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Brucellosis: serological methods compared

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SUMMARY

At least 12 persons contracted clinical, and 4 persons subclinical *Brucella melitensis* infection during a brucellosis epidemic in the spring and summer of 1983 in Southern Germany, a region which had been free of this disease for the past 20 years. All cases of illness were traced to one infected herd of sheep. The presence of antibodies against *B. melitensis* was examined in 72 sera of infected patients using the following tests: agglutination, Coomb's test, two complement fixation tests with different antigen preparations (CFT 1 and 2), IgG and IgM enzyme-linked immunosorbent assay (ELISA), and opsonophagocytosis; and the occurrence of cross-reacting antibodies against *Yersinia enterocolitica* O 9 was investigated in the agglutination and complement fixation tests. Sera from 100 blood donors and 112 other people with close contact with sheep were also examined. The results revealed the need to consider an intermediate range in the interpretation of ELISA results – due to elevated values of persons in groups at risk but without clinical signs of illness. In all other tests, however, such persons revealed the same cut-off levels as the general population. Results from all initial sera of infected persons revealed titres of optical densities above the baseline levels determined in the present study, with the exception of the Coomb's and CFT 2 tests. The agglutination test, but not brucella CFT 2, revealed complete cross-reactivity between *Y. enterocolitica* O 9 and *B. melitensis*. ELISA stood out as the only test which is suited to diagnosis of both recent and past infection, since ELISA IgM determination permits conclusions about the time of the onset of illness, and determination of IgG may still yield values above the cut-off level up to 623 days after the onset of illness. In 2 of the 16 infected persons, IgG ELISA was the only test revealing previous infection 424 and 528 days after the onset of illness. A procedural scheme is presented which may help to simplify the diagnosis of brucellosis.

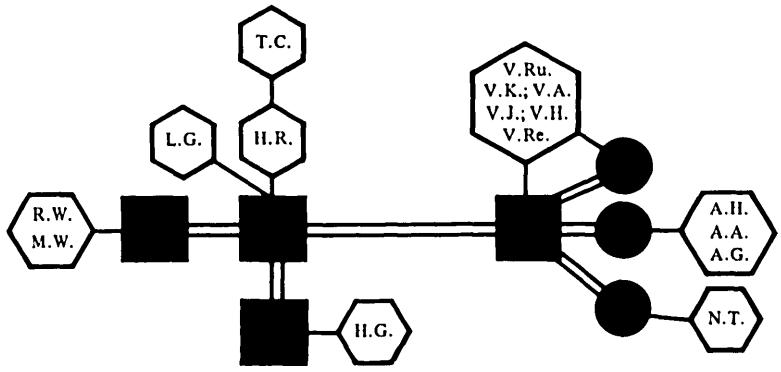

Fig. 1. Inter-relationships between infected herds of sheep and cattle, and infected persons (group 2). ■, Herds of sheep; ●, herds of cattle; ○, infected persons; ═, joint pastures or regions of movement.

INTRODUCTION

In the spring and summer of 1983, an epidemic of Malta fever broke out in Southern Germany, a region which had been kept free of brucellosis over the previous 20 years by veterinary measures. A minimum of 12 clinical and 4 subclinical infections were diagnosed in humans (Moegle *et al.* 1985). More than 3000 sheep and 40 other animals (dogs and donkeys) had to be killed.

The pathogen, *Brucella melitensis* biovar 1 (biovar was determined by the Central Veterinary Laboratory, Weybridge, UK) was isolated from three patients. All of the observed cases of disease were traced to a herd of sheep infected with *B. melitensis* (Fig. 1). This epidemic, the most severe case of which has been discussed in a case report (Heizmann, Botzenhart & Heber, 1984), led to the present serologic study in which the expediency of a recently introduced enzyme-linked immunosorbent assay was also examined.

MATERIALS AND METHODS

Patients

A total of 295 sera were examined, including 100 sera of blood donors used to determine normal levels of the brucella ELISA and a complement fixation test (CFT) in the general population. The other sera were broken down into three groups: (1) Sera from shepherds and sheepshearers; farm labourers and veterinarians prior and subsequent to extermination of infected herds; some family members of persons in group 2 (totals in group 1 : 99 people and 112 sera, including 13 paired sera). (2) Sera from shepherds, sheepshearers and some family members; all had direct or indirect contact with infected herds and revealed antibodies against brucella antigen (16 people and 72 sera). (3) Patients from the academic medical centre of the University of Tübingen who had antibodies against brucella antigen (4 people and 11 sera).

Agglutination tests

Brucella agglutination was performed with the macro method (initial 20-fold serum dilution) using antigens of *B. abortus* and *B. melitensis*, as well as a positive bovine control serum conforming to WHO standards (antigens and control serum were obtained from the Institute of Veterinary Medicine, Federal Bureau of Health, Berlin, FRG). In contrast to the procedure of Renner & McMahon (1981), however, 18 h incubation was performed at 50 °C instead of 37 °C to reduce the number of non-specific reactions (Wilson & Miles, 1975). The control serum titer then amounted to 160 rather than the titre of 320 indicated by the Institute of Veterinary Medicine.

The *Yersinia enterocolitica* agglutination test was performed with antigen and positive control serum (rabbit) of the Institute of Hygiene of the University of Tübingen. A micro method (Ansorg, Unger & Palm, 1983) was employed for this agglutination test.

Coomb's test

The Coomb's test was performed with the aid of the above-mentioned brucella antigens subsequent to agglutination testing. All tubes lacking visible agglutination were centrifuged at 1500 **g** and resuspended in 0·9 % NaCl. This washing step was repeated twice and subsequently 0·9 ml brucella suspension was incubated with 0·1 ml anti-human globulin (Fa. Molter, Heidelberg, FRG) for 24 h in a water bath maintained at 50 °C.

Complement fixation test

The antigens and control sera described under agglutination above were used for the brucella CFT1. The brucella CFT2 and the *Y. enterocolitica* O 9 CFT were performed using commerically available antigens, mainly lipopolysaccharides (LPS) and control sera (guinea-pig, Institut Virion, Würzburg, FRG). All complement fixation tests employed the micro method with long-term cold fixation (18 h, 4–6 °C; Palmer, 1980).

Enzyme-linked immunosorbent assay

The test principle of ELISA is described by Voller, Bidwell & Bartlett (1980). *B. abortus* antigen, mainly LPS (Institut Virion, Würzburg, FRG) was reconstituted in 1 ml distilled water according to the manufacturers specifications. Each well of a polystyrene microtitre plate (Institut Virion) was coated with 200 μl of the antigen (diluted 100-fold in coating buffer with 0·1 M-$NaHCO_3/Na_2CO_3$, pH 9·6) and incubated overnight at room temperature (approximately 24 °C) in a moist chamber.

The wells were rinsed three times with 0·9 % NaCl containing 0·05 % Tween 20 and 0·02 % sodium azide (washing buffer) and then filled with 200 μl of the serum to be tested (diluted 100-fold in serum-and-conjugate dilution buffer containing phosphate-buffered saline, pH 7·2; 0·1 % bovine serum albumin; 0·05 % Tween 20 and 0·02 % sodium azide). After incubation for 2 h at room temperature the wells were washed three times with washing buffer, and 200 μl of the phosphatase-labelled rabbit anti-human IgG (h+l chains) or porcine anti-human IgM (μ-chain, both

Institut Virion) were added per well. After a subsequent incubation period of 3 h at room temperature, 200 μl of substrate (1 mg *p*-nitrophenylphosphate/ml, Virion Würzburg, FRG) in buffer were added. The test was evaluated using internal standardization (Carlier, Bout & Capron, 1981); i.e., the reaction was stopped by addition of 50 μl 3 N-NaOH after the reference serum had reached a given optical density (at 405 nm). This usually occurred after approximately 30 min. At this point, all tested sera were read, taking the substrate as zero (Virion Reader, Institut Virion).

Rheumatoid factor was determined with latex reagents (Behring-Werke AG, Marburg, FRG).

Blocking test (ELISA)

LPS antigens of *B. abortus*, *Y. enterocolitica* O 9, *Y. enterocolitica* O 3 and *Bordetella pertussis* were diluted sevenfold. This antigen solution was used to dilute patient sera 50-, 100-, 200-, 400-, and 800-fold. Subsequent incubation for 18 h at 4 °C was followed by 30 min centrifugation at 1500 **g**; and ELISA was performed on the supernatant simultaneously with untreated serum.

Opsonophagocytosis test

A 48 h culture of *B. melitensis* biovar 1 (smooth colonies only) was suspended in 0·9 % saline and the density of the suspension was adjusted to McFarland > 10. The test was performed according to Jersild (1941): 0·1 ml cellular suspension was mixed with 0·1 ml patient serum (diluted 10-fold in 0·9 % NaCl) and 0·1 ml citrated blood (0·2 ml citrate + 9·8 ml blood). The resulting mixture was then incubated for 30 min at 37 °C in a water bath with slight shaking. Thereafter, smears were made and Giemsa stained.

Two controls were used: (1) blood + 0·9 % saline + brucella suspension and (2) blood + serum from various healthy persons + brucella suspension.

Subsequently, the number of bacteria engulfed by individual leucocytes was counted in 25 leucocytes, and the results were broken down as follows: leucocytes containing between 1 and 10 bacteria were scored 0·5; those with between 11 and 20 bacteria were scored 1; those with between 21 and 30 were scored 2; those between 31 and 40 were scored 3 and above 40 were scored 4. The sum of the scores of 25 leucocytes yielded the opsonophagocytosis index, with a possible maximum of 100.

RESULTS

Monitoring the level of brucella agglutination titres from routine laboratory testing in 1984 using sera from the local population revealed that 247 (97 %) of the titres were < 20. The same investigation revealed brucella CFT levels < 5.

A yersinia O 9 agglutination titre ⩾ 160 in the present laboratory is considered to constitute a strong indication of infection, whereas titres of 80 are considered worthy of being checked.

Examination of the 100 blood donor sera from a region of the FRG which is free of brucellosis revealed that an ELISA OD 405 ⩽ 0·250 (IgG: $\bar{x}+3s = 234$; IgM: $\bar{x}+3s = 236$) may be viewed as a negative reaction.

The same 100 donor sera were used to determine the cut-off level of normal titres

of the brucella CFT2 and the yersinia CFT in the general population; all donor titres were $\leqslant 10$ and may be considered to be negative.

The 112 sera (99 persons) of group 1 were examined to obtain data from a population at risk.

All brucella agglutination titres were < 20, CFT 2 < 10. Two sera revealed Coomb's test titres of 20, a CFT 1 titre of 5 was observed in one serum. Respective titres of 40 and 80 were found in two sera by yersinia agglutination; and 5 in one serum and 10 in two sera by yersinia CFT. Since these increased titres appeared in different persons with no clinical signs of disease, the above cut-off levels of normal titres were followed throughout the study; and a titre < 20 were taken as the cut-off level in the Coomb's test.

The upper cut-off level in the opsonophagocytosis test was determined to be $\bar{x}+3s = 33$ ($n = 84$).

In group 1, ELISA yielded the following optical densities: IgG: $\bar{x}+3s = 0{\cdot}360$ ($0{\cdot}038$–$0{\cdot}390, n = 112$); IgM: $\bar{x}+3s = 0{\cdot}297$ ($0{\cdot}021-0{\cdot}379, n = 112$). It was therefore necessary to consider an intermediate range of ELISA values (OD405 of 0·250–0·400) which may be found in the persons at risk. Such persons may have been exposed to antigen at a much earlier time and lack clinical signs of manifest disease. Of course sera falling in this intermediate range may also originate from patients in the incubation period or an early phase of disease.

Group 2 comprised 16 persons revealing antibodies against brucella. Since epidemiology showed very close association between these patients and infected herds, diagnosis of brucella infection can be taken to be safe in all members of the group, not just the three persons (R.W., L.G., T.C.), from the blood of whom *B. melitensis* was isolated (Table 1). Titres of initial sera were $\geqslant 40$ in brucella agglutination, $\geqslant 10$ in CFT 1, and optical density was $> 0{\cdot}700$ in brucella IgG ELISA. Initial titres of CFT 2 were > 10 in 12 sera and increased above this level in the sera of 1 patient (V.Re.) soon after the onset of observation.

Of the 16 patients in group 2, 12 were observed over a long period of time (111 to 623 days after the onset of illness); in one of these patients (V Re.) symptomatic illness was not observed.

In seven patients (R.W., N.T., A.H., A.A., V.H., V.Re. V.Ru.; see Table 1) brucella agglutination titres dropped below the cut-off level to normal values more rapidly than was the case in IgG ELISA. This indicates that brucella agglutination alone does not suffice to detect past brucella infection. The Coomb's test is also not always helpful; in three cases (R.W., A.A., V.Ru.) it was also negative.

In CFT 1 titres of 4 patients (R.W., N.T., A.H., A.A.) dropped below the cut-off level during the period of observation; in 2 other patients (A.G., V.Re.) the cut-off level was just reached. In contrast, there was a drop below the CFT 2 cut-off level in 5 patients (R.W., N.T., A.H., A.A., V.Re.), and the cut-off level was just reached in 3 other patients (T.C., V.A., V.H.) during the observation period.

IgG values were maintained at high levels over a lengthy period of time (up to 623 days after the onset of illness) in 9 of 12 patients. In 4 of the 12 patients (A.H., A.A., V.Re., R.W.) the OD405 decreased and approached the upper limit of the intermediate range. After 588 and 424 days, IgG ELISA was the only test revealing recent infection in two patients (R.W. and A.A., respectively).

Clear correlation between OD405 nm $> 0{\cdot}400$ in IgM ELISA and the onset of

Table 1. *Different serologic methods (agglutination, Coomb's test, complement fixation, ELISA, opsonophagocytosis test): comparison of results of shepherds, sheepshearers and family members with antibodies against brucella antigens (16 patients, group 2).*

			Brucella										Yersinia	
			Titre							OD_{405} ELISA		Index	Titre	
			Agglutination		Coomb's test		Complement fixation 1						*Y. enterocolitica* O9	
Name	Date	‡Days	*B. arbortus*	*B. melitensis*	*B. abortus*	*B. melitensis*	*B. abortus*	*B. melitensis*	Complement fixation 2	IgG	IgM	Opsonophagocytosis	Agglutination	Complement fixation
L.G.*	15. vii. 83	66	640	640	1280	1280	80	160	40	1·270	0·775	—	640	20
10. v. 83	20. vii. 83	71	1280	1280	n.d.	n.d.	160	640	n.d.	n.d.	n.d.	—	640	n.d.
	01. viii. 83	83	640	1280	n.d.	n.d.	80	320	n.d.	n.d.	n.d.	—	320	n.d.
	16. viii. 83	98	320	640	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	n.d.	n.d.
	22. viii. 83	104	320	640	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	n.d.	n.d.
	14. ix. 83	127	320	320	1280	2560	40	80	20	1·250	0·351	89	320	20
	21. ix. 83	134	80	320	160	640	40	80	20	1·158	0·514	—	320	20
	27. ix. 83	140	80	320	neg.	640	40	80	20	1·256	0·358	—	320	20
	12. x. 83	155	640	640	neg.	neg.	80	160	160	1·183	0·526	—	320	≧ 160
	20. x. 83	163	320	640	640	1260	160	640	usr.	1·236	0·240	—	640	usr
	26. x. 83	169	320	640	640	2560	160	640	160	1·202	0·164	—	640	80
	04. xi. 83	178	320	1280	640	2560	160	640	160	1·245	0·227	81·5	640	80
	10. xi. 83	184	320	1280	640	neg.	160	640	80	1·338	0·306	—	640	20
	17. xi. 83	191	320	1280	640	2560	80	640	40	1·266	0·345	—	640	20
	05. iii. 84	300	80	160	320	1280	40	160	80	1·252	0·082	57	160	20
H.R.	20. ix. 83		20	40	40	neg.	10	10	< 10	0·902	0·098	71·5	< 40	< 10
T.C.*	15. ix. 83	92	80	40	neg.	neg.	40	80	20	1·250	0·291	56	320	20
15. vi. 83	19. xi. 83	157	40	20	neg.	neg.	20	40	20	1·287	0·082	73·5	80	< 10
	14. xii. 83	182	20	< 20	40	40	80	80	20	1·180	0·240	63	40	10
H.G.	20. ix. 83	47	320	320	640	640	160	160	160	1·372	0·551	75	320	< 10
04. viii. 83	26. ix. 83	53	320	320	640	640	80	160	240	1·172	0·716	—	320	< 10
	05. x. 83	62	160	640	640	neg.	160	320	160	1·020	0·600	—	160	< 10
	26. x. 83	83	160	320	640	640	80	160	160	0·968	0·350	—	160	n.d.
	09. xi. 83	97	40	160	80	640	40	160	40	1·280	0·148	—	160	< 10
	23. xi. 83	111	40	80	80	160	80	160	40	1·253	0·152	74·5	80	< 10

R. W.*	02. ix. 83.	24	160	320	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
09. viii. 83	09. ix. 83	31	160	320	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	28. ix. 83	50	160	160	neg.	20	40	20	1·183	0·813	—	10
	12. x. 83	64	40	40	neg.	20	40	20	0·977	0·523	88	320	160	20
	26. x. 83	78	40	20	neg.	160	40	< 10	1·328	0·214	—	80	< 10
	09. xi. 83	92	20	< 20	40	40	40	20	1·265	0·136	—	80	< 10
	23. xi. 83	106	< 20	< 20	40	40	40	40	1·160	0·120	88	40	< 10
	09. i. 84	153	< 20	< 20	neg.	20	20	20	1·200	0·180	—	< 40	10
	30. iii. 84	234	< 20	< 20	neg.	5	20	10	0·877	0·165	35	< 40	< 10
	19. iii. 85	588	< 20	< 20	neg.	< 5	< 5	< 10	0·470	0·062	—	< 40	< 10
M. W.	29. ix. 83	16	320	320	640	40	40	n.d.	n.d.	n.d.	—	n.d.	n.d.
13. 09. 83	14. xi. 83	62	320	160	640	320	80	160	1·378	0·901	60	640	40
	25. i. 84	134	640	640	1280	1280	80	80	1·309	0·420	61	160	40
	30. iv. 84	230	80	80	neg.	neg.	20	40	1·220	0·535	—	n.d.	20
N. T.	02. i. 84	89	< 20	160	80	320	40	40	1·270	0·450	64	80	10
05. 10. 83	13. iii. 84	160	< 20	80	40	320	5	20	1·055	0·442	36·5	80	5
	08. v. 84	216	20	40	40	160	10	10	1·100	0·253	—	40	10
	06. xi. 84	398	< 20	< 20	neg.	20	< 5	n.d.	1·050	0·140	—	< 40	< 10
A. G.	21. xi. 83	111	80	40	neg.	neg.	20	< 5	1·255	0·300	63	160	< 10
02. 08. 83	27. i. 84	178	80	20	160	neg.	20	< 5	0·920	0·130	72·5	160	10
	12. iii. 84	223	80	40	160	160	20	< 5	1·171	0·049	72	160	< 10
	08. v. 84	280	80	40	neg.	80	20	20	1·218	0·420	—	80	< 10
	22. xi. 84	478	40	40	160	160	5	< 5	1·150	0·290	—	40	< 10
A. H.	21. xi. 83	91	40	80	neg.	neg.	10	40	< 10	0·758	0·478	68	80	< 10
22. 08. 83	27. i. 84	158	< 20	< 20	neg.	neg.	< 5	< 5 <	usr.	0·653	0·051	19	< 40	usr.
	12. iii. 84	203	< 20	< 20	neg.	20	< 5	10	10	1·597	0·049	31	< 40	< 10
	22. xi. 84	458	< 20	< 20	neg.	40	< 5	< 5	< 10	0·585	0·150	—	—	< 10
A. A.	21. xi. 83	57	40	< 20	neg.	40	20	40	20	1·114	0·010	74	40	< 10
25. 09. 83	27. i. 84	124	< 20	< 20	neg.	neg.	10	80	20	1·300	0·230	43	< 40	< 10
	12. iii. 84	169	< 20	< 20	neg.	neg.	5	80	20	0·679	0·008	43	< 40	< 10
	22. xi. 84	424	< 20	< 20	neg.	neg.	< 5	< 5	< 10	0·500	0·050	—	< 40	< 10
V. A.	13. x. 83	164	40	80	neg.	neg.	< 5	10	20	1·253	0·177	76	40	< 10
02.05.83														

Table 1. (*cont.*)

			Brucella									Yersinia	
			Titre							OD_{405} ELISA	Index	Titre	
			Agglutination		Coomb's test		Complement fixation 1					*Y. enterocolitica* O9	
Name	Date	‡Days	*B. arbortus*	*B. melitensis*	*B. abortus*	*B. melitensis*	*B. abortus*	*B. melitensis*	Complement fixation 2	IgG IgM	Opsonophagocytosis	Agglutination	Complement fixation
V.J.	13. x. 83	—	< 20	80	neg.	neg.	10	40	< 10	1·088 0·150	68	40	< 10
V.H.	13. x. 83	213	< 20	80	neg.	neg.	20	80	40	1·336 0·120	62	40	< 10
14. 03. 83	10. xi. 83	241	< 20	40	neg.	160	20	80	20	1·233 0·261	65	40	< 10
	22. iii. 84	374	< 20	< 20	neg.	80	40	80	20	0·840 0·325	62	< 40	< 10
	08. v. 84	421	< 20	20	20	160	20	80	40	1·200 0·286	—	< 40	< 10
	26. xi. 84	623	< 20	< 20	20	80	20	20	20	1·100 1·120	—	< 40	< 10
V.Ru.	13. x. 83	160	160	1280	640	5120	320	640	160	1·300 0·106	76	160	< 10
06. 05. 83	10. xi. 83	188	80	320	640	640	640	640	640	1·246 0·160	38	160	< 10
	22. iii. 84	321	80	320	160	2560	40	320	320	0·830 0·300	47	80	< 10
	08. v. 84	368	40	160	320	320	640	640	320	1·307 0·350	—	40	< 10
	26. xi. 84	570	< 20	< 20	80	320	80	320	80	1·290 0·720	—	< 40	< 10
V.Re	13. x. 83	0†	< 20	40	neg.	neg.	20	80	< 10	1·065 0·181	—	n.d.	< 10
	10. xi. 83	28	< 20	< 20	neg.	neg.	10	40	20	0·813 0·323	66	< 40	< 10
	22. iii. 84	161	< 20	< 20	neg.	neg.	5	40	20	0·580 0·180	53	< 40	< 10
	26. xi. 84	410	< 20	< 20	neg.	neg.	5	5	10	0·495 0·285	—	< 40	< 10
V.K.	13. x. 83	—	80	40	neg.	neg.	10	10	40	1·343 0·285	75	160	< 10

Neg., negative; n.d., not done; usr., unspecific serum reaction.

* Isolation of *Brucella melitensis* biovar 1.

† Note that V.Re. did not develop clinical symptoms during observation; days shown represent time elapsed from investigation of first serum.

‡ Days after onset of symptoms.

Date below the name indicates onset of symptoms.

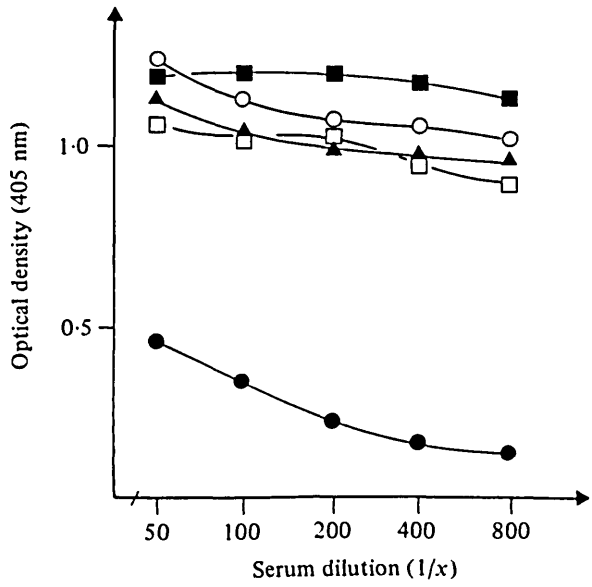

Fig. 2. Brucella ELISA (IgG) results (case H. G., 26.09.83, Table 1) after pre-absorption of the test sera with antigens: ○, without pre-absorption; ●, *Brucella abortus;* ▲, *Bordetella pertussis;* □, *Yersinia enterocolitica* O3; ■, *Yersinia enterocolitica* O9.

illness, was not evident because illness began 62 to 230 ($\bar{x} = 112$) days prior to obtaining initial sera with values at this level, whereas the onset of illness was 57 to 216 ($\bar{x} = 132$) days prior to obtaining initial sera with OD405 nm < 0·400.

If initial sera with an ELISA IgM OD405 of 0·600 are considered, however, the onset of clinical symptoms did correlate with the time of initial serum examination. The time difference between the onset of illness and the time of initial serum examination in four patients (L.G., H.G., R.W., M.W.) was 50 to 66 ($\bar{x} = 60$) days, whereas sera with OD < 0·600 revealed a time difference of 57 to 213 ($\bar{x} = 115$) days.

A remarkable finding was an increase of the IgM ELISA OD in one patient (V.Ru.) to more than 0·700 after one year in the absence of clinical symptoms of illness. Sera containing brucella-specific IgM antibodies never revealed rheumatoid factor.

With regard to the other 4 persons in group 2, from whom only 1 serum sample each was obtained, 1 patient (V.A.) complained of fatigue, fever (39 °C) and articular pain. The other persons were free of symptoms.

The specificity of the opsonophagocytosis test was tested with sera from L.G., R.W., and M.W. and Gram-negative bacteria. Average amounted to 79 for *B. melitensis*, 85 for *Y. enterocolitica* O9, 37 *Bord. pertussis*, 36 *Eschericihia coli*, 26 *Pseudomonas aeruginosa*, 18 for *Proteus vulgaris* OX19.

Since *Y. enterocolitica* infection is not uncommon in the Federal Republic of Germany, the occurrence of agglutinating and complement-fixing antibodies against this antigen was examined in all sera. The slightly elevated agglutination titres ($\leqslant 80$) found in four sera in group 1 do not constitute any indication of infection. In group 2, the yersinia agglutination titres attained the same levels as

Table 2. *Different serologic methods (agglutination, Coomb's test, complement fixation, ELISA, opsonophagocytosis test): comparison of results of four patients with brucella antibodies, from the academic medical centre of the University of Tübingen*

		Agglutination		Coomb's test		Complement fixation 1			ELISA			*Y. enterocolitica* O9	
Name	Date	*B. abortus*	*B. melitensis*	*B. abortus*	*B. melitensis*	*B. abortus*	*B. melitensis*	Complement fixation 2	IgG	IgM	Opsonophagocytosis	Agglutination	Complement fixation
TG.	17. ii. 84	80	40	160	320	< 5	< 5	< 5	0·418	0·200	47	40	20
	05. iii. 84	20	20	160	80	< 5	< 5	10	1·129	0·082	48·5	40	20
S.L.	02. iv. 84	160	80	320	160	5	20	< 5	0·600	0·428	59	320	20
	20. iv. 84	20	< 20	neg.	neg.	< 5	10	< 5	0·834	0·395	34	640	20
D.S.	20. iii. 84	40	40	neg.	neg.	n.d.	n.d.	< 5	0·225	0·335	36·5	40	5
	27. iii. 84	5120	1280	n.d.	n.d.	< 5	20	20	0·620	0·950	84	1280	40
	04. iv. 84	640	640	neg.	neg.	10	10	10	0·853	0·710	—	n.d.	40
	*16. iv. 84	160	80	n.d.	n.d.	5	5	10	1062	0·718	68	640	40
	†07. v. 84	< 20	< 20	neg.	neg.	5	5	10	0·395	0·490	30·5	80	20
B.S.	29. ix. 84	80	80	neg.	neg.	< 5	< 5	< 10	0·391	0·641	78	80	< 10
	25. xii. 84	20	< 20	neg.	neg.	5	5	usr.	0·403	0·206	67	320	usr.

Neg., negative; n.d., not done; usr. unspecific serum reaction.

* *Yersinia enterocolitica* O3a agglutination titre < 40; *Yersinia enterocolitica* O3b agglutination titre < 40.

† *Yersinia enterocolitica* O3a agglutination titre < 40; *Yersinia enterocolitica* O3b agglutination titre 160.

in brucella agglutination (Ahvonen, Jansson & Aho, 1969); early decrease in titre levels was not observed over time. Yersinia CFT titres in most (75%) of these patients were < 10.

The specificity of the ELISA used in the present study was examined using a blocking test. A patient's serum (H.G) was saturated with various antigens (Fig. 2) and the remaining optical density was subsequently determined. When saturated with *Y. enterocolitica* O9, serum optical density was not reduced compared to the nonsaturated control. Saturation with brucella antigen, however, resulted in a significant decrease in optical density. While keeping the lower initial optical density in mind, basically the same results were obtained with IgM (data not shown).

During the present period of study, *Y. enterocolitica* was isolated from the stools of a patient revealing serum antibodies. Agglutination and CFT 1 and 2 revealed cross-reactions in serum from this patient and prevented differentiation of the pathogen merely with the aid of CFT. However, the ELISA IgG and IgM values were only in the intermediate range.

Group 3 (Table 2) comprises patients of the academic medical centre of the University of Tübingen with equivocal reactions. Three patients (T.G., S.L., D.S.) had undergone surgery due to symptoms of acute appendicitis; in each case a clear change in colour of the terminal ileum was observed in the presence of a normal appendix. In one patient an enlarged mesenterial lymph node had resulted in invagination. Blocking tests (D.S.) were performed with *Y. enterocolitica* O9: repeated titre determination in ELISA, brucella and *Y. enterocolitica* agglutination as well as CFT 1 did not provide additional information helpful in identifying the pathogen because optical density and antibody titres dropped below cut-off levels.

DISCUSSION

In 1983, Malta fever broke out in a region of Southern Germany with intensive sheep herding, which had been kept free of brucellosis for the preceeding 20 years (Moegle *et al.* 1985). The cases of brucellosis presented in the present paper are therefore most probably initial infections, particularly because brucellosis was not evident from patient histories nor were non-specific symptoms reported which may be signs of chronic brucellosis. Antigen exposure is not excluded in elderly persons.

Due to the non-specific symptoms (fever, articular pain; Moegle *et al.* 1985). brucellosis was initially diagnosed 16–213 days after the onset of clinical symptoms. In the case of L.G., diagnosis lead to examination of suspected herds and the isolation of *B. melitensis* from sheep and cattle.

All patients in group 2 with or without clinical symptoms were treated with tetracycline (2 × 100 mg/day doxycycline) which was combined with streptomycin (1 × 1g/day) in some cases.

Antigen detection was attempted late in the infected patients reported in the present study. Therefore, diagnosis of Malta fever in 13 of the 16 persons found to have contracted the disease (group 2) was based on epidemiology (Fig. 1). clinical condition, and antibody detection.

In recent years, various reports have appeared on the feasibility of brucella antibody detection using radioimmunoassay (RIA; Parratt *et al.* 1977) or ELISA

(Magee, 1980; Sippel, Ayad El-Masry & Farid, 1982). Little is known about changes in antibody titres over time, however, because only Sippel, Ayad El-Masry & Farid (1982) examined sera for a certain period of time (4 sera, 1 month; 4 sera, 4–8 months). Furthermore, the previously reported data only reveal the time of onset of disease in a few cases.

In the present study, the onset of illness was 47–213 days prior to obtaining sera for all tests in group 2. The actual time at which titres began to rise therefore cannot be determined from the present data. Some conclusions about the onset of illness can be drawn from IgM ELISA OD levels $\geqslant 0{\cdot}600$, however. At this optical density, illness can be assumed to have begun within the previous 60 days. At the statistically significant cut-off level of normal values of 0·400 the same assumption cannot be made.

Since all patients were symptom free at the conclusion of the present examinations (19 March 1985), and reactivation of brucellosis was not observed in any patients, the present data do not permit conclusions about serologic alterations in reactivation of brucellosis.

The results of the serologic tests of groups 1 and 2 indicate that it is helpful to define an intermediate ELISA IgG range between OD 0·250 and 0·400 which may represent (1) persons at risk (possibly with long past antigen exposure) but without clinical symptoms, and (2) persons in the incubation period or (3) an early phase of disease. A corollary of establishing such an intermediate range is the necessity of retesting a second serum sample obtained at a later date.

Unfortunately, of the reports mentioned above (Parratt *et al.* 1977; Magee, 1980; Sippel, Ayad El-Masry & Farid, 1982) only Lindberg *et al.* (1982) discussed the problem of cross reaction of antibodies against *Brucella spp.* and *Y. enterocolitica* O 9 although this phenomenon has been known since 1969 (Ahvonen, Jansson & Aho). For this reason, both agglutinating and complement-fixing antibodies against *Y. enterocolitica* O 9 were investigated in the present study. The present results reveal complete cross-reaction in the agglutination tests (Ahvonen, Jansson & Aho, 1969; Corbel, 1975), but only incomplete cross-reaction in *Y. enterocolitica* CFT which only revealed titres above 10 in 4 of the 16 patients with brucellosis.

Despite the specificity of the present ELISA technique – which was demonstrated in the blocking experiments, and in the case of P.C. (proven *Y. enterocolitica* O 9 infection), in which the intermediate range was not exceeded – the limitations of this test should be kept in mind (patients in group 3). Exposure to brucella antigen was not detected in three patients with terminal ileitis. The clinical picture and test results of serum from D.W. (7 May 1984, Table 3) most likely suggest *Y. enterocolitica* infection. Saturation tests, which are recommended in equivocal cases (Corbel, 1975; Granfors, Viljanen & Toivanen, 1981) were of no aid to differential diagnosis of D.S. In all three cases of terminal ileitis, yersinia CFT titres were > 10. In contrast, the clinical symptoms of B.S. (lumbago, polyneuropathy, slight to moderate elevation of hepatic enzymes), who came from a region with intensive sheep raising, and the results of the serologic tests – particularly the yersinia CFT titre < 10 – would appear to make the diagnosis of chronic brucellosis quite probable.

In summary, the following conclusions can be reached on the diagnostic value of the serologic tests used in the present study: (1) The opsonophagocytosis test

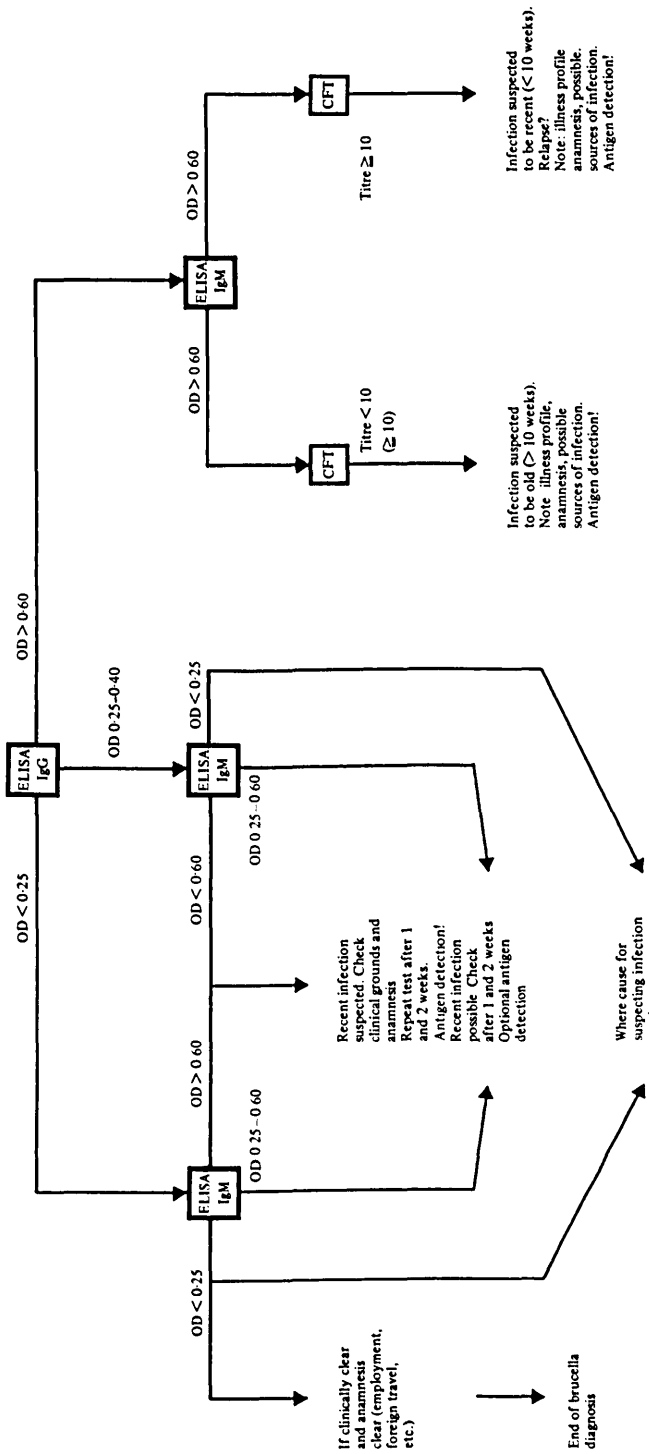

Fig. 3. In all cases where results are positive or doubtfully positive, investigation should be carried out simultaneously for antibodies against *Yersinia enterocolitica* O9.

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is quick and easy to perform; evaluation is quite time-consuming, however, and viable antigen must be used. Furthermore, differentiation between brucellosis and *Y. enterocolitica* infection is impossible. The test therefore appears to be unsuitable for brucellosis diagnosis and is rightly no longer used for this purpose. (2) Brucella agglutination titres may drop below cut-off levels more quickly than in other tests – which may prevent diagnosis of the illness when the test is used long after the onset of infection. (3) Incomplete antibodies are not always detected; they were only observed in a portion of infected patients. In three patients both the agglutination reaction and the Coomb's test were negative at the end of the observation period. (4) Diagnosis of past infection using the brucella CFT is also incomplete; CFT 1 titres began to drop below cut-off levels in infected patients after 158 days, CFT 2 titres after 91 days. (5) Brucella ELISA is the only serologic test which permitted diagnosis of both recent and past infection in the present study; i.e., determination of IgM antibodies permitted conclusions about the onset of illness; and IgG levels remained significantly elevated ($> 0{\cdot}400$) in all brucellosis patients observed for more than a year (in one case up to 623 days). In two patients (A.A., R.V.), the past infection was only detectable with IgG ELISA 424 and 588 days, respectively, after the onset of illness. If high optical density ($> 0{\cdot}600$) is confirmed in a second serum obtained from an interval of 3 weeks during the acute phase of the disease, brucellosis can be diagnosed with considerable reliability: only T.G. appears to represent an exception to this rule. The present ELISA is relatively easy to perform and conclusions about serologic parameters are forthcoming within 6 h. (6) Yersinia agglutination reveals complete cross-reaction with sera of brucellosis patients. There is no difference in the rate of change in titre dynamics.

Differentiation between infection due to *B. melitensis* and *Y. enterocolitica* O 9 succeeded with 10 of 16 patients when the range of error of one titre step in the yersinia CFT cut-off ($\leqslant 10$) was taken into account. Thus, differential diagnosis of brucellosis may be simplified by initial yersinia CFT and repetition of IgG ELISA, a procedure which may lead to identification of the pathogen without the necessity of performing time-consuming blocking tests.

In 3 of the 4 patients in group 3 (T.G., S.L., D.S.), which we consider to have suffered from yersiniosis on the basis of the clinical symptoms and the lack of contact with infected animals, yersinia CFT titres were $\geqslant 20$. In the fourth patient (B.S.), which we considered to have chronic brucellosis, the titre was < 10. A suggested diagnostic scheme is outlined in Fig. 3.

The present report presents a comparison of all methods currently available for brucellosis diagnosis and takes into account the serologic cross-reaction with *Y. enterocolitica* O 9. In contrast to earlier studies (Magee, 1980; Lindberg *et al.* 1982; Sippel, Ayad El-Masry & Farid, 1982), the present investigation included serum from patients involved in a well-documented *B. melitensis* epidemic. The results indicate that the present ELISA test is superior to conventional methods due to great sensitivity, the possibility of distinguishing brucella-specific classes of immunoglobulins, and simple and easy performance.

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