

## Enzyme immunoassay of the antibody response to *Brucella* and *Yersinia enterocolitica* 09 infections in humans

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### SUMMARY

An enzyme immunoassay, with phenol-water-extracted lipopolysaccharide (LPS) from *Brucella abortus* as antigen, was used to detect the class-specific antibody response in sera from 173 patients with *B. abortus*, *B. melitensis* or *B. suis* infection. Sera from 30 patients with salmonellosis, yersiniosis or tularaemia and from 25 healthy individuals served as controls. The *B. abortus* LPS antigen permitted a safe diagnosis of acute and chronic brucellosis with high IgM and rising IgG titres in sera collected in the acute stage of the disease, and with elevated IgG titres only in the chronic stage. The *B. abortus* LPS antigen also permitted a specific diagnosis with the exception of the high titres estimated in sera from patients with *Yersinia enterocolitica* 09 infection. The problem with that well-known reciprocal cross-reactivity was overcome by using two additional antigens: *Y. enterocolitica* 09 native and periodate oxidized and borohydride reduced LPS preparations. In sera from patients with brucellosis high titres were estimated against all three antigens, whereas in sera from patients with yersiniosis caused by serotype 09 high titres were measurable only with the *B. abortus* and the *Y. enterocolitica* native LPS antigens. These data suggest that the *B. abortus* and *Y. enterocolitica* 09 LPS share one antigenic determinant resistant to periodate oxidation and borohydride reduction, and that in addition the *Y. enterocolitica* 09 LPS has a determinant which is sensitive to periodate oxidation and borohydride reduction.

### INTRODUCTION

The clinical diagnosis of brucellosis in man and animals is often difficult to establish. The laboratory diagnosis is equally difficult: the incidence of positive cultures is low, and therefore the diagnosis often has to rest on the detection and titration of *Brucella* antibodies in serum.

There are a number of serological tests in common use: tube agglutination, card agglutination, precipitation-plate agglutination, complement fixation (FAO/

WHO, 1971). The multiplicity of tests indicates that they have a number of limitations in sensitivity as well as in specificity. During recent years attempts have been made to use more sensitive tests such as enzyme-linked immunosorbent assays (Carlsson, Hurvell & Lindberg, 1976; Lamb *et al.* 1979; Magee, 1980) and radioimmunoassays (Parratt *et al.* 1977), and also to employ better-defined antigenic preparations (Carlsson *et al.* 1976; Berman *et al.* 1980).

We earlier reported on the increased sensitivity and specificity for detection of anti-*Brucella* antibodies in experimental rabbit sera using phenol-water-extracted lipopolysaccharide as antigen in an enzyme-immunoassay (Carlsson, *et al.* 1976). In the present communication the method has been applied to human sera collected from cases with acute and chronic brucellosis. Furthermore, the problems with the differential diagnosis between brucellosis and yersiniosis because of the known cross-reaction between *Brucella* and *Yersinia enterocolitica* 09 have been overcome by using as an additional antigen a chemically modified LPS from a *Y. enterocolitica* 09 strain.

## MATERIALS AND METHODS

### *Bacterial strains*

*Brucella abortus* strain 19, *Yersinia enterocolitica* 03 strain 482 and 09 strain 486 were from the strain collection at the Department of Bacteriology, National Bacteriological Laboratory.

### *Cultivation and extraction of lipopolysaccharide*

The *Brucella* and *Yersinia* strains were cultivated in a tryptone yeast medium as described earlier (Lindberg & Holme, 1972), except that the *Y. enterocolitica* 03 and 09 strains were grown at +25° to ensure a minimal fraction of rough forms in the culture (Niléhn, 1969). *Brucella abortus* strain 19 was grown in submerged culture by Jensen-Salsberg Laboratories, Kansas City, Kansas 66103, USA. All cultures were killed by addition of formaldehyde to a final concentration of 1 per cent; the whole cells were then extracted with hot phenol-water and the aqueous phase collected (Lindberg & Holme, 1972). The LPS preparations were purified by ultracentrifugation (105000 g, 4 h) until no specific absorbance at 260 nm could be detected. If necessary, remaining ribonucleic acid was eliminated by treatment with ribonuclease (5 × crystalline, Nutritional Biochemical Corp., Cleveland, Ohio, USA) as described before (Lindberg & Holme, 1972).

### *Chemical modification of Y. enterocolitica 09 LPS*

The LPS was periodate oxidized and borohydride reduced using the conditions described earlier (Hammarström *et al.* 1975).

### *Sources of human sera*

Sera were obtained from 173 patients with a diagnosis of acute or chronic brucellosis. The clinical diagnosis was supported by laboratory tests such as isolation of the causative organism and serological assays (tube agglutination,

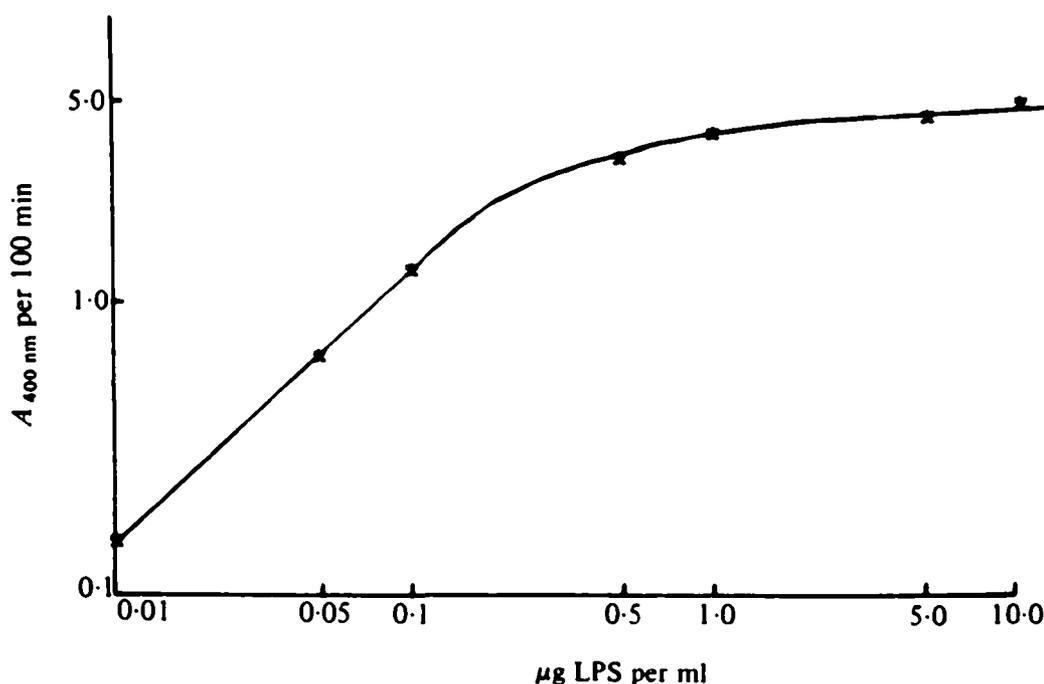


Fig. 1. Titration of *Brucella abortus* LPS for optimal coating of plastic tubes in ELISA. Tubes coated with varying concentrations of LPS were incubated for 4 h with a convalescent serum, diluted 1:1000. The serum was collected from a patient who had recovered from a *B. melitensis* infection.

complement fixation). As a reference material serum samples were collected from 25 healthy persons, 10 patients each with verified salmonellosis (Karlsson *et al.* 1980), tularaemia (Carlsson *et al.* 1979) or *Yersinia enterocolitica* 0 type 3 (unpublished data) infections.

#### *Immunological reagents*

Swine anti-human IgM- and IgG-specific alkaline phosphatase conjugates were purchased from Orion Diagnostica Oy, Helsinki, Finland. Polyspecific sheep anti-human immunoglobulin, purified by affinity chromatography, conjugated with alkaline phosphatase (calf intestinal mucosa, Sigma Chemical Company, St Louis, Mo., USA) was prepared as described by Engvall & Perlmann (1971).

#### *Enzyme-linked immunosorbent assay (ELISA)*

The ELISA test was performed as described before (Engvall & Perlmann, 1972; Carlsson, Lindberg & Hammarström, 1972) using 1 M-diethanolamine-HCl (pH 9.8) as substrate buffer.

## RESULTS

#### *Optimal antigen dose for coating of plastic tubes in ELISA*

Plastic tubes were incubated with solutions of phenol-water-extracted LPS from *Brucella abortus* strain 19 at different concentrations. A convalescent serum collected from a patient who had recovered from a *B. melitensis* infection, proved by positive blood cultures, was used as antibody source. The absorbance value

Table 1. Rank correlation coefficient between end-point and relative ELISA titres for IgM, and IgG and Ig conjugates

Antibody class titre estimated	Rank correlation coefficient between end-point and relative titre of serum diluted <sup>a</sup>			
	1:100	1:1000	1:10000	1:100000
IgM	0.87	0.92	0.95	0.95
IgG	0.72	0.89	0.96	0.98
Ig (IgM, IgG, IgA)	0.85	0.79	0.92	0.99

<sup>a</sup> Nineteen different sera collected from patients with brucellosis [and collected] in the acute or convalescent phase of the infection were used. The rank correlation coefficient was determined according to Spearman.

increased with increasing antigen concentrations up to about 1.0 µg LPS/ml where the curve levelled off (Fig. 1). A concentration of 0.5 µg/ml, just before the curve levelled off, was chosen as the coating dose for subsequent assays.

#### *End-point and relative ELISA titre determinations*

On 19 sera collected from patients with brucellosis the end-point titres for IgM, IgG and an Ig conjugate (measuring IgM, IgG and IgA) were estimated using serial tenfold dilution steps. The end-point titre values were then plotted against the relative titre estimated for each dilution and the rank correlation coefficient determined (Table 1). For all dilutions tested the correlation between the end-point and the relative ELISA titre was statistically highly significant ( $P < 0.001$ ) for each type of antibody class. For most antibody titre estimates the 1:1000 dilution was used since it favoured detection of early antibody rises. At the same time it allowed estimation of high titre in convalescent sera although a linear relationship was not seen with very high-titred sera (data not shown). The following titres are based on measurements on the 1:1000 dilution and the titres are expressed as absorbance at 400 nm per 100 min multiplied by the dilution factor ( $10^3$ ) and are referred to as relative ELISA titres.

#### *ELISA antibody titres to Brucella abortus lipopolysaccharide antigen*

Two or more serum samples were collected from 6 patients who had fallen ill with brucellosis, verified by positive blood cultures, in Sweden during 1978–80: one patient had a *B. suis* infection whereas five had *B. melitensis* infections.

The *B. suis* infection was seen in a two-year-old child who had suffered from a relapsing febrile illness for nine months. The maximum relative IgM and IgG titres were 160 and 3530, respectively. Seven weeks later, after chemotherapy for four weeks, the corresponding titres were 90 and 930, respectively.

Sera from all five patients with *B. melitensis* infection gave high relative titres: the peak median IgM and IgG values were 1120 and 1780, respectively. The IgM titres reached their peak levels early in the infection and stayed high until the beginning of treatment, after which the IgM titres fell (illustrated in Fig. 2 for three of the five patients).

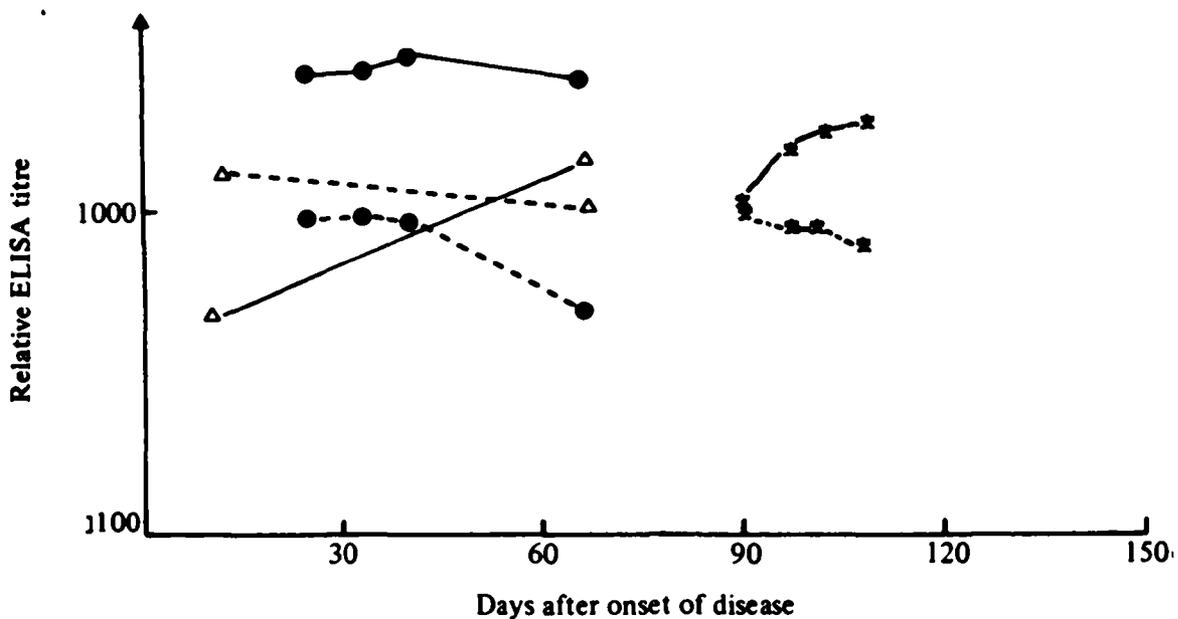


Fig. 2. Relative ELISA IgM and IgG titres against *Brucella abortus* LPS antigen in 10 serum samples, obtained from three of the five patients with *B. melitensis* infection. The diagnosis of brucellosis based on isolation of the bacteria from the blood was made at approximately the same time as the first serum sample was collected. Symbols:  $\Delta$ , patient no. 1;  $\bullet$ , patient no. 2;  $\blacksquare$ , patient no. 3; —, IgG titre; ----, IgM titre.

The peak IgG titres were, as expected, reached later, and often not until treatment had been initiated. A serum sample collected one year after termination of therapy had an unchanged IgG titre, 1890 and 1930, respectively.

#### *ELISA antibody titres to Brucella abortus, Yersinia enterocolitica 03 and 09 LPS antigens*

The usefulness of the *B. abortus* LPS antigen for detection of the antibody response after brucellosis was further tested on sera collected from humans where the diagnosis had been established on clinical signs and serology (tube agglutination, Wright test, etc.). One serum from each patient was investigated. At the same time the antibody response detectable with the cross-reacting *Y. enterocolitica* type 09 LPS was estimated, as well as that seen with the *Y. enterocolitica* type 03 LPS (Table 2).

Sera collected in England, France, Italy and Sweden were drawn during the convalescent stage after a *B. abortus* or *B. melitensis* infection. The median relative IgM and IgG titres ranged between 600–1380 and 1830–2500, respectively. The titres were significantly higher than in sera collected from healthy Swedish controls. Elevated titres were also seen against the *Y. enterocolitica* 09 LPS antigen, the median relative IgM and IgG titres ranged between 260–530 and 300–1300, respectively. Thus the median titres were half or less of those seen against the *B. abortus* LPS antigen. However, in individual sera the *Y. enterocolitica* 09 LPS titres were occasionally almost as high as the *B. abortus* LPS titres making a differential diagnosis based on serology doubtful. The titres estimated against the *Y.*

Table 2. *ELISA IgM and IgG titres on sera from patients with brucellosis using Brucella abortus, Yersinia enterocolitica types 03 and 09 LPS as antigens*

Source of serum samples	No. of sera assayed	Relative ELISA titre (median and range) against*					
		<i>B. abortus</i> LPS		<i>Y. enterocolitica</i> 09 LPS		<i>Y. enterocolitica</i> 03 LPS	
		IgM	IgG	IgM	IgG	IgM	IgG
England	11	1380 (490-3200)	2080 (720-4320)	530 (160-2100)	700 (60-4180)	130 (40-270)	60 (40-210)
France	3	1150 (300-1700)	2500 (2150-4200)	260 (200-980)	1300 (800-1920)	100 (80-130)	100 (60-540)
Iran	48	70 (0-2200)	580 (0-3900)	80 (10-1810)	180 (10-3000)	40 (10-180)	60 (10-2470)
Italy	14	600 (20-2000)	2300 (30-4800)	260 (130-470)	300 (50-6000)	60 (40-140)	80 (20-210)
Poland	91	20 (10-2150)	750 (10-3400)	40 (10-1810)	520 (10-3200)	40 (10-170)	50 (10-930)
Sweden	6	830 (160-1700)	1830 (520-3530)	270 (90-810)	540 (270-980)	150 (20-300)	130 (30-610)
Controls	25	40 (10-80)	50 (10-110)	30 (10-90)	70 (10-140)	90 (10-250)	210 (10-970)

\* The coating doses were 0.5 µg/ml for *B. abortus* LPS antigen and 0.5 µg/ml for *Y. enterocolitica* 03 and 09 LPS.

*enterocolitica* 03 LPS antigen were in all instances low, and not higher than those seen in sera from controls.

Sera collected in Iran and Poland were in most instances from patients suffering from chronic brucellosis. In both groups the median relative IgM titres were low (20–70) whereas the corresponding IgG titres were elevated (580–750) compared to the control group. This suggests that the ELISA test using class-specific conjugates can be a useful diagnostic aid for detecting chronic cases of brucellosis. Within both groups, however, large variations in IgM as well as IgG titres were seen. The titres seen with the *Y. enterocolitica* LPS antigens showed the same tendency as in the groups discussed above.

*Differentiation between B. abortus and Y. enterocolitica 09 infection using a modified Y. enterocolitica 09 antigen*

A previous investigation (Hurvell, Lindberg & Carlsson, 1979) using the ELISA inhibition system with LPS from *B. abortus* and *Y. enterocolitica* 09 as inhibitors of the interaction between rabbit *B. abortus* antibodies and *B. abortus* LPS had revealed that periodate oxidation followed by borohydride reduction completely removed the inhibitory properties of the *Yersinia* LPS while the *B. abortus* LPS was unaffected. No attempt was, however, made to see if the use of a *Y. enterocolitica* 09 LPS, which had been chemically modified, could improve the differential diagnosis between *Brucella* and *Yersinia* type 09 infection. The hypothesis was first tested using phenol–water-extracted LPS from *B. abortus* and *Y. enterocolitica* 09, native and chemically modified, together with rabbit antisera against *B. abortus* and *Y. enterocolitica* 09 (Fig. 3 A, B). The titration curves reveal that, in assays using rabbit anti-*B. abortus* serum, *B. abortus* LPS at lower concentrations measured the antibody content more efficiently than the two *Y. enterocolitica* 09 LPS preparations, which were equally effective (Fig. 3 A). In assays using the rabbit anti-*Y. enterocolitica* 09 serum the *Y. enterocolitica* 09 LPS was most efficient followed by the *B. abortus* LPS. The periodate oxidized, borohydride-reduced LPS preparation from *Y. enterocolitica* 09 was tenfold less efficient than the untreated LPS in detecting the *Y. enterocolitica* 09 antibodies. These results suggest that antibodies against *B. abortus* are detected by a periodate-resistant antigenic determinant in the *Y. enterocolitica* 09 LPS. However, in rabbit serum against *Y. enterocolitica* 09 a periodate-sensitive antigenic determinant in the *Y. enterocolitica* 09 LPS was the most efficient antigen for diagnostic purposes.

We used this observation to test for the antibody titres in sera from patients with *B. abortus* and *Y. enterocolitica* 09 infections employing as antigens *B. abortus* LPS and *Y. enterocolitica* 09 LPS, native and periodate-oxidized and borohydride-reduced (Table 3). The end-point titres seen in sera from patients with brucellosis were equally high using either the native or the periodate-oxidized and borohydride-reduced *Y. enterocolitica* 09 LPS. However, when sera from patients with a known *Y. enterocolitica* 09 infection were tested high titres were seen only against the native *Y. enterocolitica* 09 LPS. The titres measured with the chemically modified LPS antigen were only one-tenth of those seen with the native LPS. These results demonstrate that the use of periodate-oxidized and borohydride-reduced

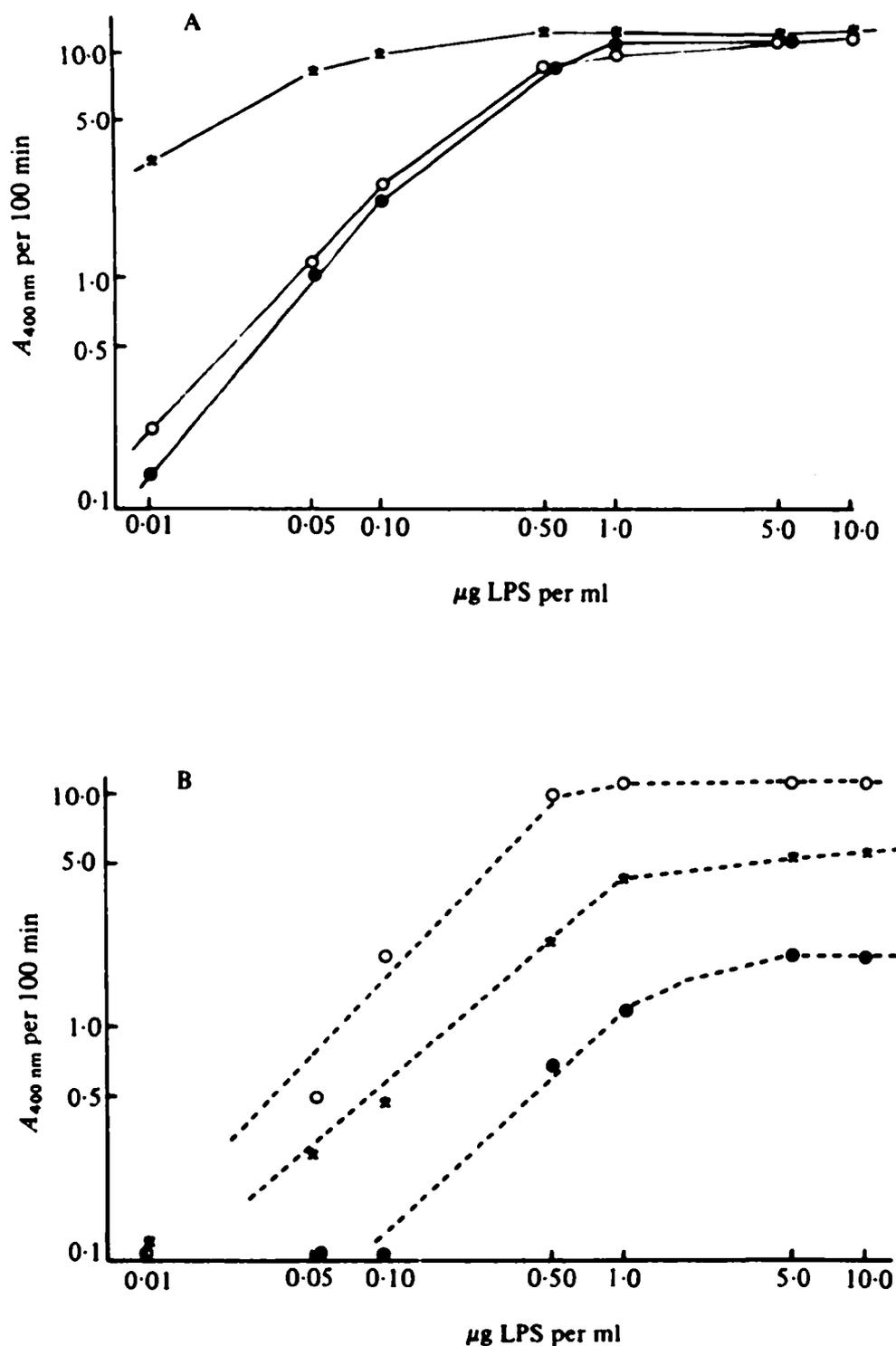


Fig. 3. Titration of *Brucella abortus* and *Yersinia enterocolitica* type 09 LPS (before and after periodate oxidation and borohydride reduction) against (A) rabbit *B. abortus* and (B) *Y. enterocolitica* type 09 specific antisera. The tubes were incubated with varying concentrations of LPS and rabbit antisera diluted 1:1000. Symbols: x, *B. abortus* LPS; o, *Y. enterocolitica* type 09 LPS; ●, *Y. enterocolitica* type 09 LPS periodate oxidized and borohydride reduced.

**Table 3.** *ELISA titres in sera from patients with Brucella abortus or Y. enterocolitica 09 infection using B. abortus and Y. enterocolitica 09 LPS, native and periodate oxidized*

Serum	ELISA end-point titre $\times 10^{-3}$ against <sup>a</sup>		
	<i>B. abortus</i> LPS	<i>Y. enterocolitica</i> 09 LPS	<i>Y. enterocolitica</i> 09 chemically modified LPS
<i>B. abortus</i>			
No. 1	39	7	10
No. 2	1400	52	87
No. 3	7	4	3
<i>Y. enterocolitica</i> 09			
No. 1	4	24	2
No. 2	7	30	2

<sup>a</sup> A polyspecific sheep anti-human immunoglobulin conjugate (detecting IgA, IgG and IgM class antibodies) was used. For LPS coating doses see Table 2.

**Table 4.** *Median relative titres of antibody to lipopolysaccharides from Brucella abortus and Yersinia enterocolitica 09, native and chemically modified, as determined by enzyme-linked immunosorbent assay, in sera from patients with brucellosis or yersinia type 09 infection*

Type of infection	No. of sera assayed	Median titre and range against <sup>a</sup>		
		<i>B. abortus</i> LPS	<i>Y. enterocolitica</i> 09 LPS	<i>Y. enterocolitica</i> 09 chemically modified LPS
Brucellosis	23	1040 (320–1760)	930 (270–1630)	1010 (290–1690)
Yersiniosis 09	9	950 (30–1090)	1500 (330–2200)	180 (10–230)

<sup>a</sup> The same conjugate and coating doses as in Table 3 were used.

*Y. enterocolitica* 09 LPS antigen will permit a differential diagnosis between brucella and yersinia type 09 infections.

This observation was further tested by making titre determinations on 23 of the sera collected from patients with brucellosis in England, Italy and Iran (Table 4). The median relative ELISA titres against the three different antigens were equally high in all instances. In all individual sera the titre estimated with the chemically modified *Y. enterocolitica* 09 LPS antigen was as high as that seen with the native LPS antigen. When nine sera from patients with *Y. enterocolitica* 09 infections were assayed only the native *Y. enterocolitica* 09 LPS antigen efficiently detected the antibody response (Table 4).

Table 5. *Median (and range of) relative titres of antibody to Brucella abortus lipopolysaccharide in sera collected from patients with known tularaemia, salmonellosis or Yersinia enterocolitica 03 infection, and from healthy controls*

Origin of serum	No of sera assayed	Median titre and range against <sup>a</sup> <i>B. abortus</i> LPS
Tularaemia	10	40 (30-140)
Salmonellosis	10	60 (30-230)
<i>Y. enterocolitica</i> 03	10	50 (20-180)
Healthy controls	25	20 (0-90)

<sup>a</sup> The same conjugate and coating dose as in Table 3 was used.

### *Specificity of B. abortus LPS antigen*

The specificity of the *B. abortus* phenol-water-extracted LPS antigen was tested in an experiment using 55 different sera collected from 25 healthy normal controls and 30 patients with infections verified by positive bacteriological cultures: 10 with tularaemia, 10 with salmonellosis and 10 with *Y. enterocolitica* type 03 infections. In the sera collected from patients the homologous titres in ELISA assays were > 1500 using a sheep anti-human Ig conjugate. When the *B. abortus* LPS was used as antigen no individual titre above 230 was seen and all median titres were ≤ 60 (Table 5). This demonstrates that the LPS antigen used is specific for antibodies elicited during *Brucella* infections, with the exception of antibodies elicited during *Y. enterocolitica* type 09 infections.

## DISCUSSION

The specificity of the antigen preparation used is of crucial importance in order to take full advantage of the increased sensitivity offered by enzyme immunoassay (Carlsson & Lindberg, 1978). Antibody determinations for the diagnosis of brucellosis, like the direct and mercapto-ethanol agglutination tests, complement fixation tests and Coombs' test (Kerr *et al.* 1968) all employ whole bacterial cells with their multitude of antigenic determinants. The results of these tests have not always been easy to interpret.

The results presented in this study suggest that the use of a phenol-water-extracted LPS from *B. abortus* strain 19 as antigen permits a safe serological diagnosis of the antibody response after *B. abortus*, *B. melitensis* and *B. suis* infections. Thus high titres significantly above the levels found in normal healthy controls were seen in patients where the diagnosis had been verified by positive blood cultures (Fig. 2, Table 1). Also in sera collected from patients, where the bacteria had not been isolated but the diagnosis was considered safe on clinical and laboratory findings, the titres were high. As most sera were collected in an

early convalescent stage IgM as well as IgG titres were high (Table 1). As expected the IgM titres were high during the acute phase of the illness and did not decline until the bacteria were eradicated by antibiotic therapy (Fig. 2, Swedish cases). In sera collected from 139 cases of chronic brucellosis in Iran and Poland the median IgM titre was low, and at the same level as in Swedish controls (Table 2).

The persistence of the IgG titre varied. In some of the sera collected one year after the infection (Swedish patients) the IgG titres were still high, and well above titres seen in the control material. However, in sera from some patients the IgG titre within months had dropped to 'background' levels seen in sera from healthy controls. Not unexpectedly high as well as low IgG titres were seen in the 139 sera from patients with chronic brucellosis. The median titre (720) was, however, much higher than that seen in sera from the controls (50).

The *B. abortus* LPS antigen also seemed to permit a specific diagnosis of brucellosis. None of the convalescent phase sera collected from patients with salmonellosis, yersiniosis type 03 or tularaemia, where isolation of the causative organism had been made, showed titre values above the level of sera from healthy controls (Table 5).

The cross-reaction between *B. abortus* and *Y. enterocolitica* 09 is well established (Ahvonen, Jansson & Aho, 1969; Hurvell & Lindberg, 1973), and can lead to difficulties in differential diagnosis in areas where both brucellosis and yersiniosis are common. Attempts to circumvent this cross-reactivity, such as using the presence of the enterobacterial common antigen in *Y. enterocolitica*, and its absence in *B. abortus*, have met with difficulties because of the presence of low titres of anti-enterobacterial common antigen in sera from cattle (Mittal, Ricciardi & Tizard, 1980). Our studies show that the use of phenol-water-extracted LPS from *B. abortus* and *Y. enterocolitica* 09 improved the specificity of the serological assay. Thus the *B. abortus* titre was higher than the *Y. enterocolitica* 09 titre in sera from patients with brucellosis, and vice versa (Tables 2-4). Based on median titres the difference was up to sevenfold. In individual sera, however, the difference was occasionally non-existent, making a differential diagnosis impossible. Therefore an additional method was devised based on the susceptibility of the *Y. enterocolitica* 09 LPS to periodate oxidation and borohydride reduction (Hurvell *et al.* 1979). The use of the chemically modified *Y. enterocolitica* 09 LPS antigen together with its native LPS showed that antibody titres against brucella were equally well detected by both antigens in experimental rabbit sera and in human sera (Fig. 2, Tables 3 and 4). In sera with specificity for *Y. enterocolitica* 09 the native LPS was about 10 times more effective in detecting the antibody response than the chemically modified antigen. These data suggest that the antigenic determinants in the *B. abortus* and *Y. enterocolitica* 09 antigens are not identical but only partly so. The homology should be found in a region resistant to periodate oxidation and borohydride reduction, whereas the *Y. enterocolitica* 09 0-antigen should have a region, i.e. determinant, susceptible to chemical treatment. This determinant also appears to be the site which predominantly detects the antibody response after a *Y. enterocolitica* 09 infection.

The chemical structure of the 0 polysaccharide chain in either *B. abortus* or

*Y. enterocolitica* 09 is still unknown. LPS extracted from both organisms contains only small amounts of carbohydrate (Hurvell & Lindberg, 1973; Berman *et al.* 1980). Glucose and galactose have been found to be monosaccharides common to both bacteria, and also in terminal non-reducing positions (Hurvell & Lindberg, 1973). An added interesting feature which, however, has complicated structural analyses, is the fact that in *B. abortus* protein appears to be covalently attached to the O polysaccharide chain (Berman *et al.* 1980). It is evident that knowledge of the structure(s) of the antigenic determinants will be required before we can establish absolutely safe serological analyses.

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