooth, attenuated, living strain) on day 1; number 4 was vaccinated with strain 19 as a calf but was subsequently infected with *B. abortus*, and serum samples were taken from the day after birth of infected calf; number 5 was vaccinated with strain 19 as a calf but became infected with *B. abortus* during the course of the experiments (acute infection); number 6 was vaccinated with strain 19 as a calf but subsequently became infected with *B. abortus* (chronic infection).

A pool of sera from vaccinated and from infected animals was prepared from individual bleeds taken from animals 3 and 6, respectively. A pool of serum from noninfected, nonvaccinated cattle was also used.

Antigen extracts. Sodium dodecyl sulfate (SDS) extracts were prepared from a 10% (vol/vol) suspension of *B. abortus* strain 544/W (19).

Immunization of rabbits. Immunodiffusion plates were prepared by using 1% purified agar (Oxoid) in 0.15 M saline. A series of troughs were cut 10 mm apart, and alternate troughs were filled with SDS extract of *B. abortus* strain 544/W and pooled infected bovine serum. After incubation for 3 days at 4°C, two discrete precipitation lines, designated B and C, were present between each pair of troughs. Each line was cut out of the agar and washed with frequent changes of 0.15 M saline for 2 days and then emulsified in Freund complete adjuvant to give a total volume of 3 ml. New Zealand white rabbits were immunized intramuscularly with 1 ml of emulsion and boosted with similar injections until satisfactory antisera were obtained.

Preparation of immunoadsorbents. Immunoadsorbents were prepared by coupling appropriate material to Sepharose CL4B activated with sodium metaperiodate (18). The following immunoadsorbents were used.

(i) Sepharose CL4B-normal bovine serum.

Sepharose CL4B-normal bovine serum was used for the removal of antibovine activity from rabbit antisera raised against precipitation lines B and C before evaluation in two-dimensional immunoelectrophoresis (2D-IE), using an intermediate gel technique (4). Coupling was performed at a concentration of 25 mg of protein per g of gel.

(ii) Sepharose CL4B-immunoglobulin G fraction of rabbit anti-B or -C. Sepharose CL4B-immunoglobulin G fraction of anti-B or -C was used for purification of antigens from crude 544/W extracts. Five milligrams of protein per gram of gel was used for coupling.

(iii) Sepharose CL4B-purified antigens. Sepharose CL4B-purified antigens were prepared with appropriate concentrations of affinity chromatography-purified antigens from the 544/W extract.

Immunoadsorbents ii and iii were packed into columns over an equal volume of Sephadex G-25 to facilitate rapid separation of eluted antigens from eluent buffer during purification by affinity chromatography.

Affinity chromatography. Removal of antibovine activity from rabbit antisera was achieved by applying samples to Sepharose-normal bovine serum columns and washing through with 10 mM phosphate-buffered saline, pH 7.5. Antigen and antibody purification was performed by passing the crude 544/W extract or infected bovine serum pool through appropriate Sepharose-antibody or Sepharose-antigen immunoadsorbent columns. After emergence of unadsorbed material, the columns were eluted with 2 M sodium thiocyanate in 10 mM phosphate buffer (pH 6.6) to remove bound components. Both washes and eluted material from each column were collected, concentrated, and examined by the techniques described in Results.

Gel diffusion techniques. (i) 2D-IE. The specificity of rabbit antisera and the purity of antigens and antibodies eluted from immunoadsorbent columns were assessed by 2D-IE, using 1% agarose in 50 mM Veronal buffer (25). For preliminary investigations of antigen purity, separation in the first dimension was followed by electrophoresis in the second dimension into agarose containing 12.5% pooled infected bovine serum.

(ii) 2D-IE with intermediate gel. Identification of individual precipitation peaks obtained with different antigen preparations and evaluation of the specificity of rabbit antisera were performed by using an intermediate gel technique (4). In this method, antigen preparations were electrophoresed in the second dimension through an intermediate gel containing the test antiserum into the "reference" gel containing pooled infected bovine serum, thus enabling investigation of immunological relationships between the antigen preparation and antibodies present in each gel.

(iii) Immunodiffusion. Immunological identity between components in crude SDS extracts and purified antigen, and between different antisera, was investigated by Ouchterlony double immunodiffusion in 1% purified agar containing 50 mM Veronal buffer, pH 8.6.

Sensitization of SRBCs. Sheep erythrocytes (SRBCs) were initially fixed with pyruvic aldehyde and formaldehyde, using the method of Prince et al. (16) but substituting 10 mM phosphate-buffered saline, pH 7.6, for 5% sodium citrate buffer. The cells were further treated with tannic acid followed by sensitization with an appropriate dilution of antigen preparation or with the immunoglobulin fraction of pooled infected bovine serum for detection of antibody or antigen, respectively.

HA tests. Test sera or antigen preparations were titrated from an initial dilution of 1:5 in V-well microtiter plates (Dynatech Ltd.), using 0.1 M phosphate-buffered saline (pH 7.2) containing 1.5% normal rabbit serum preabsorbed with 10% fixed SRBCs. An equal volume of a 1% suspension of the appropriately sensitized SRBCs in the same buffer was added. After gentle agitation, the plates were covered and allowed to stand at room temperature for 18 h before endpoint agglutination titers were recorded.

Bacterial tube agglutination tests. Tube agglutination tests were performed by the method of Alton et al. (3), using *B. abortus* strain 99-stained suspension (Wellcome Reagents Ltd.). Endpoint agglutination titers were recorded, avoiding the need for scoring degrees of reaction.

RESULTS

Reactivity of crude extracts in gel diffusion techniques. Analysis by 2D-IE of 544/W extract against pooled infected bovine serum produced three separate precipitation peaks. These peaks were designated α, β, and γ (Fig. 1). No peaks were obtained in similar tests against vaccinated or negative bovine serum pools. Ouchterlony immunodiffusion tests on 544/W extract against pooled infected bovine serum produced at least three precipitation lines, but when parallel troughs were cut in gel 10 mm apart and filled alternately with extract or infected serum, only two discrete lines, designated B and C, were observed.

Analysis of rabbit antisera. Antisera from rabbits hyperimmunized with precipitation lines B and C gave strong reactions in gel diffusion against 544/W extract and normal bovine serum. The activity against bovine immunoglobulins present in the precipitation lines used for immunization was removed by affinity chromatography on the Sepharose CL4B-normal bovine serum immunoadsorbent. Reactivity against 544/W extract was retained after this treatment. Immunodiffusion tests with selected absorbed rabbit antisera (B5 and C9) showed clear nonidentical precipitation lines, confirming that they contained antibody against two immunologically distinct antigens present in 544/W extract.

To identify the antigens involved, antisera B5 and C9 were tested in intermediate gel 2D-IE; the results are shown in Fig. 1. A reduction in the size of a single peak and an extension of the precipitation line forming this peak into the

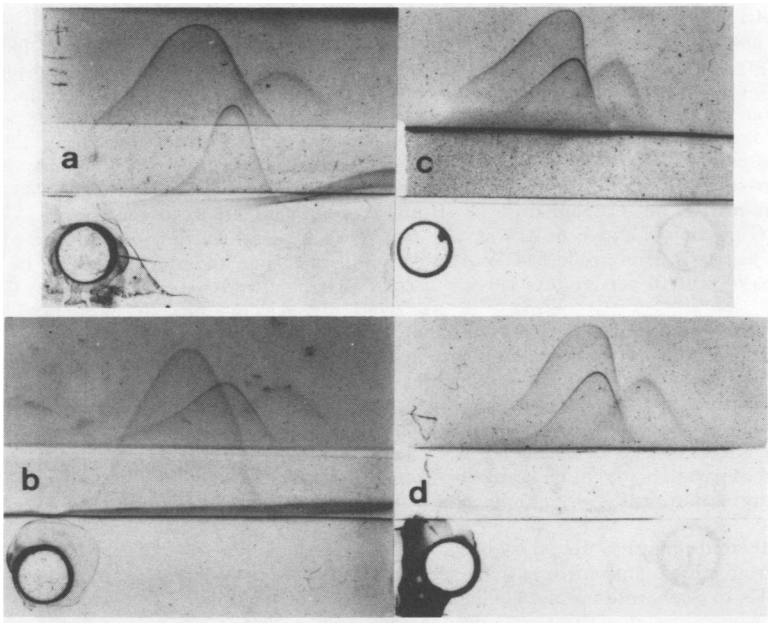

FIG. 1. *Wells contained 544/W extract. Reference gels contained a 12.5% pool of infected bovine serum. Intermediate gels contained: (a) 10% absorbed antiserum against β line (rabbit B5); (b) 30% absorbed immunoglobulins from anti-γ serum (rabbit C9); (c, d) normal rabbit serum.*

intermediate gel indicate that the intermediate and reference gels contain antibody of identical specificity. These tests show that antisera B5 and C9 contain specific reactivity against the antigens forming peaks β and γ, respectively.

Purification of antigens. Purification of β and γ antigens from 544/W extract was performed by affinity chromatography, using immunoadsorbents prepared from antisera B5 and C9 as described above. Similar elution profiles were obtained with both columns. Some fractionation of nonadsorbed components in 544/W extract occurred during passage through the column (wash), presumably due to the molecular-sieve effect of the Sephadex G-25. The peak of purified antigen obtained after elution with sodium thiocyanate was also separated from the eluent buffer by passage through the Sephadex G-25 portion of the column.

2D-IE using intermediate gels containing rabbit antisera B5 and C9 was performed on concentrated washes and eluates from each column in order to identify the components present. The immunoadsorbent columns effectively adsorbed the corresponding antigens as the eluate peaks contained selectively purified immunologically distinct antigenic components β and γ (Fig. 2).

2D-IE tests on the washes from each column showed variable results depending on the volume and concentration of the sample applied. Application of large samples resulted in detectable levels of specific antigens appearing in the first peak of the wash, indicating overloading of the column. If the sample volume was too small, specific antigen was not detectable in the eluate, emphasizing the need for optimizing the volumes used. The optimal sample volume in this system was approximately 2% of the column volume.

HA tests with affinity chromatography-purified antigens. SRBCs sensitized with affinity chromatography-purified β and γ antigen preparations (SRBC-β, SRBC-γ) and crude 544/W extract (SRBC-544/W) were tested for HA activity against titrations of infected and vaccinated bovine serum pools and B5 and C9 rabbit antisera. The results in Table 1 show that similar discrimination was obtained between infected and vaccinated bovine serum pools irrespective of the antigen preparation used for sensitization. Minimal titers were observed with B5 and C9 antisera even against SRBCs sensitized with the homologous antigen, indicating that insignificant levels of β and γ antigens were coupled and as a consequence were unlikely to contribute towards the serological differences observed. This apparent dissociation of precipitating antigen (β and γ) activity and HA activity was confirmed by absorption experiments.

Samples of B5 and C9 antisera were exhaustively absorbed with SRBCs sensitized with β and γ antigen preparations and crude 544/W extract and then tested for HA activity and

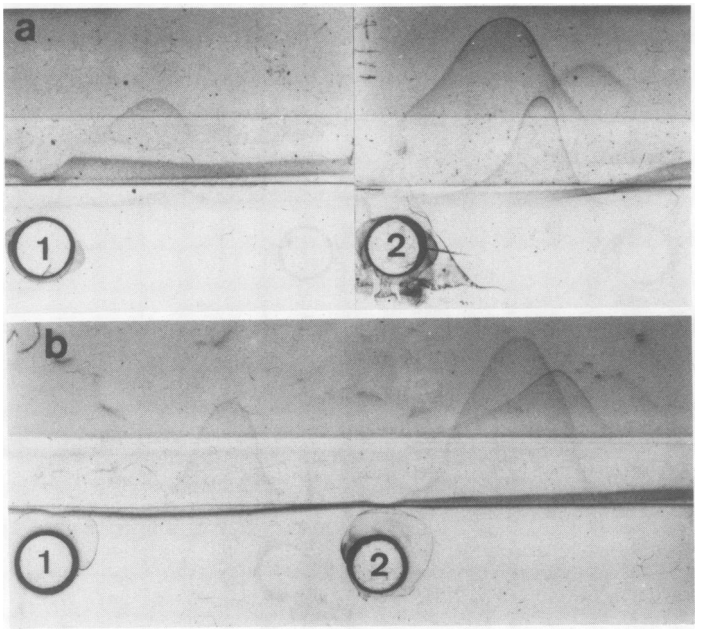

FIG. 2. *Reference gels contained a 12.5% pool of infected bovine serum. (a) Well 1, Eluate from B5 column (β antigen); well 2, 544/W extract. Intermediate gel was 10% absorbed B5 antiserum. (b) Well 1, Eluate from C9 column (γ antigen); well 2, 544/W extract. Intermediate gel was 30% absorbed C9 immunoglobulins.*

TABLE 1. *HA studies using SRBCs sensitized with affinity chromatography-purified β and γ antigen preparations and crude 544/W extract*

Antiserum	Reciprocal of HA titer against SRBCs sensitized with:			
	β antigen prepn	γ antigen prepn	544/W extract	No antigen
Infected bovine serum pool	20,480	20,480	20,480	<5
Vaccinated bovine serum pool	80	80	80	<5
B5 antiserum	40	40	40	<5
C9 antiserum	5	5	5	<5
Nonimmune rabbit serum	<5	<5	<5	<5

precipitating antibody activity in 2D-IE with 544/W extract. Absorption removed all HA activity, but strong precipitating activity against β and γ antigens remained in the antisera, thus confirming the failure of β and γ antigens to bind to SRBCs.

Similar experiments were performed by using immunoadsorbent columns containing Sepharose coupled to affinity chromatography-purified β and γ antigen preparations. Samples of B5 and C9 rabbit antisera and the infected bovine serum pool were absorbed through each column. Washes and eluates were tested by 2D-IE against 544/W extract and for HA activity against SRBC-β, SRBC-γ, and SRBC-544/W. The results in Table 2 show that precipitating activity against β or γ antigens was confined to column washes whereas HA reactivity was present predominantly in column eluates. These data provide evidence that β and γ antigens were neither coupled to Sepharose beads nor responsible for the discriminatory HA reactivity.

Antigens responsible for discriminatory HA activity. The presence of additional antigen(s), specifically bound by both B5 and C9 Sepharose immunoadsorbents and tanned SRBCs, was investigated by passing β and γ antigen preparations through the heterologous C9 and B5 immunoadsorbent columns, respectively. The washes and eluates were tested for HA activity against SRBCs sensitized with immunoglobulins from the infected bovine serum pool. Most of the HA activity present in the applied sample was bound by both B5 and C9 immunoadsorbent columns and present in the eluate, showing that the antigenic components responsible for HA discrimination between pools of bovine sera from infected and vaccinated animals were bound reversibly by both B5 and C9 immunoadsorbent columns and were therefore present in both affinity chromatography-purified β and γ preparations. These components were designated antigen X. Since this antigen adsorbed equally to B5 and C9 antibody-Seph-

arose columns, attempts were made to determine whether the interaction was due to immunological or non-immunological binding. Immunological binding might have been due to the presence of "nonspecific" antibodies in B5 and C9 antisera, and non-immunological binding might have been due to direct adsorption of this antigen by Sepharose gel.

Control columns were prepared with nonimmune rabbit immunoglobulin coupled to activated Sepharose CL4B and with unactivated noncoupled Sepharose CL4B overlaid on Sephadex G-25. Passage of 544/W extract through these columns showed that similar amounts of HA-reactive material (antigen X) were bound and eluted to that obtained from B5 and C9 immunoadsorbent columns. These results indicate that antigen X was bound by non-immunological adsorption directly to Sepharose CL4B. Precipitating antigens β and γ were not bound by these control columns.

These results indicate that discrimination between infected and vaccinated bovine serum pools by HA was not due to the precipitable α, β, or γ antigen, but rather to another component in the 544/W extract, designated antigen X, which was also present in affinity chromatography-purified β and γ antigen preparations. The data in Tables 1 and 2 indicate that tanned SRBCs and activated Sepharose beads selectively bind antigen X from crude extract and affinity chromatography-purified antigen preparations. If this were so, then similar HA activity would be seen with SRBC-β, SRBC-γ, and SRBC-544/W. Ninety-two characterized bovine sera from infected and vaccinated animals were therefore coded and tested by HA against these sensitized SRBCs.

Figure 3 shows that excellent discrimination between sera from infected and vaccinated cattle was obtained irrespective of the antigen preparation used for erythrocyte sensitization, confirming the major involvement of antigen X in the discriminatory ability of this system.

Correlation between HA and bacterial tube agglutination tests. The 92 bovine sera

TABLE 2. *HA and precipitating activity in bovine and rabbit antisera before and after passage through Sepharose-antigen immunoadsorbent columns*[a]

Sample	Serum adsorbed					
	B5		C9		Infected bovine pool	
	HA titer	Lines in 2D-IE	HA titer	Lines in 2D-IE	HA titer	Lines in 2D-IE
544/W control	40	β	5	γ	10,240	α, β, γ
β column wash	<5	β	<5	γ	<10	α, β, γ
β column eluate	20	None	<5	None	2,560	None
γ column wash	<5	β	<5	γ	2,560	α, β, γ
γ column eluate	20	None	<5	None	2,560	None

[a] Sera were passed through Sepharose-β antigen and Sepharose-γ antigen immunoadsorbent columns. The wash containing unadsorbed antibody and the eluate containing affinity chromatography-purified antibody were tested in HA and 2D-IE. HA reactivity was tested with SRBCs sensitized with crude extracts and with β or γ antigens. Analogous titers were found with each group of sensitized SRBCs.

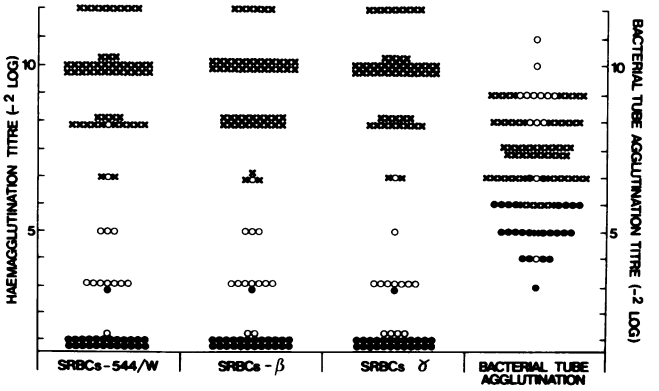

FIG. 3. *Sera from infected* (×), *strain 19-vaccinated* (○), *or strain 45/20-vaccinated* (●) *cattle.*

examined in the HA system were also tested by bacterial tube agglutination. Figure 3 shows that whereas HA titers were consistently low with sera from vaccinated animals, these samples were distributed throughout the titer range in bacterial agglutination tests, showing lack of correlation between the two test procedures.

DISCUSSION

Several authors have demonstrated precipitation lines, similar to those reported in this study, with a variety of *Brucella* extracts (10, 12, 13, 23, 24). The differential precipitation response seen with infected and vaccinated cattle indicated that the use of selected antigens in a diagnostic system might enable improved discrimination between these two groups of sera (19). Purification of selected antigens from SDS extracts of *B. abortus* was attempted by using immunoadsorbents prepared from rabbit antisera raised against individual precipitation lines.

Affinity chromatography enabled purification of antigens which were reactive in gel precipitation tests and, when bound to SRBCs, enabled excellent differentiation of infected from vaccinated bovine sera by passive HA. Subsequent experiments showed a disassociation of precipitating and HA activity, indicating that the β and γ antigens were not involved in the discriminatory ability of the HA system. 2D-IE and HA tests on bovine antibodies separated by affinity chromatography confirmed that the β and γ antigens were not coupled to sensitized SRBCs or activated Sepharose beads. The differential reactivity of bovine sera in both HA and immunofluorescence (19) systems was therefore due to another component in the 544/W extract which was selectively bound by tanned SRBCs and activated Sepharose beads. The presence of this component, designated antigen X, in affinity chromatography-purified β and γ preparations was due to its non-immunological adsorption from the crude extract (by Sepharose CL4B) and elution by sodium thiocyanate. Antigen X has not been described previously, probably because most authors have used immunoprecipitation procedures for examination of antigenic components in extracts. Antigen X did not form a precipitation line in gel tests with bovine antisera despite its extremely high activity in passive HA systems. Non-precipitation may have been due to the presence of SDS, which can prevent the occurrence of immunological reactions (8, 11, 17), although this seems unlikely as SDS neither interfered with the reactivity of antigen X in HA systems nor prevented precipitation of other antigens in the 544/W extract. Furthermore, no differences were observed between the precipitation characteristics of β and γ antigens in 544/W extract which contained 0.5% SDS and affinity chromatography-purified antigen preparations which contained no free SDS.

The preferential binding of antigen X from crude 544/W extract or affinity chromatography-purified β and γ preparations indicates that it is the major component responsible for the improved discriminatory activity seen both in the immunofluorescence system described previously (19) and in this HA test. The mechanism of antigen binding to fixed, tanned SRBCs is not known. However, the reactions involved in coupling to sodium metaperiodate-activated Sepharose gels have been described by Sanderson and Wilson (22). Aldehyde groups created by oxidation of sugar subunits of the agarose gel condense with amino groups present on the antigen molecule. Thus, a high concentration of amino groups on antigen X might encourage preferential binding by activated Sepharose. The presence of amino groups might also be responsible for reversible adsorption of this antigen to nonactivated Sepharose and activated Sepharose coupled to nonimmune rabbit immunoglobulins, as all cross-linked Sepharose gels contain a small number of charged groups which can cause adsorption of basic proteins (15).

HA tests enabled accurate identification of the infection status of animals in 95% of bovine sera tested, a considerable improvement over current bacterial tube agglutination or complement fixation tests (6, 7). Only five sera gave inconclusive HA titers; two of these were samples taken from an animal 14 and 28 days after strain 19 vaccination, and the other three were from an acute case taken within 6 weeks of actual contact with another actively infected animal. These results suggest that serological discrimination between infected and vaccinated cattle should be possible by systems utilizing antigen X from 6 weeks after inoculation with the virulent or vaccine organism.

The improved discrimination obtained with serological systems involving antigen X may be due to differential distribution of this component on the surface of virulent and vaccine strains of *B. abortus* or differences in the immune response to infection with brucellosis and vaccination against the disease. Studies are in progress to clarify which of these mechanisms is responsible.

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LITERATURE CITED

1. **Allan, G. S., R. J. Chappel, P. Williamson, and D. J. McNaught.** 1976. A quantitative comparison of the sensitivity of serological tests for bovine brucellosis to different antibody classes. J. Hyg. **76**:287-298.
2. **Alton, G. G.** 1978. Recent developments in vaccination against bovine brucellosis. Aust. Vet. J. **54**:551-557.
3. **Alton, G. G., L. M. Jones, and D. E. Pietz.** 1975. Laboratory techniques in brucellosis, 2nd ed. W.H.O. Monogr. Ser. No. 55.
4. **Axelson, N. H.** 1973. Intermediate gel in crossed and in fused rocket immunoelectrophoresis. Scand. J. Immunol. **2**(Suppl. 1):71-78.
5. **Beh, K. J.** 1974. Quantitative distribution of brucella antibody amongst immunoglobulin classes in vaccinated and infected cattle. Res. Vet. Sci. **17**:1-4.
6. **Chappel, R. J., D. J. McNaught, J. A. Bourke, and G. S. Allan.** 1978. Comparison of the results of some serological tests for bovine brucellosis. J. Hyg. **80**:365-371.
7. **Chappel, R. J., D. J. McNaught, J. A. Bourke, and G. S. Allan.** 1978. The diagnostic efficiency of some serological tests for bovine brucellosis. J. Hyg. **80**:373-384.
8. **Crumpton, M. J., and R. M. E. Parkhouse.** 1972. Comparison of the effects of various detergents on antigen-antibody interactions. FEBS Lett. **22**:210-212.
9. **Diaz, R., P. Garatea, L. M. Jones, and I. Moriyon.** 1979. Radial immunodiffusion test with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle. J. Clin. Microbiol. **10**:37-41.
10. **Diaz, R., L. M. Jones, D. Leong, and J. B. Wilson.** 1968. Surface antigens of smooth *Brucellae*. J. Bacteriol. **96**:893-901.
11. **Dimitriadis, G. J.** 1979. Effect of detergents on antibody-antigen interactions. Anal. Biochem. **98**:445-451.
12. **Freeman, B. A., J. R. McGhee, and R. E. Baughn.** 1970. Some physical, chemical and taxonomic features of the soluble antigens of *Brucellae*. J. Infect. Dis. **121**: 522-527.
13. **Hinsdill, R. D., and D. T. Berman.** 1967. Antigens of *Brucella abortus*. I. Chemical and immunoelectrophoretic characterization. J. Bacteriol. **93**:544-549.
14. **Ianelli, D., R. Diaz, and T. Manlio-Bettini.** 1976. Identification of *Brucella abortus* antibodies in cattle serum by single radial immunodiffusion. J. Clin. Microbiol. **3**: 203-205.
15. **Pharmacia Fine Chemicals, Inc.** 1979. Sepharose CL. *In* Gel filtration: theory and practice, p. 22. Pharmacia Fine Chemicals, Inc., Piscataway, N.J.
16. **Prince, A. M., H. Ikram, D. Chicot, R. Wright, J. Vnek, R. Neurath, A. Lippin, and S. Swiss.** 1975. A new reversed passive haemagglutination test for detection of HBsAg. Vox Sang. **29**:319-329.
17. **Qualtiere, L. F., A. G. Anderson, and P. Meyers.** 1977. Effects of ionic and non-ionic detergents on antigen-antibody reactions. J. Immunol. **119**:1645-1651.
18. **Raybould, T. J. G., and S. Chantler.** 1979. Comparison of activation and blocking procedures in the use of defined antigen substrate spheres (DASS) for quantitative and visual assessment of conjugate activity. J. Immunol. Methods **27**:309-318.
19. **Raybould, T. J. G., and S. Chantler.** 1979. Serological differentiation between infected and vaccinated cattle by visual and quantitative immunofluorescence using *Br. abortus* antigen coupled Sepharose beads. J. Immunol. Methods **30**:37-46.
20. **Rice, C. E., D. Cochrane, and J. Tailyour.** 1966. Electrophoretic studies of sera from cattle vaccinated or naturally infected with *Br. abortus*. Can. J. Comp. Med. Vet. Sci. **30**:161-168.
21. **Rice, C. E., D. Cochrane, and J. Tailyour.** 1966. Ultracentrifugal studies of sera from cattle vaccinated or naturally infected with *Br. abortus*. Can. J. Comp. Med. Vet. Sci. **30**:270-278.
22. **Sanderson, C. J., and D. V. Wilson.** 1971. A simple method for coupling proteins to insoluble polysaccharides. Immunology **20**:1061-1065.
23. **Schurig, G. G., L. M. Jones, S. L. Speth, and D. T. Berman.** 1978. Antibody response to antigens distinct from the smooth lipopolysaccharide complex in *Brucella* infection. Infect. Immun. **21**:994-1002.
24. **Stemshorn, B., and K. Nielsen.** 1977. Bovine immune response to *Br. abortus*: water soluble antigen precipitated by sera of some naturally infected cattle. Can. J. Comp. Med. **41**:152-159.
25. **Weeke, B.** 1973. Crossed immunoelectrophoresis. Scand. J. Immunol. **2**(Suppl. 1):47-56.
26. **Wood, W. A., and M. J. Corbel.** 1973. Concentrations of bovine serum protein classes in relation to reactivity in serological tests for brucellosis. J. Comp. Pathol. **83**: 143-150.