Acta vet. scand. 1972, *13*, 472—483.

From the National Veterinary Institute, Stockholm, Sweden.

SEROLOGICAL CROSS-REACTIONS BETWEEN DIFFERENT BRUCELLA SPECIES AND YERSINIA ENTEROCOLITICA

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS

By

B. Hurvell

HURVELL, B.: *Serological cross-reactions between different Brucella species and Yersinia enterocolitica. Immunodiffusion and immunoelectrophoresis.* Acta vet. scand. 1972, *13*, 472—483. — By immunodiffusion and immunoelectrophoresis tests in agarose serological cross-reactions were demonstrated between Yersinia enterocolitica type IX and Brucella strains from four species (Brucella abortus, Brucella melitensis, Brucella suis and Brucella neotomae). No qualitative differences between these strains in their tendencies to cross-react with Yersinia enterocolitica type IX were observed. Brucella canis and Brucella ovis, which have nonsmooth colonial morphology, gave no demonstrable cross-reaction with Yersinia enterocolitica type IX.

The results of absorption tests and qualitative staining reaction of the obtained precipitation lines suggest that the antigenic determinants common to Brucella and Yersinia enterocolitica type IX seemed to be associated with the outer layer and in the lipopolysaccharide complex of the respective bacteria. By immunodiffusion and immunoelectrophoresis it was possible to identify in hyperimmune sera those antibodies that derive from Brucella and Yersinia enterocolitica type IX.

serological cross-reaction; Brucella species; Yersinia enterocolitica serotype IX; immunodiffusion; immunoelectrophoresis.

A strong serological cross-reaction between Yersinia enterocolitica type IX and Brucella abortus and melitensis has been demonstrated by agglutination test (*Ahvonen et al.* 1969) and by both complement fixation and agglutination tests (*Hurvell et al.* 1970, 1971). In the latter two studies a cross-reaction between Yersinia enterocolitica type IX and Brucella suis was also observed. *Diaz et al.* (1970) also reported that Brucella abortus and

Brucella melitensis share in common antigenic determinants with Yersinia enterocolitica type IX.

The objects of the present work were to examine the antigenic relationships between different Brucella species and Yersinia enterocolitica type IX, using immunodiffusion and immunoelectrophoresis, and to find out whether the various Brucella species differed qualitatively in their tendencies to cross-react with Yersinia enterocolitica type IX.

MATERIAL AND METHODS

Bacterial strains

Yersinia enterocolitica type IX (Y.e.). A representative strain (M.Y. 79) was used in a previous study (*Hurvell et al.* 1971).

Brucella abortus 544 (B.a.), Brucella melitensis 16M (B.m.), Brucella suis 1330 (B.s.), Brucella neotomae 5K33 (B.n.), Brucella canis R.M. 6/66 (B.c.) and Brucella ovis 63/90 (B.o.). B.a., B.m., B.s. and B.n. are of smooth colonial morphology. Evidence of smoothness was obtained by the oblique light technique (*Henry* 1933) and the acriflavine test (*Braun & Bonestell* 1947). B.c. and B.o. are of nonsmooth colonial morphology. All the Brucella strains are FAO/WHO reference strains and were obtained from Dr. W. J. Brinley Morgan, Central Veterinary Laboratory, Weybridge, England.

Media

Subcultures of smooth Y.e. were grown in Roux flasks with meat extract agar (0.5 % Bacto Beef Extract, Difco; 10 % Peptone, Merck) and incubated for 48 hrs. at 22°C.

The Brucella strains were grown in Roux flasks with serum-dextrose agar (5 % horse serum, 1 % dextrose) for three-four days at 37°C. B.c. organisms grown on this medium were difficult to suspend in saline. The addition of 2 % normal rabbit serum instead of horse serum to the dextrose agar eliminated this drawback (*Diaz et al.* 1968).

B.a. and B.o. require an atmosphere of 10 % CO_2 for good growth. The bacterial cultures were harvested with 0.15 M-NaCl. The cells were sedimented by centrifugation at 3000 × g for 40 min., resuspended in cold 0.15 M-NaCl and washed twice with distilled water.

Antigens

Soluble test antigens. An amount of 1 ml of packed viable cells was suspended in 4 ml of distilled water (*Baughn & Freeman* 1966). The cells were disintegrated by the use of a 20 kc 60-W MSE Ultrasonic Disintegrator (Measuring & Scientific Equipment Ltd, London) operating with full power. The breakage of the cells was achieved at 3°C in 10 min. The effectivity of the disintegration was checked microscopically. The disrupted organisms were centrifuged at 5500

× g for 1 hr. at 5°C. To effect sterility, formalin was added to a final concentration of 1 %. The protein-nitrogen contents were determined by micro-Kjeldahl analysis (*Miller & Houghton* 1945). The conventional factor of 6.25 was used in order to convert the nitrogen value to protein content. The antigen suspensions were adjusted so as to contain 20—25 mg of protein per ml.

Antigens for the preparation of antisera. One volume of washed and packed cells was suspended in four volumes of distilled water. Three volumes of chilled acetone were added to one volume of cell suspension. The mixture was stored overnight at —20°C and the cells were then washed three times in cold acetone and dried in desiccator over $CaCl_2$ (*Diaz et al.* 1967).

Antigens to the absorption tests. Washed and packed cells were suspended overnight in phosphate-buffered saline (pH 7.2) with 0.5 % formalin. The cells were then washed three times in phosphate-buffered saline (pH 7.2).

Antisera

Specific antisera against the different strains were prepared in rabbits weighing, on the average, 3½ kg. The preimmunization sera contained no antibodies to Yersinia, Brucella or Pasteurella tested by immunodiffusion and immunoelectrophoresis in agarose. The rabbits were immunized with a preparation of 10 mg of acetone-killed organisms per ml of Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan, USA). One ml of this emulsion was used for subcutaneous injections twice weekly for seven weeks and the rabbits were bled one week after the last injection. Two rabbits were immunized with each strain. The sera were preserved with 1:10000 merthiolate and stored at —20°C.

Absorptions of antisera

Five ml of each serum were absorbed with 1 ml of the packed formalin-killed and washed cells. The mixtures were placed on a shaker at 37°C for 2 hrs. and were then stored at 5°C for 18 hrs. The precipitates were removed by centrifugation at 4000 × g. The sera were stored at 5°C. If the homologous absorption was incomplete, the procedure was repeated.

Immunological methods

Immunodiffusion. A 1 % solution of agarose (L'Industrie Biologique Francaise, S.A., Gennevilliers, France) in veronal buffer, pH 8.6 (LKB produkter AB, Stockholm, Sweden) was melted and pipetted in 3-ml quantities on to level microscope slides (2.6 by 7.5 cm). Wells, 2 mm in diameter and 3 mm apart, were punched out. The diffusion was performed at 37°C for 24 hrs. in a moist chamber.

Immunoelectrophoresis. The medium and the preparation of the glasses were the same as for the immunodiffusion test. A template with one antigen well, 2 mm in diameter and two antiserum trenches,

2 mm in width, was employed. The test antigens were electrophoresed in a Gelman Electrophoresis Chamber, model 51170 (Gelman Instrument Company, Michigan, USA), using a regulated power supply. In the vessels of the electrophoresis chamber was used Gelman High Resolution Barbituric Buffer, pH 8.8, ionic strength 0.05. The electrophoresis was applied in a cold room (5°C) for 90 min. to two frames with 12 glasses at 500 v and 27—29 ma. After the electrophoretic separation, the diffusion was performed at 37°C for 24 hrs. in a moist chamber.

Slides from the immunodiffusion and immunoelectrophoresis tests were photographed, using black and white Kodak Panatomic X film.

Staining procedures

Before staining, the slides were washed overnight in 0.15 M-NaCl and then in distilled water for 8 hrs. Thereafter the slides were dried at room temperature.

Light green SF was employed, as described by *Clausen* (1969) for staining proteins. Sudan black B was used for staining lipids (*Crowle* 1961). To indicate polysaccharides, the staining reaction was performed as described by *Uriel & Grabar* (1961), except that the oxidation with periodic acid was prolonged to 2½ hrs.

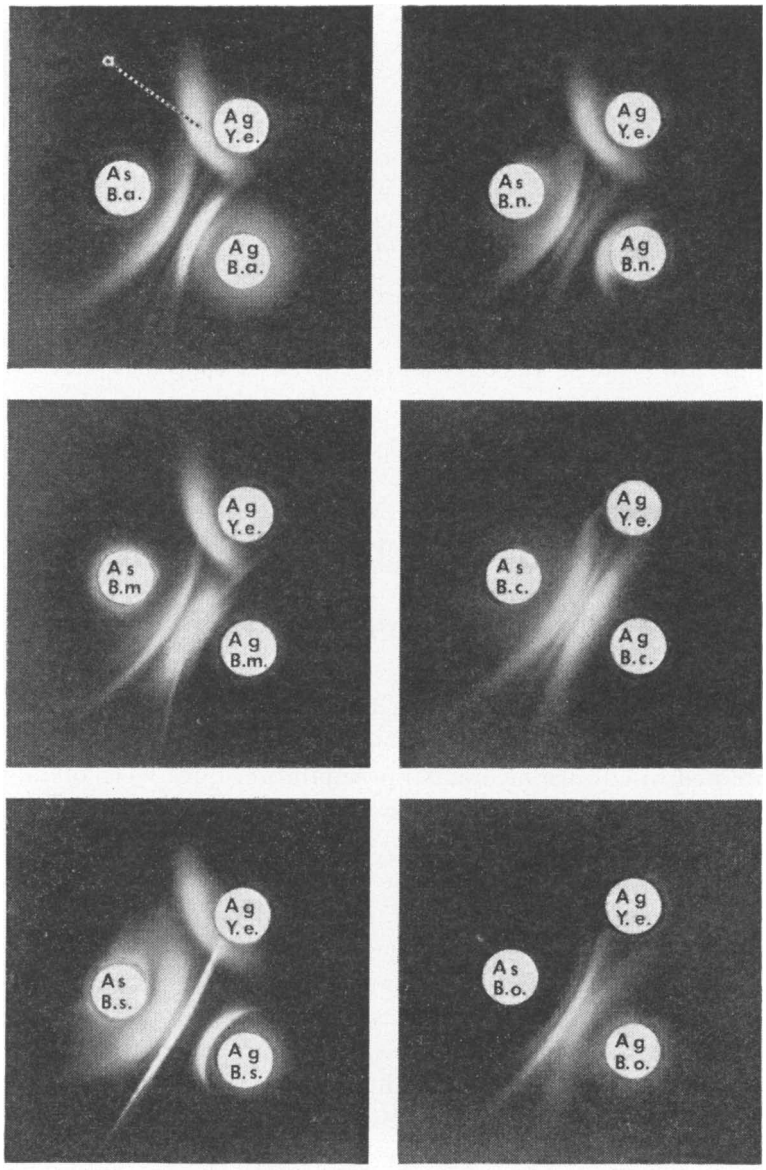
RESULTS

Unabsorbed antisera

All the antisera were tested against Freund's incomplete adjuvant and against preparations consisting of the ingredients contained in culture media. No precipitation lines were obtained, either by the immunodiffusion or by immunoelectrophoresis tests.

The results from the immunodiffusion tests with antisera to the different Brucella species and antigens from Y.e. and from homologous Brucella strains are shown in Fig. 1. The number of precipitation lines between homologous antigen and antisera to the various Brucella strains ranged from 5 to 9. Y.e. antigen gave a marked precipitation line (a) with antiserum to B.a., B.m., B.s. and B.n. No precipitation lines could be seen when Y.e. was tested against antiserum to B.c. and B.o., both of which are rough cultures. Antisera to all the tested smooth Brucella reference strains did not seem to differ qualitatively with respect to the cross-reactions with Y.e. antigen. The strains from these four species gave one precipitation line with Y.e.

Fig. 2 shows the results of the immunodiffusion tests with Y.e. antiserum tested against antigen from the respective Brucella



F i g u r e 1. Immunodiffusion. Antiserum (As) to the different Brucella species tested against homologous and Y. enterocolitica type IX antigens (Ag).

species. At the heterologous cross-reaction with B.a., B.m., B.s. and B.n. one precipitation line (b) was obtained. The cross-reacting precipitation lines were much weaker than the corresponding lines between antisera against the respective Brucella strains and Y.e. antigen. No qualitative difference was observed in the precipitation reaction between antiserum to Y.e. and the various cross-reacting Brucella antigens from B.a., B.m., B.s. and B.n. The precipitation lines for the different smooth Brucella strains showed identity. It will be seen from Fig. 2 that no visible precipitation lines could be detected when the two rough strains B.c. and B.o. were tested against antiserum to Y.e.

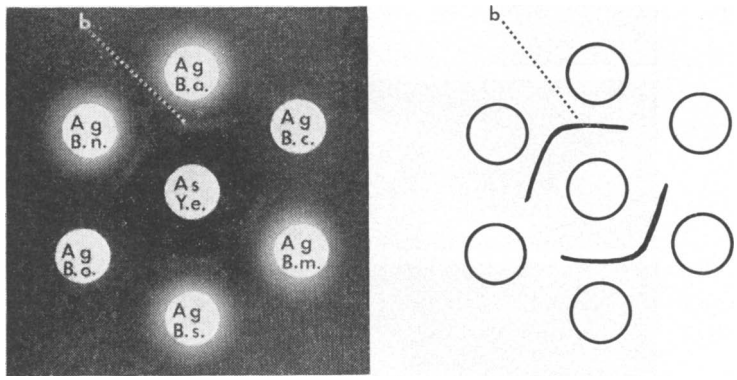


Figure 2. Immunodiffusion. Antiserum (As) to Y. enterocolitica type IX tested against antigen (Ag) of the different Brucella species.

The results of the immunoelectrophoretic study of Y.e. antigen are illustrated in Fig. 3. Y.e. antigen gave 15 precipitation lines with homologous antiserum, and with antiserum to B.a., B.m., B.s. and B.n., respectively, it gave one marked precipitation line. This line (I in Fig. 3) was distinctly defined and showed identity with the corresponding precipitation line between Y.e. antigen and homologous Y.e. antiserum. No qualitative differences between antisera to the tested smooth Brucella reference strains were observed with respect to their cross-reactions with Y.e. antigen. Antisera to B.c. and B.o. did not give rise to any visible precipitation lines when tested against Y.e. antigen, as will be seen from Fig. 3.

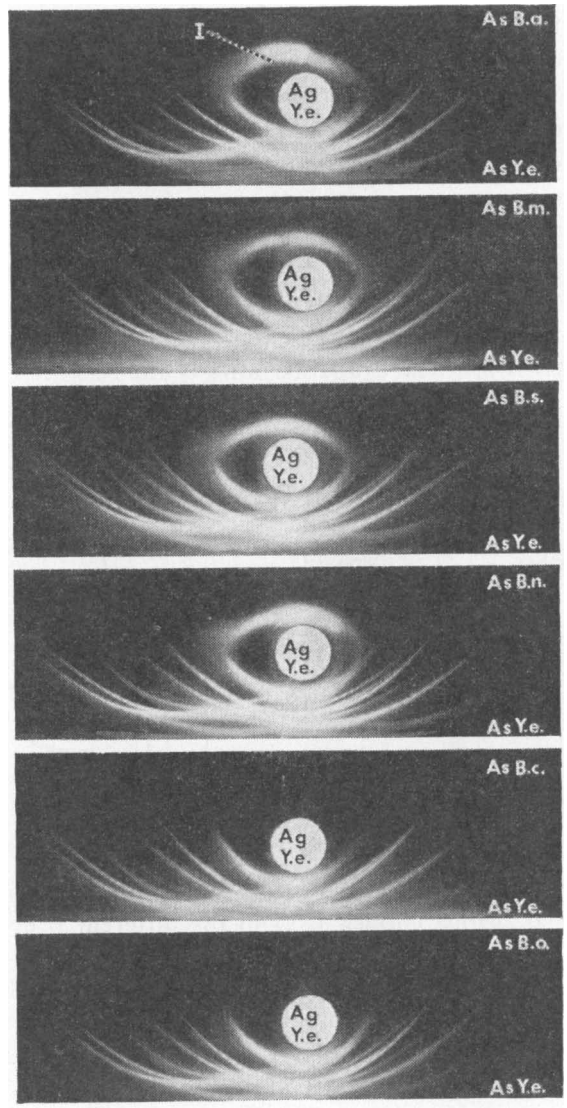


Figure 3. Immunoelectrophoresis. Antigen (Ag) of Y. enterocolitica type IX tested against homologous and different Brucella species antiserum (As).

The results of the immunoelectrophoretic studies of antigen from the respective Brucella strains are shown in Fig. 4. The number of precipitation lines between homologous antigen and antiserum to these strains ranged from 9 to 19. Antiserum to Y.e. gave one precipitation line with antigen from all smooth

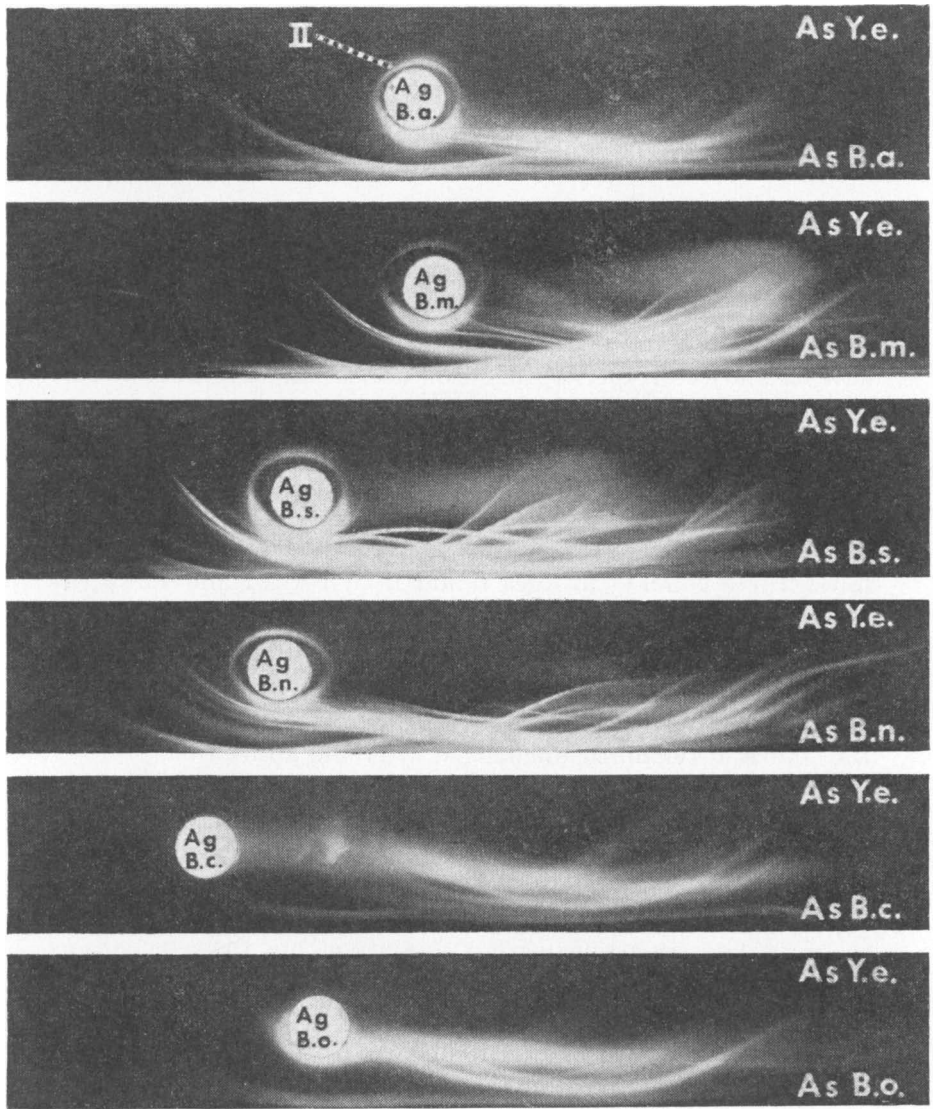


Figure 4. Immunoelectrophoresis. Antigen (Ag) of the different Brucella species tested against homologous and Y. enterocolitica type IX antisera (As).

Brucella types. This line (II in Fig. 4) showed identity with the corresponding precipitation line between homologous antigen and antiserum to the respective smooth Brucella strains. No precipitation lines were seen when B.c. and B.o. antigens were tested against Y.e. antiserum.

Staining reactions

The staining reactions indicated the presence of proteins, polysaccharides and lipids in the obtained precipitation lines (a and b in Figs. 1 and 2; I and II in Figs. 3 and 4) at the cross-reactions between Y.e. and B.a., B.m., B.s. and B.n.

Absorbed antisera

After absorption of antiserum to B.a., B.m., B.s. and B.n. with Y.e. antigen, no cross-reacting precipitation lines (corresponding to lines a and I in Figs. 1 and 3, respectively) between Y.e. antigen and the respective Brucella antiserum were obtained by the immunodiffusion and immunoelectrophoresis tests. There was no qualitative difference between the different antisera to these four smooth Brucella strains.

After absorption of Y.e. antiserum with antigen from B.a., B.m., B.s. and B.n., respectively, the cross-reacting precipitation lines (corresponding to lines b and II in Figs. 2 and 4, respectively) failed to appear. No qualitative differences were observed between the four strains.

When Y.e. antiserum was absorbed with antigen from B.c. or B.o., its ability to cross-react with antigen from B.a., B.m., B.s. and B.n. remained unchanged after the absorption.

DISCUSSION

The results of the immunodiffusion and immunoelectrophoresis tests confirm the observation that a manifest serological cross-reaction occurs between Y.e. and smooth Brucella strains from various species. This agrees with the results of previous studies on cross-reactions between Y.e. and B.a., B.m. and B.s. in which agglutination and complement fixation tests were used (*Hurvell et al.* 1971). No qualitative differences between the employed smooth reference strains in their tendencies to cross-react with Y.e. could be demonstrated by the methods used in the present study. No cross-reaction between Y.e. and B.c. or B.o. was obtained by immunodiffusion or electrophoresis. Nor was any cross-reaction obtained by agglutination tests with Y.e. and these Brucella strains (*Hurvell,* unpublished data). Both type-strains for B.c. and B.o. have nonsmooth colonial morphology and have none or very little of the lipopolysaccharide complex that is associated with agglutinogen in smooth Brucella species

(*Leong et al.* 1970). This may be the reason why these Brucella strains do not cross-react with Y.e. According to this hypothesis, the antigenic determinants in Brucella which cross-react with Y.e. would be present in the lipopolysaccharide complex in the cell walls of the bacteria. The obtained qualitative staining reactions indicated the presence of proteins, lipids and polysaccharides in the obtained precipitation lines at the cross-reactions between Y.e. and the various Brucella strains. As regards the protein reaction, an unequivocal answer to the question whether this reaction comes from the antigenic component cannot be given, because the immunoglobulins in the antisera which were involved in the precipitation reaction also yield positive protein staining. The lipid and the polysaccharide reactions would in all probability arise from the antigenic component. The diffuse appearance of the precipitation lines, both in the immunodiffusion and on immunoelectrophoresis and the limited diffusion ability and mobility of the antigen in both tests suggest that the antigenic component would be a high-molecular substance. Intact bacterial cells react in vitro with antibodies to their surface antigen alone, since these bacteria are impermeable to antibody molecules (*Baughn & Freeman* 1966). The absorptions of Y.e. antiserum with the respective smooth Brucella strains in the present study suggest that the antigenic determinants in Brucella species which react to Y.e. seem to be associated with the outer layers of the bacteria. The absorption of antiserum to the various smooth Brucella species with Y.e. antigen would also imply that the antigenic determinants which cross-react with the various Brucella species are associated with outer layers of the Y.e. organisms.

Thus the results of the present tests indicate that the antigenic determinants common to Brucella and Y.e. would be present in the lipopolysaccharide layer of the respective bacteria. Studies with extractions of antigenic fractions from the organisms concerned are in progress and confirm this presumption. At a recent congress, *Diaz et al.* (1970) reported that the determinants common to Brucella and Yersinia seem to be present in a protein-lipopolysaccharide complex obtained by phenol-water fractionation of the respective organisms.

The results obtained by immunodiffusion and electrophoresis tests indicate that it might be possible to identify in hyperimmune sera the antibodies that derive either from Brucella or Y.e.

REFERENCES

Ahvonen, P., E. Jansson & K. Aho: Marked cross-agglutination between Brucellae and a subtype of Yersinia enterocolitica. Acta path. microbiol. scand. 1969, *75*, 291—295.

Baughn, R. E. & B. A. Freeman: Antigenic structure of Brucella suis spheroplasts. J. Bact. 1966, *92*, 1298—1303.

Braun, W. & A. Bonestell: Independent variation of characteristics in Brucella abortus variants and their detection. Amer. J. vet. Res. 1947, *8*, 386—390.

Clausen, J.: Immunochemical Techniques for the Identification and Estimation of Macromolecules. North-Holland Publishing Company, Amsterdam 1969, 531.

Crowle, A. J.: Immunodiffusion. Academic Press, New York and London 1961, 306.

Diaz, R., L. M. Jones, D. Leong & J. B. Wilson: Differences between Brucella antigens involved in indirect hemagglutination tests with normal and tanned red blood cells. J. Bact. 1967, *94*, 499—505.

Diaz, R., L. Jones & J. B. Wilson: Antigenic relationship of the gram-negative organism causing canine abortion to smooth and rough Brucellae. J. Bact. 1968, *95*, 618—624.

Diaz, R., R. Lacalle, M. P. Medrano & D. Leong: Immunobiological activities of the endotoxin from Yersinia enterocolitica strain M.Y. 79. Proc. Vth Int. Congr. Infectious Diseases, Vienna 1970, 11—17.

Henry, B. S.: Dissociation in the genus Brucella. J. infect. Dis. 1933, *52*, 374—402.

Hurvell, B., P. Ahvonen & E. Thal: Serological cross-reactions between different Brucella species and Yersinia enterocolitica. Proc. 11th Nord. Vet. Congr., Bergen 1970, 282.

Hurvell, B., P. Ahvonen & E. Thal: Serological cross-reactions between different Brucella species and Yersinia enterocolitica. Agglutination and complement fixation. Acta vet. scand. 1971, *12*, 86—94.

Leong, D., R. Diaz, K. Milner, J. Rudbach & J. B. Wilson: Some structural and biological properties of Brucella endotoxin. Infect. Immun. 1970, *1*, 174—182.

Miller, L. & J. A. Houghton: The micro-Kjeldahl determination of the nitrogen content of amino acids and proteins. J. biol. Chem. 1945, *159*, 373—383.

Uriel, J. & P. Grabar: A new technique for direct detection of glycoproteins and polysaccharides after electrophoresis or immunoelectrophoresis in agar gel. Analyt. Biochem. 1961, *2*, 80—82.

SAMMANFATTNING

Serologiska korsreaktioner mellan olika Brucella species och Yersinia enterocolitica.

Immunodiffusion och immunoelektrofores.

Med hjälp av immunodiffusion och immunoelektrofores i agarose har en serologisk korsreaktion påvisats mellan Yersinia enterocolitica typ IX och Brucella stammar från 4 olika species (Brucella abortus, Brucella melitensis, Brucella suis och Brucella neotomae). Några kvalitativa skillnader för dessa stammar med avseende på deras benägenhet att korsreagera med Yersinia enterocolitica typ IX har inte kunnat påvisas. Brucella canis och Brucella ovis, som är kolonimorfologiskt av R-typ, har icke givet någon påvisbar korsreaktion med Yersinia enterocolitica typ IX.

Resultaten från gjorda absorptionsförsök och kvalitativa färgningsreaktioner av erhållna precipitationslinjer tyder på att de gemensamma antigena determinanterna för Brucella och Yersinia enterocolitica typ IX torde finnas hos respektive bakteriecellers ytskickt och i deras lipopolysackaridkomplex. Immunodiffusion och immunoelektrofores visar en möjlighet att i hyperimmunsera kunna identifiera de antikroppar som härrör från Brucella respektive Yersinia enterocolitica typ IX.

(Received September 20, 1971).

Reprints may be requested from: Bengt Hurvell, Department of Bacteriology, National Veterinary Institute, S-10405 Stockholm 50, Sweden.