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Evaluation of in-house and commercial immunoassays for the sero-diagnosis of brucellosis in a non-endemic low prevalence population

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Summary The Brucella Reference Unit (BRU) at the University Hospital Aintree offers a national *Brucella* sero-diagnosis service for England, Wales, Eire and Northern Ireland. The United Kingdom is a non-endemic area with a very low prevalence of infection. The objective of this study was to evaluate new CE marked assays, Brucellacapt (Vircell) and *Brucella* IgG and IgM ELISAs (Vircell), against the standard set of in-house serological assays used at BRU. These include a micro-agglutination (MAG) assay, in-house IgG and IgM assays and a complement fixation test (CFT).

One hundred and forty-three archived serum samples were re-tested by both the commercial and in-house assays. Samples were divided into four distinct groups based on the most common clinical patterns of serological profiles seen (negative, clinically significant and two forms of clinical indeterminate results). The kappa test was calculated to determine the level of agreement between the commercial and in-house results.

The kappa coefficient for Brucellacapt and MAG assays was 0.90 (95% CI 0.85, 0.95) giving a very good level of agreement. Discrepancies between positive MAG and Brucellacapt assay results (5.7%) occurred only in sera with weakly reactive MAG titres of <1:160.

Similarly the kappa coefficient calculated for the IgG assays was 0.81 (95% CI 0.75, 0.87) also indicating good agreement. However, the kappa coefficient for the commercial and in-house IgM assays was poor at 0.38 (95% CI, 0.30, 0.46). The weak IgM correlation was associated in some instances, with a lack of use of IgG sorbent in the in-house assay resulting in false-negative results. In a low prevalence population, the combination of in-house and commercial immunoassays offers improvements in the sero-diagnosis of brucellosis.

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Introduction

Brucellosis is primarily a zoonotic disease that can also be transmitted to humans. It has a high prevalence in certain geographic areas such as the Mediterranean, Middle East,

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Latin America and Asia.¹ The clinical findings of the disease are non-specific and highly variable. Thus, the diagnosis requires confirmation by either isolation of the bacteria from blood or tissue samples or demonstrating the presence of *Brucella* antibodies by serological tests or *Brucella* DNA detection by PCR amplification assays. The sensitivity of blood culture varies from 15% to 70% depending on individual laboratory practices, stage of the disease, *Brucella* species and culture medium used.² Even with automated blood culture systems, subcultures need to be performed for at least four weeks. PCR diagnosis remains promising for the rapid diagnosis of acute but not chronic brucellosis since bacteraemia is present only in the acute stages of infection. Hence, serological investigation remains the mainstay for diagnosis.

Standard serological tests for the diagnosis of brucellosis include the standard agglutination test (SAT), Coombs test and enzyme immuno-assays (EIAs). Despite a high sensitivity ranging from 65% to 95%, these methods have a number of limitations. A high prevalence of *Brucella* antibodies in the healthy population decreases the specificity of these tests in endemic areas. Though a SAT titre of 1:160 or above may be considered diagnostic in conjunction with compatible clinical features, titres of 1:320 or greater may be more specific in endemic areas. Also, in patients with chronic disease who have a long period of evolution of the disease, the immune response can be reduced giving false-negative results in standard agglutination assays. The Coombs test, which is used to detect non-agglutinating or incomplete antibodies, is a useful test for SAT-negative cases of chronic brucellosis if the laboratory does not have access to EIAs. However, EIAs are now considered the test of choice for chronic brucellosis when other tests are negative. Serological tests also have a low sensitivity for early diagnosis of a possible relapse and are not useful for patient follow-up as serum titres decline slowly.

Annual reports by the UK Department of Environment Food and Rural Affairs indicate that cases of human brucellosis are rarely reported from this country. Though some cases are acute (reflecting travel to endemic areas and ingestion of infected, unpasteurized animal-milk products) and diagnosed by blood culture and/or serology, the majority are chronic infections. These cases reflect occupational exposure to infected cattle and are usually only diagnosed serologically.

The *Brucella* Reference Unit (BRU) at University Hospital Aintree, Liverpool offers a *Brucella* sero-diagnosis service for England, Wales, Eire and Northern Ireland. The service was established approximately five years ago, following transfer from the Public Health Laboratory at Portsmouth. Routine methods for sero-diagnosis include four in-house assays, micro-agglutination (MAG) assay and complement-fixation test (CFT) together with specific IgG and IgM EIAs. Though broadly comparable with the MAG test, CFTs have been used as an additional screening test since detectable antibodies may occur slightly earlier compared to agglutination tests. All serum samples submitted to the BRU for *Brucella* serology are routinely examined by the four tests mentioned above. The BRU receives approximately 2500 sera per year of which less than one percent have a MAG titre of 1:160 or greater, reflecting the very low prevalence of brucellosis in the UK.

Brucellacapt (Vircell S.L., Spain) is a CE marked single-step immunocapture assay for the detection of total anti-*Brucella* antibodies, which has been recently launched in the UK through Quest Biomedical (Solihull, West Midlands).

Previous evaluations have suggested good correlation with Brucellacapt, SAT and Coombs tests.³⁻⁵ Similarly CE marked commercial *Brucella* ELISA IgG and IgM assays (Vircell S.L., Spain) are now available. In the hope of incorporating such CE marked assays into the existing sero-diagnostic repertoire at BRU, we present an evaluation of these immunoassays in comparison with our standard laboratory methods.

Materials and methods

A number of archived sera were re-tested in parallel by both in-house and Vircell commercial assays. Sera were chosen to allow for investigation of the most common clinical patterns of serological results seen at BRU. They are as follows:

- Sera testing negative in all in-house assays (Group A).
- Sera with clinically significant MAG titres (greater than or equal to 1:160) and positive antibody titres in other in-house assays (Group B).
- Sera with clinically indeterminate MAG titres (more than 1:20 but less than 1:160) with negative antibody titres in other in-house assays (Group C).
- Sera with clinically indeterminate MAG titres (less than 1:160) but with positive antibody titres in other in-house assays (Group D).

The in-house MAG assay measures total *Brucella* antibody (IgG, IgM and IgA classes). Serum is incubated with safranin stained whole cell *Brucella abortus* antigen strain S99 obtained from the Veterinary Laboratory Agency (VLA), Weybridge, UK. The assay is performed in a V-bottomed plate at a dilution range of 1:20–1:2560, which is incubated at 37 °C for 24 h and then read by naked eye. A negative result (less than 1:20) is indicated by a button of red-stained bacteria in the centre of the well with a surrounding pink halo. If *Brucella* antibody is present, antigen–antibody complex formation results in a mat of stained cells covering the bottom of the well or by a diminished button of bacterial cells in the centre of the well with a surrounding slightly opaque diluent. A positive result was defined as a titre of 1:20 or greater. The end-point titre is derived from the well with the greatest dilution showing agglutination.

A titre of greater than or equal to 1:160 was considered to be clinically significant whilst a titre greater than 1:20 but less than 1:160 was of clinically indeterminate significance.

Like the MAG assay, the CFT measures total *Brucella* antibody and uses the same source of *Brucella* antigen. A standard CFT methodology is performed. The assay is undertaken in a U-bottomed microtitre plate at a dilution range of 1:4–1:256. If *Brucella* antibodies are present, an antigen–antibody complex is formed which 'fixes' added complement. If complement has been 'fixed' then when sensitised sheep red blood cells (RBCs) are added, lysis will not occur. However, if complement has not been fixed,

it is free to lyse the sensitised RBCs. The percentage lysis of RBCs in each well is interpreted as a lysis score. A positive result is indicated by a button of RBCs on the bottom of the wall (lysis score zero) and a negative result by complete lysis of RBCs (lysis score four). For a positive sample, the titre of the test serum is derived from the dilution showing 50% or more fixation (i.e. lysis score of two). A negative result is indicated by a complete lysis at 1:4 dilution. A positive result is indicated by a titre of greater than 1:4.

The in-house IgG and IgM enzyme immunoassays (EIAs) use VLA *B. abortus* bacterial antigen suspension, horse-radish peroxide labelled conjugate (anti-human IgG or IgM depending on which assay is performed) and enzyme substrate in a standard EIA format. For the purpose of the evaluation, a screening dilution of 1:20 was used with a titration range of up to 1:10,240. A positive result was indicated by an optical density (OD) value of more than 0.8. The titre of the test serum is derived as the highest dilution with an OD of more than 0.8. A titre of less than 1:20 was considered a negative result. Titres greater than or equal to 1:20 were considered positive results.

Methods for the Brucellacapt assay and *Brucella* IgG and IgM ELISAs (Vircell) have been previously described.^{3–5} The ELISAs use purified lipopolysaccharide (LPS) antigen of *B. abortus*, as opposed to whole cell preparations used in the in-house EIAs. After discussion with the manufacturer, a Brucellacapt titre of greater than or equal to 1:320 was considered to be of clinical significance for the purpose of an evaluation in a non-endemic area. The specificity of the Brucellacapt assay decreases slightly when titres of <1:320 are used for sero-diagnosis.⁴ For the study, the Brucellacapt assay was performed at a dilution range of 1:20–1:5120. An antibody titre of greater than or equal to 1:20 indicated a positive result. The Vircell IgG and IgM ELISAs are supplied as positive/negative qualitative assays using a screening dilution of 1:20. An antibody index of less than 9 is interpreted as a negative result, 9–11 as equivocal and more than 11 as positive. A negative result was indicated by an antibody index of <9 at a dilution of a titre of 1:20, a positive result was indicated by an antibody index of >11 at a dilution of \geq greater than or equal to 1:20. For

the purpose of the evaluation, equivocal antibody indexes were treated as a positive result.

For discrepant IgG and IgM results (though not stated in the product insert) the Vircell IgG and IgM ELISAs were also used in a quantitative format with a dilution range of 1:20–1:2560.

For statistical analysis, the simple percentage level of agreement was calculated for the comparison of the MAG test with Brucellacapt and for the comparison of the EIAs. The kappa coefficient, a measure of chance corrected agreement, was also calculated for each test comparison.^{6,7} The kappa coefficient was calculated for MAG and Brucellacapt comparison and the EIA comparison where there were two outcome measures, positive and negative. Ninety-five percent confidence intervals (95% CI) for each kappa coefficient were calculated.

Results

One hundred and forty-three archived sera were re-tested with the in-house and Vircell commercial assays. The numbers of sera in groups A, B, C and D were 35, 38, 35 and 35, respectively. An overall comparison of the results for the different immunoassays for each sero-diagnostic group is presented in Table 1.

For Group A sera, all 35 samples proved negative both in all in-house assays and the Brucellacapt and *Brucella* ELISA IgM assays. In the comparison of IgG EIAs, the Vircell commercial assay gave two (5.7%) discrepant positive results.

For Group B sera, there was also 100% agreement between MAG and Brucellacapt positive results though Brucellacapt titres were up to more than fourfold higher (Table 2). There was 94% (32/34) level of agreement between the in-house and *Brucella* ELISA IgG assays. Of the two discrepant results, both were negative in the in-house assay and positive in the commercial IgG ELISA. The level of agreement between the in-house and commercial *Brucella* ELISA IgM assays was lower at 63% (22/35). Of the 13 discrepant sera, all were negative in the in-house assay whilst positive in the commercial test.

Table 1 Summary of clinical interpretation of in-house and commercial immunoassays for sero-diagnosis of brucellosis

Clinical sero-groups	Total sera tested	Positive results (number)						
		MAG (\geq 1:20)	Brucellacapt (\geq 1:20)	CFT (\geq 1:4)	In-house IgG assay (\geq 1:20)	Commercial IgG assay (\geq 1:20)	In-house IgM assay (\geq 1:20)	Commercial IgM assay (\geq 1:20)
A–D	143							
A (negative MAG group)	35	0	0	0	0	2	0	0
B (clinically significant MAG group)	38	38	38	38	32	34	22	35
C (clinically indeterminate MAG group with negative antibody titres in other in-house assays)	35	35	35	0	0	8	0	13
D (clinically indeterminate MAG group with positive antibody titres in other in-house assays)	35	30	35	35	35	34	3	20

Table 2 Comparison of end point titres for Micro-agglutination and Brucellacapt titres by sero-diagnostic groups

Test	Antibody titre range										Sero-diagnostic group
	≥5120	2560	1280	640	320	160	80	40	20	<20	
MAG Brucellacapt	5	5	6	9	11	2	–	–	–	–	B
	27	7	3	0	0	0	–	–	–	–	
MAG Brucellacapt	–	–	–	0	0	0	1	12	22	–	C
	–	–	–	1	0	2	18	12	2	–	
MAG Brucellacapt	–	–	–	0	0	0	4	13	13	5	D
	–	–	–	15	3	10	6	0	1	0	

MAG = micro-agglutination test.

For Group C sera, the agreement between low positive MAG titres (more than 1:20 but less than 1:160) and positive Brucellacapt titres was 100% (35/35). However, only one serum sample had a clinically significant positive Brucellacapt titre (1:640) with a corresponding MAG titre of 1:80, CFT of less than 1:4 and negative in-house and commercial IgG and IgM assays. In the EIA IgG comparison 77% (27/35) of sera showed an agreement between negative in-house and commercial assays. Of the eight discrepant sera, all were negative in the in-house IgG assays, whilst positive in the commercial Vircell assay. The corresponding level of agreement for negative IgM assays was lower at 63% (22/35). Of the 13 sera showing discrepant results, all were negative in the in-house IgM assay, but positive in the commercial IgM assay.

For Group D sera, 86% of samples (30/35) had positive MAG titres though all were positive by the Brucellacapt assay. There was 97% (34/35) agreement between positive in-house IgG results and the commercial IgG assay. Only one sample was negative in the commercial Vircell IgG assay but positive in the in-house assay. The level of agreement between positive in-house and commercial IgM assays was again low at 15% (3/20). Of the 17 discrepant results all were negative in the in-house assay.

Though 113 sera in sero groups B, C and D were positive by MAG and Brucellacapt assays, titres were generally greater than fourfold higher in the Brucellacapt Test (Table 2). These end-points were also clearer and easier to read by naked eye.

Eighteen selected samples, where there was a discrepancy between in-house EIAs and Vircell assays, were re-tested independently by Vircell. Vircell performed a Brucellacapt and IgG/IgM ELISA using a quantitative format. For the Brucellacapt assay results were similar. Two samples, reported *Brucella* IgG ELISA positive at 1:20 by BRU, were negative by Vircell. Nine sera, where the in-house BRU IgM assay was negative at 1:20 but the Vircell *Brucella* IgM ELISA was positive at 1:20, were re-tested by the in-house method using commercial IgG sorbent in addition. In eight cases, the IgM result became positive with titres ranging from 1:20 to 1:1280.

The overall level of agreement between positive MAG and Brucellacapt sera was 96.5% (138/143) as shown in Table 3. The exact agreement between MAG and Brucellacapt positive sera was 95.4%. The kappa coefficient was 0.90 (95% CI 0.85, 0.95) indicating very good agreement.

The results of the comparison between the in-house and commercial Vircell IgG ELISA are shown in Table 4. The exact agreement between the two assays was 90% (129/143). The kappa coefficient was 0.81 (95% CI, 0.75, 0.87) indicating good agreement. The results of the comparison between the in-house and commercial IgM EIA are shown in Table 5. The exact agreement between the two assays was 69% (100/143). The kappa coefficient was 0.38 (95% CI, 0.30, 0.46) indicating that the agreement was poor and not much better than chance.

Discussion

Due to its protean clinical features and lack of truly diagnostic tests, brucellosis remains a difficult disease to diagnosis particularly in non-endemic countries with a low prevalent population. Furthermore, though BRU encourages users of the service to complete a detailed clinical questionnaire (see Health Protection Agency [HPA] website), often serological results have to be interpreted with only minimal clinical information. As a result, it is clearly of paramount importance that the repertoire of serological assays is of high sensitivity. In non-endemic countries such as the UK, specificity is less of a concern since the BRU has a low threshold for simply requesting a follow serum and reporting one-off low level positive titres as 'non-diagnostic'.

Commercial *Brucella* IgG and IgM EIA assays have been available for a number of years.^{8–11} Indeed, EIAs are considered an excellent method for screening sera for *Brucella* antibodies⁸ and detection of IgM specific antibody may be sufficient for the diagnosis of brucellosis in a high

Table 3 Overall comparison of Brucellacapt and micro-agglutination results

		MAG		Total
		Positive	Negative	
Brucellacapt	Positive	103	5	108
	Negative	0	35	35
Total		103	40	143

MAG: micro-agglutination, positive titre ≥ 1:20;
Brucellacapt: positive titre ≥ 1:20.

Table 4 Overall comparison of in-house *Brucella* IgG and commercial *Brucella* IgG ELISA results

		In-house IgG assay		Total
		Positive	Negative	
Commercial IgG <i>Brucella</i> ELISA	Positive	66	13	79
	Negative	1	63	64
	Total	67	76	143

proportion of patients presenting with symptoms suggestive of acute brucellosis.^{9–12} Commercial EIAs are also generally considered to have a high degree of sensitivity and specificity.^{8–11} However, IgM antibody cannot be detected reliably in some patients with brucellosis. One study found that up to 11% of patients with brucellosis did not have detectable levels of specific IgM by immunoblot assay.¹³

The current comparison of BRU's in-house assays with an array of sero-diagnostic kits from a new commercial CE marked supplier has been useful in assessing its sero-diagnostic performance. Such an evaluation has not been previously performed in a non-endemic country. For seronegative *Brucella* sera (Group A), there was complete agreement with MAG, CFT and Brucellacapt results. The EIA results were also very similar though it is likely that there were two false-positive Vircell IgG results. For seropositive sera, with MAG titres $\geq 1:160$ (Group B), the evaluation again demonstrated comparability between in-house MAG, CFT and Brucellacapt results which confirms previous assessments.^{3,4} However, the Vircell IgM assay proved far more sensitive than the in-house IgM assay probably due to the failure to include human IgG sorbent in the in-house test. For IgM testing, human IgG sorbent is recommended since false-negative results may be obtained due to an excess of IgG antibodies. False-positive results may also be obtained due to the presence of rheumatoid factor. Unfortunately, BRU did not have the benefit of the original validation data for the in-house assays when the service was transferred to Liverpool nor has it proved easy to establish an external quality assurance scheme with an overseas laboratory. Hence, the close collaboration with Vircell on this point has been very useful. A positive IgM result acts as a clinical trigger for BRU to make immediate contact with the referring laboratory not only to discuss the patient's current management but also to prevent laboratory exposure to brucellosis if an unsuspected Gram-negative isolate is under investigation. The disease remains

Table 5 Overall comparison of in-house *Brucella* IgM and commercial *Brucella* IgM ELISA results

		In-house IgM assay EIA		Total
		Positive	Negative	
Commercial <i>Brucella</i> IgM ELISA	Positive	25	43	68
	Negative	0	75	75
	Total	25	118	143

one of the commonest recognized causes of laboratory-transmitted infection.¹⁴ EIA IgG results were comparable for the in-house and commercial assays. However, there have been no previous published evaluations on the Vircell IgG ELISA.

For the 70 clinically indeterminate sera examined (groups C and D), results were less clear cut. All Group D sera had positive CFT results suggesting that this assay may be more sensitive than the MAG test for samples with significant but low levels of *Brucella* agglutinating antibody. However, all samples were also Brucellacapt positive. IgG assays were comparable and a discrepancy with IgM results was again noted.

For Group C sera, which may reflect false-positive low level MAG results, there were no positive CFT results suggesting that the CFT assay also has superior specificity over the MAG assay. However, there was again 100% agreement between MAG and Brucellacapt assays. The eight Vircell positive IgG results may reflect false-positive reactions in the context of the other assay results, though specificity should be better with an LPS antigen preparation. Again, the discrepancy in IgM results was noted.

Overall, the Brucellacapt assay proved easy to perform and simple to interpret. Higher end-points were noted for each sero-diagnostic group suggesting improved sensitivity over the MAG assay. Indeed the Brucellacapt assay may offer the same advantage noted with traditional combined SAT and Coombs testing.

Since December 2003, it has been mandatory for diagnostic manufacturers to comply with the European Union directive on in vitro diagnostic devices.¹⁵ As a result, all diagnostic kits now marketed in the UK must be CE marked. Though the CE mark shows that the manufacturer has submitted its product for third party assessment and approval, this should not be taken as a substitute for an independent comparative evaluation as undertaken in this study. Ideally, in the UK the HPA's 'Microbiological Diagnostic Assessment Service' is best placed to provide such evaluations. However, the specialist nature of *Brucella* serological investigations lends itself to assessment by a national centre such as the BRU. Furthermore, this particular evaluation has also demonstrated the wisdom of incorporating appropriate CE marked assays into an already well-established in-house service.

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