# Differentiation of Serological Responses to *Yersinia enterocolitica* Serotype O9 and *Brucella* Species by Immunoblot or Enzyme-Linked Immunosorbent Assay Using Whole Bacteria and *Yersinia* Outer Membrane Proteins

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Serum samples from 134 patients showing by the microagglutination test serological cross-reactivity between *Yersinia enterocolitica* serotype O9 and *Brucella* spp. were analyzed by immunoblot and enzyme-linked immunosorbent assay techniques for the presence of antibodies directed against plasmid-encoded, yersinia-associated outer membrane proteins (OMPs). Since these OMPs are exclusively expressed in pathogenic strains of *Yersinia* spp., this characteristic was chosen for serological differentiation of infections caused by these bacteria. The presence of antibodies against plasmid-encoded OMPs of pathogenic *Yersinia* spp. in patient sera appeared to be a suitable means to identify acute or recent infection with *Y. enterocolitica* serotype O9, whereas the failure to detect such antibodies indicated an acute or recent infection with *Brucella* spp.

Serodiagnosis of acute and recent infections with *Yersinia enterocolitica* serotype O9 and *Brucella* spp. on the basis of the commonly used microagglutination assay is seriously impaired by the well-documented, strong serological cross-reactivity between these bacteria (1, 2, 4, 6, 12, 16). Caroff et al. (5) have identified the cross-reacting determinant as residing in the 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units of the O-antigenic chains of the lipopolysaccharides (LPS) of the bacteria.

In the past, considerable efforts have been aimed at the development of assay systems for the unambiguous serological identification of yersiniosis and brucellosis (6–8, 12–14, 16, 17). Up to now, none of them has been generally accepted for unequivocal distinction between the two infections, because of limitations with respect to sensitivity, specificity, or practicability.

Recent studies have revealed that virulence of *Yersinia* spp. is associated with the presence of a yersinia-specific 40- to 46-megadalton (MDa) plasmid (pYV) which codes for the temperature-dependent expression of at least four antigenic outer membrane proteins (OMPs) of molecular masses of approximately 25, 36, 47, and 200 kDa (11, 18). Therefore, sera from patients suffering from yersiniosis always harbor, in addition to anti-LPS antibodies, antibodies directed towards these OMPs (3, 9, 20). We hence reasoned that by means of the immunoblot technique utilizing lysates of whole bacteria as antigens or the enzyme-linked immunosorbent assay (ELISA) using OMPs from *Y. enterocolitica* serotype O9 as antigens, it should be possible to distinguish anti-LPS antibodies from antibodies that react with plasmid-encoded OMPs of *Yersinia* spp. Such a strategy should allow the differentiation between infections with *Y. enterocolitica* serotype O9 and those with *Brucella* spp. The purpose of the present study was to assess the suitability of both immunoblotting and ELISA to provide reliable means for the differential diagnosis of yersiniosis and brucellosis.

## MATERIALS AND METHODS

**Antigens.** The smooth *Brucella abortus* S19 was cultured on blood agar plates in a 10% $CO_2$ atmosphere at 37°C for 48 h. The bacteria were collected, washed twice in phosphate-buffered saline (PBS; 82 mM NaCl, 42 mM $Na_2HPO_4$, 10 mM $KH_2PO_4$, pH 7.3) and adjusted spectrophotometrically in PBS to an optical density (OD) of 1.0 at 436 nm. The plasmid (pYV$^+$) harboring *Y. enterocolitica* serotype O9, strain Ye O9/5 (22), was grown on blood agar plates at 22°C for 48 h and subsequently incubated overnight at 37°C in TYG-MOX broth (tryptone-yeast-glucose broth, containing 20 mM $MgCl_2$ and 20 mM $Na_2C_2O_4$) for optimal expression of the OMPs (19). Bacteria were harvested by centrifugation, washed twice in PBS, and adjusted to an OD of 1.0. The cured plasmid-free *Y. enterocolitica* serotype O9, strain Ye O9/6, and *Y. enterocolitica* serotype O3, strain Ye O3/15 (Institute of Clinical Microbiology, Erlangen, Federal Republic of Germany), were cultured on blood agar plates at 22°C for 48 h. Isolation of the released plasmid-encoded proteins of *Y. enterocolitica* serotype O9 (released OMPs) was performed as described by Heesemann et al. (10). Briefly, an overnight culture of Ye O9/5 bacteria (brain heart infusion medium [Difco Laboratories, Detroit, Mich.], 26°C) was diluted 1:20 with fresh brain heart infusion broth and then incubated with shaking (120 rpm) at 37°C for 90 min. After 10 mM EGTA was added, incubation was continued at 37°C for 90 min. Bacteria were then separated by centrifugation, and the released OMPs were precipitated from the culture supernatant by the addition of ammonium sulfate (40 g/100 ml of supernatant). Precipitated OMPs were dissolved in $H_2O$, purified by dialysis, and then lyophilized.

**Patient sera.** Eight or nine serum samples originated from patients suffering from bacteriologically proven yersiniosis or brucellosis, respectively. A further 134 serum samples were taken from patients presenting with clinical symptoms of *Yersinia* or *Brucella* infection and were sent to our diagnostic laboratory for routine antibody tests. In addition, eight serum samples originated from patients with *Y. enterocolitica* serotype O3-positive stool cultures.

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TABLE 1. Microagglutination test[a] of sera of patients with bacteriologically proven infections and of two hyperimmune sera

| No. | Serum Source | Antibody titer of O antigen from: | | |
|---|---|---|---|---|
| | | *Y. enterocolitica* serotype: | | *B. abortus* |
| | | O9 | O3 | |
| 1 | *Y. enterocolitica* serotype O9 (pYV$^+$)[b] antiserum (rabbit) | 1:160 | ≤1:40 | 1:160 |
| 2 | *Y. enterocolitica* serotype O9 infection (human)[c] | 1:640 | ≤1:40 | 1:1,280 |
| 3 | *Y. enterocolitica* serotype O9 infection (human)[c] | 1:160 | ≤1:40 | 1:160 |
| 4 | *B. abortus* S19 antiserum (rabbit) | 1:640 | ≤1:40 | 1:640 |
| 5 | *B. abortus* infection (human)[c] | 1:640 | ≤1:40 | 1:640 |
| 6 | *B. abortus* infection (human)[c] | 1:320 | ≤1:40 | 1:640 |
| 7 | *Y. enterocolitica* serotype O3 infection (human)[c] | ≤1:40 | 1:1,280 | ≤1:40 |
| 8 | Serum from a healthy person | ≤1:40 | ≤1:40 | ≤1:40 |

[a] Done with plasmid-free *Y. enterocolitica* serotypes O9 and O3 and *B. abortus* as antigens.

[b] pYV, Virulence plasmid of *Yersinia* sp.

[c] Serum from bacteriologically proven infection. Data from representative sera are given.

Ten serum samples from healthy persons were used as negative controls. Some of the sera tested were kindly provided by E. Protz, Bundesgesundheitsamt, Berlin, Federal Republic of Germany, and W. Heizmann, Hygiene Institut, Tübingen, Federal Republic of Germany.

**Microagglutination assay.** Heat-killed bacteria of the plasmid-free *Y. enterocolitica* serotype O9, strain Ye O9/6, and *B. abortus* S19 were used as antigens, as described in detail recently (23). Briefly, each well of a flat-bottom microculture plate (Dynatech, Plochingen, Federal Republic of Germany) received $10^8$ heat-killed (100°C) bacteria in 50 μl of PBS and 50 μl of serum in serial dilutions, beginning with a dilution of 1:40. The test was read after an overnight incubation at 37°C. In our laboratory, sera with agglutination titers of 1:80 or higher were considered to indicate an acute or recent infection, whereas sera with titers below 1:80 were regarded as indicating either no infection or an infection in the past.

**Antisera.** Hyperimmune sera against *Y. enterocolitica* serotype O9 and *B. abortus* were raised by immunization of rabbits with three doses of $10^9$ live plasmid-positive Ye O9/5 bacteria or live *B. abortus* S19 bacteria given intravenously at weekly intervals.

**Immunoblot analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (15), and the immunoblot was set up according to a modification of the method of Towbin et al. (21), as recently described (23). Briefly, 50 μl of a whole-cell bacterial suspension (OD, 1.0) or 100 μg of the released proteins of the plasmid-carrying Ye O9/5 strain per 10 μl was heated with sample buffer (62.5 mM Tris hydrochloride, pH 6.8, containing 10% glycerol, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 0.001% bromphenol blue) at 100°C for 5 min. Disrupted cell products, i.e., proteins and LPS, or the released proteins were separated electrophoretically for 120 min on a vertical sodium dodecyl sulfate-polyacrylamide slab gel at a constant current of 60 mA, with a stacking gel of 3% acrylamide and a running gel of 10% acrylamide. The separated components were immediately transferred onto nitrocellulose sheets (pore size, 0.45 μm) at a constant current of 250 mA overnight. After blocking of nonspecific binding sites with 3% gelatin in Tris-saline, the nitrocellulose sheets were incubated for 3 h with the sera of the patients diluted 1:100 in Tris-gelatin-saline or rabbit hyperimmune sera diluted 1:300, followed by incubation for 1 h with horseradish peroxidase-labeled goat anti-human immunoglobulin polyvalent antiserum (no. 2393; Medac, Hamburg, Federal Republic of Germany) or goat anti-rabbit immunoglobulin G antiserum (no. 170-6515; Bio-Rad Laboratories, Munich, Federal Republic of Germany) diluted 1:2,000. Development of the nitrocellulose was carried out with 4-chloro-1-naphthol solution.

**ELISA.** As the antigen, 5 μg of the released OMPs of Ye O9/5 per well was adsorbed onto flat-bottom Immulon Microelisa plates (Dynatech) and blocked by incubation at 37°C with 0.5% bovine serum albumin in PBS for 2 h. Sera were diluted 1:500 in PBS–0.05% Tween 20 and added in 100-μl volumes to the plates. The plates were incubated at 37°C for 2 h, washed three times with PBS-Tween 20, and then incubated at 37°C with polyvalent alkaline phosphatase-conjugated goat anti-human immunoglobulin (1:600) (Medac) for 4 h. After three further washing steps, 100 μl of freshly prepared substrate, i.e., 1 mg of *p*-nitrophenylphosphate (Sigma, Deisenhofen, Federal Republic of Germany) per ml of diethanolamine $MgCl_2$ buffer, was added to each well and incubated at 22°C for 30 min. The reaction was stopped by the addition of 100 μl of 1 N NaOH. The OD of the colored product was measured in a Titertek Multiscan MC enzyme immunoassay reader (Flow Laboratories, Meckenheim, Federal Republic of Germany) by using dual wavelengths (414 and 492 nm).

## RESULTS

In an initial series of experiments, sera were subjected to the classical microagglutination assay using heat-killed bacteria of the plasmid-negative *Y. enterocolitica* serotype O9 and *B. abortus* S19 as antigens in order to demonstrate the well-known cross-reactive antibody titers. For this purpose, we used sera from eight patients with bacteriologically proven infections with *Y. enterocolitica* serotype O9 and from nine patients with *B. abortus* infections, 18 control serum samples, 8 of which originated from patients with *Y. enterocolitica* serotype O3-positive stool cultures and the other 10 of which were from healthy persons, as well as 2 rabbit hyperimmune serum samples raised against either *Y. enterocolitica* serotype O9 or *B. abortus* S19. In Table 1, representative results for each of these serum groups are given. Subsequently, all of these sera were studied with the immunoblot technique by using, as antigens, lysates of plasmid-positive Ye O9/5, plasmid-negative Ye O9/6, and *B. abortus* S19 bacteria as well as released OMPs from plasmid-positive Ye O9/5 (Fig. 1). From the data given in Table 1 and Fig. 1, the following points became obvious. Cross-reactivity observed in the agglutination assay with sera 1 to 6 fully correlated with the typical smear-type staining (4, 23) of the LPS of both *Y. enterocolitica* serotype O9 and *B.*
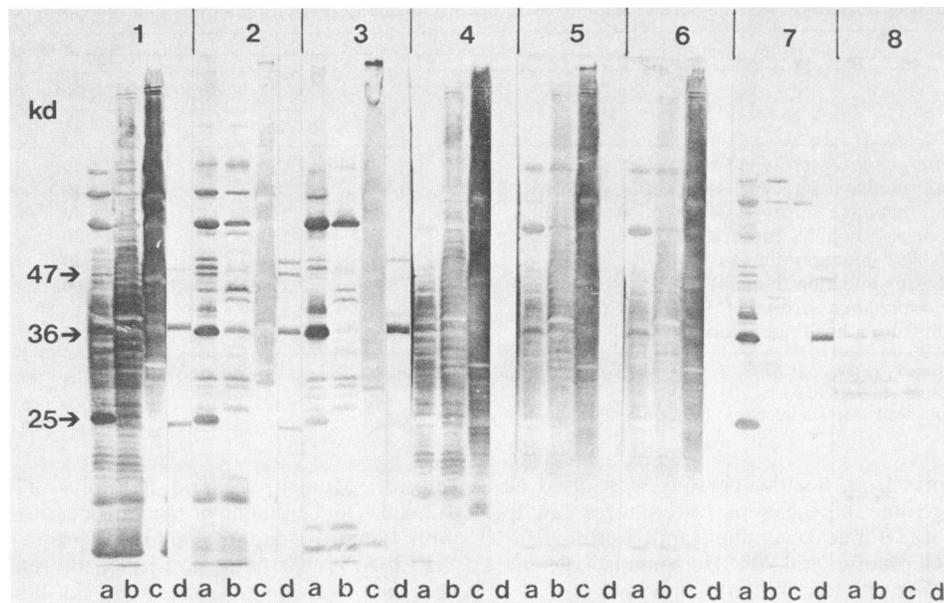
FIG. 1. Immunoblot analysis of rabbit hyperimmune sera raised against plasmid-positive *Y. enterocolitica* serotype O9 and *B. abortus* S19 and of sera from patients suffering from proven yersiniosis (sera 1 to 3) or brucellosis (sera 4 to 6) with whole-bacteria antigen Ye O9/5 containing the virulence plasmid (lanes a), plasmid-free Ye O9/6, (lanes b), *B. abortus* S19 (lanes c), or released OMPs of Ye O9/5 (lanes d). Serum 7 originated from a patient with a *Y. enterocolitica* serotype O3 infection, and serum 8 originated from a healthy person. Arrows point to the most prominent plasmid-encoded OMP antigens of 25, 36, and 47 kDa. The agglutination antibody titers of the sera tested are given in Table 1.

*abortus*. All sera of proven *Y. enterocolitica* serotype O9 infection, apart from anti-LPS antibodies, also contained antibodies that were directed against the distinct plasmid-encoded OMPs, strongly staining the respective proteins of relative molecular masses of 25, 36, and 47 kDa. This characteristic was seen with lysates of plasmid-positive Ye O9/5 bacteria (Fig. 1, lanes a) as well as with the released OMP preparation (lanes d) but not with lysates of the plasmid-negative Ye O9/6 bacteria (lanes b). Serum 7, from a patient with *Y. enterocolitica* serotype O3 infection, reacted with neither the *Y. enterocolitica* serotype O9 LPS nor with the *Brucella* LPS but clearly stained the plasmid-encoded *Yersinia* OMPs, as expected.

These data thus show that the identification of antibodies against *Yersinia* OMPs with the immunoblot allows the differentiation between acute or recent infections with *Y. enterocolitica* serotype O9 and *Brucella* spp. This prompted us to select 134 serum samples that in the agglutination assay had shown cross-reacting antibodies to *Y. enterocolitica* serotype O9 LPS and *Brucella* LPS with titers of 1:80 or higher and to subject these sera to immunoblot and ELISA. Efforts to isolate *Yersinia* or *Brucella* spp. from the sera had failed, thus leaving the serological diagnosis ambiguous. Of these 134 serum samples, 129 were identified as having originated from patients with *Y. enterocolitica* serotype O9 infection, because of their strong positive staining of three of the *Yersinia* OMPs, i.e., the 25-, 36-, and 47-kDa OMPs. Only five serum samples did not react with these *Yersinia* OMPs but showed strong smear-type staining with the LPS from *Y. enterocolitica* serotype O9 as well as from *B. abortus*. These sera were therefore considered to indicate an acute or recent *Brucella* infection. Figure 2 shows the immunoblot analysis of 4 representative serum samples from 134 patient serum samples (the agglutination titers of which

are given in Table 2) and a one-to-one mixture of serum 2, identified as yersiniosis serum, and serum 4, identified as brucellosis serum. Rabbit hyperimmune serum against *Y. enterocolitica* serotype O9 was used as a control. The staining characteristics of sera 2 to 5 unequivocally reflect either acute or recent yersiniosis (sera 2 and 3) or brucellosis (sera 4 and 5). The staining pattern of the 1:1 mixture of sera 2 and 4, however, indicates the limitation of the method used: an acute or recent double infection by *Y. enterocolitica* serotype O9 and *Brucellae* spp. cannot be distinguished from a monoinfection caused by *Y. enterocolitica* serotype O9.

As an alternative method for serological distinction of yersiniosis and brucellosis, we applied the ELISA procedure. As the antigen, we used a preparation of OMPs released from plasmid-carrying Ye O9/5 that was devoid of the cross-reactive LPS antigen, as demonstrated in the immunoblots (Fig. 1 and 2). Under these conditions, all sera that contained OMP-specific antibodies, as previously assessed by immunoblotting, showed OD values between 0.1 and 1.0, whereas sera from patients with brucellosis or negative controls showed OD values between 0.02 and 0.05. OD values between 0.05 and 0.1 were not observed (Fig. 3). Therefore, this ELISA procedure was also considered to be suitable for the identification of sera of yersiniosis patients and for their differentiation from sera obtained from patients with brucellosis.

## DISCUSSION

The results demonstrate that both methods, immunoblot and ELISA, are suitable to clearly identify antibodies specific for OMPs of *Yersinia* spp., the immunoblot allowing, in addition, the demonstration of the cross-reacting anti-LPS antibodies. Of the 134 serum specimens tested, which were
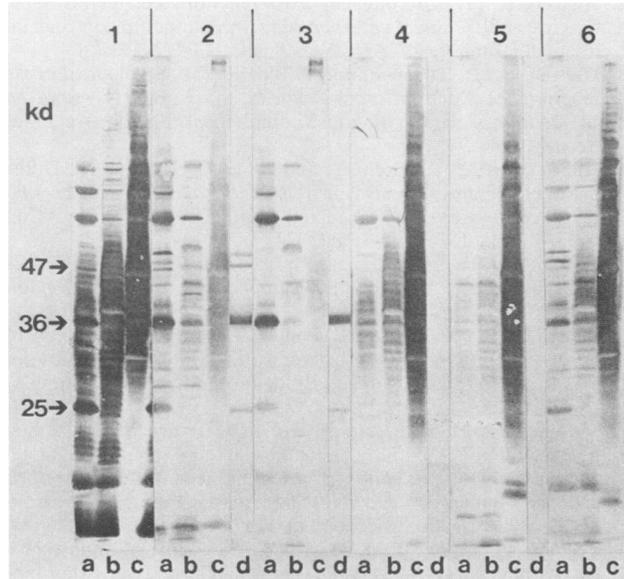
FIG. 2. Immunoblot analysis of 4 of 134 serum specimens obtained from patients showing clinical symptoms of yersiniosis (sera 2 and 3) or brucellosis (sera 4 and 5) without cultural confirmation and revealing cross-reactivity in the agglutination test against *Y. enterocolitica* serotype O9 and *B. abortus* antigen. The antigens applied in lanes a to d were the same as those described in the legend to Fig. 1. Serum 1 was a rabbit antiserum raised against the plasmid-positive Ye O9/5 strain, and serum 6 was a 1:1 mixture of sera 2 and 4. The agglutination antibody titers of the sera tested are given in Table 2.



FIG. 3. ELISA adsorbance values of sera from patients with bacteriologically proven yersiniosis or brucellosis (O) and of sera with cross-reacting agglutination antibody titers of $\geq$1:80 ($\bullet$).

taken from patients with clinical evidence for infection with *Y. enterocolitica* serotype O9 or *Brucella* spp., 129 strongly displayed antibodies against *Yersinia* OMPs and thus were considered to have been induced by *Y. enterocolitica* serotype O9. The remaining five serum specimens consistently failed to show a positive reaction with the OMPs of *Yersinia* spp. in the immunoblot and in the ELISA; these sera were considered to indicate a *Brucella* infection. In all five cases, the histories of the patients and the clinical picture indeed were strongly compatible with an acute or recent brucellosis.

On the basis of this study, we now routinely use the immunoblot or ELISA as a confirmatory and discriminative test in situations in which the result of the microagglutination antibody titer requires a differential diagnosis between acute or recent yersiniosis and brucellosis. However, this procedure does not allow the detection of infections that occurred in the past because in such cases, the agglutination antibody

TABLE 2. Microagglutination test[a] on sera from patients presenting clinical symptoms of yersiniosis or brucellosis without cultural confirmation

| Serum no. | Antibody titer of O antigen from: | |
| --- | --- | --- |
| | *Y. enterocolitica* serotype O9 | *B. abortus* |
| 2 | 1:2,560 | 1:1,280 |
| 3 | 1:80 | 1:160 |
| 4 | 1:160 | 1:320 |
| 5 | 1:80 | 1:320 |

[a] Done as described for Table 1. Data for representative sera are given. Sera 2 and 3 were identified by immunoblot as yersiniosis sera, and sera 4 and 5 were identified as brucellosis sera (Fig. 2).
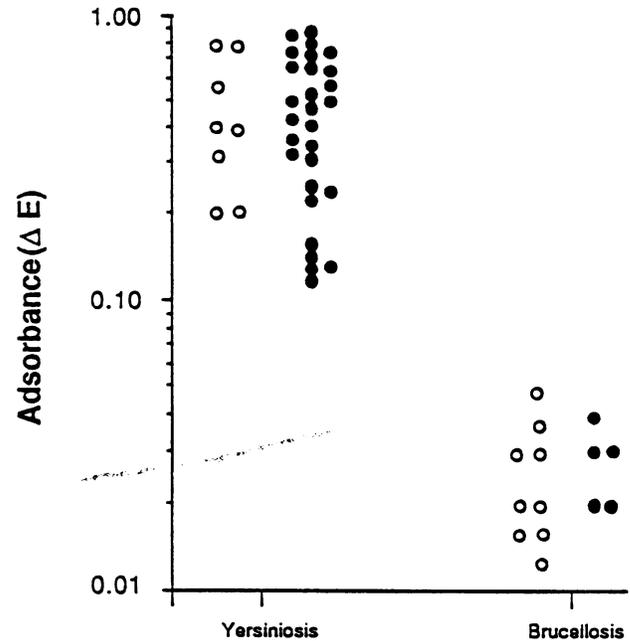
titers are often below the threshold of 1:80 and thus, in our procedure, would not be selected for the differential tests. In this laboratory, the levels of the agglutination antibody titers and their kinetics form the basis to decide whether either an acute or recent infection is present or we are dealing with an infection from the past. Only sera with antibody titers of 1:80 or higher are considered to reflect acute or recent infections and thus have been analyzed in this study.

It has been demonstrated that antibodies directed against *Yersinia* OMPs can be found in about 39% of healthy persons (9). Since it could be argued that this high background may interfere with our strategy to differentiate between the two infections studied, we examined by immunoblot 68 serum samples from healthy persons, which showed *Y. enterocolitica* serotype O9 agglutination antibody titers of $\leq$1:40 for the presence of antibodies directed against yersinia-specific OMPs by using goat anti-human immunoglobulin G diluted 1:500 as the second antibody. With 43% of these sera, weak staining of yersinia-specific OMPs, mainly the 36-kDa OMP, was obtained without staining of the LPS. However, under the conditions used in this study (i.e., the particular antigen concentration used and the goat anti-human immunoglobulin polyvalent serum in dilution 1:2,000), no significant staining of OMPs was detected in these 68 serum specimens (C. Schoerner et al., unpublished data). Thus, the above-mentioned complication does not appear to interfere with the procedure as carried out in this study.

The fact that an acute or recent double infection with *Y. enterocolitica* serotype O9 and *Brucella* spp. cannot be identified by the proposed methods and would mistakenly be considered as an infection with only *Yersinia* spp. does not cause a significant limitation, because such double infections occur very rarely.

## LITERATURE CITED

1. Ahvonen, P., E. Jansson, and K. Aho. 1969. Marked cross-agglutination between *Brucellae* and a subtype of *Yersinia enterocolitica*. Acta Pathol. Microbiol. Scand. **75**:291–295.

2. Akkermans, J. P. W. M., and W. K. W. Hill. 1971. *Yersinia enterocolitica* serotype 9-infectie als storend element bij de serologische diagnostiek van brucella-infecties bij het varken. Tijdschr. Diergeneeskd. **24**:1654–1662.

3. Bölin, I., D. A. Portnoy, and H. Wolf-Watz. 1985. Expression of the temperature-inducible outer membrane proteins of yersiniae. Infect. Immun. **48**:234–240.

4. Bundle, D. R., M. A. J. Gidney, M. B. Perry, J. R. Duncan, and J. W. Cherwonogrodzky. 1984. Serological confirmation of *Brucella abortus* and *Yersinia enterocolitica* O:9 O-antigens by monoclonal antibodies. Infect. Immun. **46**:389–393.

5. Caroff, M., D. R. Bundle, and M. B. Perry. 1984. Structure of the O-chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O9. Eur. J. Biochem. **139**:195–200.

6. Corbel, M. J., and C. A. Dag. 1973. Assessment of fluorescent antibody absorption procedure for differentiation of the serological response to *Yersinia enterocolitica* serotype IX and *Brucella abortus* in cattle. Br. Vet. J. **129**:67–71.

7. Corbel, M. J., F. A. Stuart, and R. A. Brewer. 1984. Observations on serological cross-reaction between smooth *Brucella* species and organisms of other genera. Dev. Biol. Stand. **56**:341–348.

8. Granfors, K., M. K. Viljanen, and A. Toivanen. 1981. Measurement of immunoglobulin M, immunoglobulin G, and immunoglobulin A antibodies against *Yersinia enterocolitica* by enzyme-linked immunosorbent assay: comparison of lipopolysaccharide and whole bacterium as antigen. J. Clin. Microbiol. **14**:6–14.

9. Heesemann, J., C. Eggers, and J. Schröder. 1987. Serological diagnosis of yersiniosis by immunoblot technique using virulence-associated antigen of enteropathogenic yersiniae. Contrib. Microbiol. Immunol. **9**:285–289.

10. Heesemann, J., U. Gross, N. Schmidt, and R. Laufs. 1986. Immunochemical analysis of plasmid-encoded proteins released by enteropathogenic *Yersinia* sp. grown in calcium-deficient media. Infect. Immun. **54**:561–567.

11. Heesemann, J., C. Keller, R. Morawa, N. Schmidt, H. J. Siemens, and R. Laufs. 1983. Plasmids of human strains of *Yersinia enterocolitica*: molecular relatedness and possible importance for pathogenesis. J. Infect. Dis. **147**:107–115.

12. Hurvell, B. 1972. Serological cross-reactions between *Brucella* species and *Yersinia enterocolitica*. Immunodiffusion and immunoelectrophoresis. Acta Vet. Scand. **13**:472–483.

13. Hurvell, B., P. Ahvonen, and E. Thal. 1971. Serological cross-reactions between different *Brucella* species and *Yersinia enterocolitica*. Agglutination and complement fixation. Acta Vet. Scand. **12**:86–94.

14. Hurvell, B., A. A. Lindberg, and H. E. Carlsson. 1979. Differentiation of antibodies against *Brucella abortus* and *Yersinia enterocolitica* by enzyme-linked immunosorbent assay. Contrib. Microbiol. Immunol. **5**:73–79.

15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

16. Marx, A., R. Sandulache, A. Pop, A. Cerbu, and A. Pop. 1974. Serological cross-reaction between *Brucella abortus* and *Yersinia enterocolitica*. J. Hyg. Epidemiol. Microbiol. Immunol. **18**:429–431.

17. Mittal, K. R., I. D. Ricciardi, and I. R. Tizard. 1980. Indirect hemagglutination employing enterobacterial common antigen and *Yersinia* somatic antigen: a technique to differentiate brucellosis from infections involving cross-reacting *Yersinia enterocolitica*. J. Clin. Microbiol. **11**:149–152.

18. Portnoy, D. A., and R. J. Martinez. 1985. Role of a plasmid in the pathogenicity of *Yersinia* species. Curr. Top. Microbiol. Immunol. **118**:29–51.

19. Portnoy, D. A., H. Wolf-Watz, I. Bölin, A. B. Beeder, and S. Falkow. 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43**:108–114.

20. Stahlberg, T. H., K. Granfors, and A. Toivanen. 1987. Immunoblot analysis of human IgM, IgG and IgA responses to plasmid-encoded antigens of *Yersinia enterocolitica* serovar O3. J. Med. Microbiol. **24**:157–163.

21. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.

22. Weninger, J., C. Schoerner, K. Wartenberg, and M. Röllinghoff. 1988. Detection of cross-reacting epitopes on plasmid-encoded outer membrane proteins of enteropathogenic *Yersinia* by monoclonal antibodies. Med. Microbiol. Immunol. **178**:45–51.

23. Weninger, J., K. Wartenberg, and M. Röllinghoff. 1988. Immunological characterization of *Yersinia enterocolitica* O:9 and O:3 LPS antigens by monoclonal antibodies. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **269**:298–313.