

## ORIGINAL ARTICLE

# Comparison Of a Novel Immunocapture Assay With Standard Serological Methods in the Diagnosis of Brucellosis

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## SUMMARY

**Background:** Microbiological culture methods and immunological assays currently available are technically challenging, difficult to interpret even in non-endemic areas. They are also time consuming leading to misdiagnosis, treatment delay, and severe morbidity and mortality. Therefore, the development of a simple and accurate diagnostic assay which could be performed even in small laboratories is a pressing need. This has prompted us to evaluate an assay based on the immunocapture technique in a region where brucellosis is prevalent.

**Methods:** The immunocapture test was evaluated for diagnostic efficacy on 211 patients with suspected brucellosis. Standard tube agglutination test (SAT), 2-mercaptoethanol (2-ME) agglutination, Coombs, immunocapture tests, and blood cultures were performed on these 211 blood samples. 190 sera belonging to healthy blood donors of endemic and non-endemic areas and 43 sera obtained from non-brucellosis patients were also subjected to SAT, 2-ME, Coombs, and immunocapture tests. A total of 15 blood cultures belonging to blood donors of endemic area and non-brucellosis cases were done.

**Results:** SAT picked up only 21(9.9 %), Coombs established the diagnosis in 69 (32.7 %), while the immunocapture test confirmed the diagnosis in 76 (36 %;  $p < 0.001$ ) patients with brucellosis, including 48 culture-confirmed cases. Sensitivity and specificity of the immunocapture technique were 97.29 % and 97.08 % respectively. SAT could not exclude the diagnosis in 55 cases as they were confirmed in most cases by the Coombs test and in all by immunocapture.

**Conclusions:** Our results clearly show that immunocapture is superior to SAT in all stages of illness but is not significantly superior to Coombs. It also seems to be a useful tool in diagnosing a relapse. Immunocapture and Coombs tests were found to be more sensitive eliminating the ambiguity in the interpretation of the results for diagnosing brucellosis. The Coombs test is laborious, subjective in interpretation and demanding on skills. The immunocapture technique does not have the subjective reading errors, is simple to perform, and the results of the immunocapture technique seem to be reproducible. Thus we recommend the immunocapture technique especially for brucellosis-endemic countries. The Coombs, immunocapture, and 2-ME tests may also be considered useful tools in assessing treatment outcome.

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## KEY WORDS

Brucellosis; endemic area; blood culture; SAT; 2-ME; Coombs test; immunocapture test

## INTRODUCTION

*Brucellae*, the etiologic agents of brucellosis, are pathogenic to a wide variety of domesticated and wild animals and also are known to produce a severe human infection. There are eight classic pathogens of which *B. melitensis*, *abortus*, *suis*, *canis*, *pinnipediae*, and *cetaceae* are well recognized human pathogens. This re-emerging zoonotic disease impacts public health and agricultural economies globally on account of its high infectivity rate (1). *Brucellae* have also long been considered a potential biological weapon (2). Human brucellosis is endemic in many regions of the world, including the Mediterranean region, Latin America, Asia, and Africa due to limited availability of animal vaccines, expensive animal vaccination programmes, very expensive animal therapy, and herd culling to limit transmission, a lack of vaccines for human use (1,3), low infectious dose (as few as 10-100 organisms) for humans and due to general inexperience with this devastating illness. Diagnosis of brucellosis is clinically and microbiologically challenging. Human brucellosis is characterized by its protean manifestations.

Thus, brucellosis which presents itself in a variety of clinical manifestations, could be missed frequently by clinicians if not suspected and if it is not considered seriously in making a differential diagnosis. Since the symptoms are exceedingly protean, with few characteristic physical signs, many cases of chronic brucellosis have undoubtedly been missed (4). This has been a matter of experience everywhere and has been confirmed from time to time in the published literature. Therefore laboratory diagnosis is indispensable. Isolation of the etiological agent by culture followed by its identification is the best way to establish the diagnosis of brucellosis. However, identification of the organism is complex and time consuming and not suitable for large scale screening exercises. Thus, a positive diagnosis is more frequently made serologically involving the detection of *Brucella*-specific antibodies. Although a variety of serological techniques have been developed to identify these antibodies, the Rose Bengal plate agglutination test (RBPT), SAT, 2-ME, and the Coombs tests are still in common use worldwide. The development of a simple, rapid, and precise diagnostic assay which could be performed even in small laboratories is a pressing need. This has prompted us to evaluate an assay based on the immunocapture technique which is easy to carry out and interpret. We evaluated patients clinically suspected of having brucellosis with a novel single-step immunocapture (Brucellacapt)-agglutination assay. This study evaluated the diagnostic accuracy of this method with culture and standard serologic techniques such as SAT, 2-

ME, and Coombs which are the currently accepted armamentarium of tests used for microbiological confirmation in a region where brucellosis is prevalent.

## MATERIALS AND METHODS

Two hundred eleven patients reporting to Belgaum Institute of Medical Sciences (BIMS) Hospital, Belgaum, Karnataka, India during a period from February 2008 to May 2009 with clinical suspicion of brucellosis were enrolled in the study. Clinical diagnosis was made on symptoms, signs, and epidemiological characteristics compatible with brucellosis reported earlier (5). Patients were categorized into acute, subacute, and chronic groups according to the duration of symptoms (5).

The control group comprised 96 consecutive healthy blood donors from BIMS Hospital, Belgaum - an endemic area for brucellosis, 94 consecutive healthy blood donors were selected from Manipal Hospital Transfusion Services, Bangalore - a metropolitan city and a non-endemic area for brucellosis, and sera obtained from patients diagnosed with illnesses other than brucellosis ( $N = 43$ ) from BIMS, Belgaum including enteric fever/typhoid fever ( $N = 15$ ), malaria ( $N = 10$ ), pyrexia of unknown origin ( $N = 9$ ), rheumatoid arthritis ( $N = 5$ ), pulmonary tuberculosis ( $N = 2$ ), toxoplasmosis ( $N = 1$ ), and hepatitis B virus infection ( $N = 1$ ).

Information obtained from each patient included demographic data, symptoms, duration of illness from the onset of symptoms, treatment history, and an epidemiological profile such as occupational history, and a history of ingestion of raw milk and contact with domestic animals. The findings of the clinical examination were also recorded and analyzed.

Serological methods: For antibody assays, clotted blood samples were centrifuged at 3000 x g for 10 minutes. The serum was then divided into aliquots and stored at -20 °C until required for processing. Serum specimens of all 211 patients with suspected brucellosis and 233 blood specimens belonging to the control groups were subjected to SAT, 2-ME, Coombs, and immunocapture agglutination tests. *B. abortus* plain antigen for SAT and 2-ME tests was supplied by the Indian Veterinary Research Institute (IVRI), Izatnagar, India. Equal volumes of serial dilutions of the serum (from 1 : 10 to 1 : 1280) and *B. abortus* plain antigen were mixed in test tubes and incubated in an incubator at 37 °C for 24 hours. The 2-ME agglutination test was done with the identical antigen except that the 2-ME solution was added to each tube to a final concentration of 0.05 M (6). The highest dilution of serum yielding  $\geq 50$  % agglutination for both SAT and 2-ME tests was considered a positive end point. The Coombs test was carried out according to routine laboratory procedures with anti-human globulin (Span Diagnostics, Surat, India). Immunocapture testing was performed using reagents obtained by Vir-cell SL, Santa Fe, Granada, Spain and results were interpreted as specified by the manufacturer.

Immunocapture titre  $\geq 1 : 320$  was considered a positive diagnosis for brucellosis.

Paired sera available from 48 suspected brucellosis patients were subjected to SAT, 2-ME, Coombs, and immunocapture-agglutination tests.

However, multiple sera were reevaluated during follow-up at different time intervals after diagnosis and onset of therapy using SAT, 2-ME, Coombs, and immunocapture-agglutination tests in 17 of the suspected brucellosis group. The follow-up period ranged from 14 to 180 days with a median follow-up of 84 days. These patients were also followed-up clinically for resolution of symptoms and signs.

### Blood culture

Twenty milliliters of venous blood was collected (at one time) from each patient of the suspected brucellosis group and four cultures were done by inoculating five milliliters aseptically into the broth phase of each Castaneda's biphasic medium consisting of brain heart infusion agar and broth (Hi Media, Mumbai, India). 2-10 mL of venous blood was obtained for pediatric patients and inoculated as described above. The media were incubated at 37 °C in an incubator and observed for bacterial growth once a day for 30 days allowing the broth-blood mixtures to run over the solid phase every day.

Additional samples were obtained for blood culture from 3 relapsed patients.

The blood cultures were performed in 6 of the antibody positive (by more than one assay with < diagnostic levels) blood donors of Belgaum control group.

The blood cultures were performed in 9 non-brucellosis patients who were positive for *Brucella* antibodies (by more than one assay with < diagnostic levels).

All serologic and culture specimens from the same patient were processed simultaneously. Identification of culture isolates at the species level was made applying methods described previously elsewhere (7). Clinical isolates were sent to IVRI, Izatnagar, India, for confirmatory identification.

### Statistics

Statistical significance was determined by the Chi-square test with Yates correction. Statistical tests were done using Epi Info version 6.02 software (CDC, USA and WHO, Geneva, Switzerland). Receiver operating characteristic (ROC) curves were used to evaluate and compare the results and area under the curve (AUC) was calculated for each diagnostic test employed in the study with a corresponding 95 % confidence interval (95 % CI) using MedCalc version 11.2.1 software. The *p* values less than 0.05 were considered significant.

A diagnosis of brucellosis was established when *Brucella* were isolated from blood or if SAT revealed a titre of *Brucella* antibodies  $\geq 1 : 160$  or if blood showed brucellar incomplete (Coombs test) or immunocapture antibodies to titres  $\geq 1 : 320$  together with a compatible clinical presentation. Patients with uncomplicated brucel-

losis were given therapy consisting of a combination of doxycycline and rifampicin orally for 45 days for patients  $\geq 8$  years old while co-trimoxazole was given in place of doxycycline for children < 8 years and extending treatment to 90 days for complicated brucellosis. Repeat course was given to treat the relapsed patients.

## RESULTS

The patients were between 1 - 70 years of age, mean age 25.74 years, with Standard Deviation [SD]  $\pm 16.55$ . 142 (67.3 %) patients were adults (> 14 years) and 69 (32.7 %) were children giving an adult to child ratio of 2 : 1. Of 211 patients, 119 were males (56.4 %) [mean age 27.21  $\pm$  SD 16.93 years] and 92 (43.6 %) females [mean age 23.87  $\pm$  SD 15.84 years]. Thus the male to female ratio was 1.29 : 1. Distribution of cases by age and sex showed that brucellosis principally infected working-age adolescents and adults both males and females as men and women between the ages 15 and 70 years accounted for 142 (67.3 %) of the 211 cases.

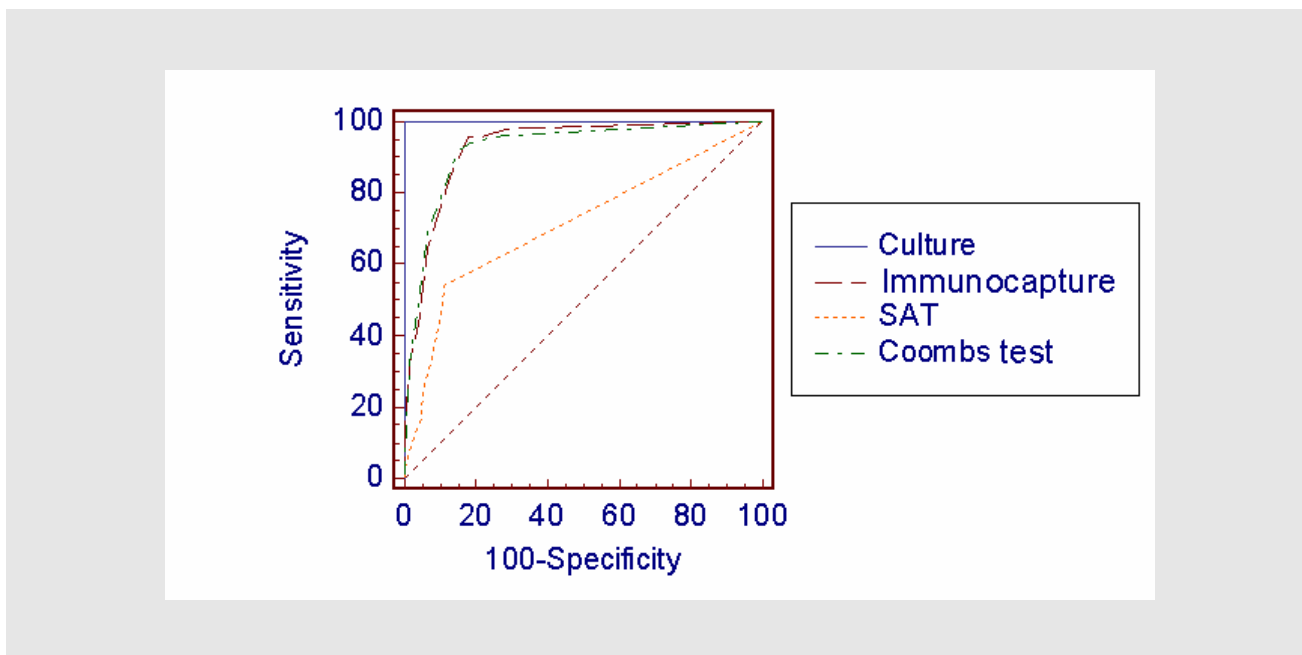
One hundred fifty eight (75.9 %) patients presented with acute symptoms, sub acute illness was noted in 11 (5.2 %), while 42 were chronically infected patients (19.9 %). The patients presented with fever, joint pain, low back ache, weight loss, headache, nausea, vomiting, abdominal pain, and testicular pain. Fever was the common symptom. 23 (10.9 %) had fever as the only complaint.

A combination of fever and arthritis was very common. Arthritis involved mainly the larger joints. The most frequent affected joint was the knee followed by hip, ankle, shoulder, and elbow. In most of these, it was monoarthritis. On physical examination, hepatosplenomegaly was seen in 48 patients and it was the most common finding on presenting to the hospital. Splenomegaly alone was found in 17 patients, whereas hepatomegaly alone in 8 patients, and lymphadenopathy alone in 5 patients were recorded. In addition, there were three cases of neurobrucellosis, two cases of epididymo-orchitis, two cases of endocarditis, and one case each of cellulitis, severe anemia, infertility, and splenic abscess. Three (1.4 %) patients experienced a relapse after therapy. Two patients relapsed in the first month and the remaining one in the second month. Each patient showed only one episode and management outcome was remarkable. Farmers / farm labourers (76 patients), shepherds (53 patients) and veterinarians (9 patients) were the major occupational groups affected. Consumption of unboiled milk and direct contact with domestic animals were recognized as major transmission risk factors of brucellosis in the present series. The possible source of infection was unknown in 32 (15.1 %) cases.

A positive family history for brucellosis was recorded in 21 (9.9 %) patients. Nine cases of brucellosis were found from individuals infected with human immunodeficiency virus (HIV).

**Table 1. Results of SAT, Coombs, and immunocapture tests performed on 211 patients with clinical suspicion of brucellosis.**

Total No. of patients	No. of patients positive by	No. of patients positive by	No. of patients positive by
	SAT(n (%))	Coombs test(n (%))	Immunocapture test( n (%))
211	21 (9.9)	69 (32.7)	76 (36)



**Figure 1. AUCs for the ROC curve for the immunocapture test, the Coombs test, and the SAT when the blood culture was considered the gold standard. The diagonal line running from lower left to upper right represents the relationship between sensitivity and specificity. The AUCs for the immunocapture and Coombs test were very similar (0.928 and 0.923 respectively;  $p = 0.697$ ) but considerably higher than that for the SAT (0.712;  $p < 0.001$ ).**

The diagnosis of brucellosis was confirmed in 76 (36 %) of 211 suspected brucellosis patients. *B. melitensis* biotype 1 was isolated from blood cultures of 48 (22.7 %) cases out of 211 thereby confirming 48 cases bacteriologically and 163 culture unconfirmed cases. Table 1 demonstrates a comparison of sero-diagnostic methods with the yields. SAT tested positive in 21 (9.9 %) cases and 2-ME in 20 ( $\geq 1 : 80$ ; 9.4 %) only, whereas Coombs picked up 69 (32.7 %) and immunocapture 76 (36 %) cases. When the culture was considered the gold standard, the AUCs for the ROC curve (Figure 1) for immunocapture, Coombs test, and SAT were 0.928, 0.923, and 0.712, respectively. No significant difference was observed between immunocapture and the Coombs test (difference 0.005;  $p = 0.697$ ), but the difference between SAT and immunocapture / Coombs tests was statistically significant ( $p < 0.001$ ). The sensitivity and specificity of the immunocapture

technique were 97.29 % and 97.08 %, respectively. The diagnosis was established by all three serological procedures (SAT, Coombs, immunocapture) in 21 (9.9 %) patients. The combination of Coombs and immunocapture yielded a diagnosis of brucellosis in 69 (32.7 %) cases. Single technique (immunocapture) alone was able to establish the diagnosis in 7 (3.3 %) cases. A statistically significant difference was noticed in the performance of Coombs and immunocapture agglutination tests over traditional agglutination tests in the diagnosis of brucellosis ( $p < 0.001$ , Chi-square Yates corrected = 25.6 for Coombs and  $p < 0.001$ , Chi-square Yates corrected = 31.18 for immunocapture). However, the difference was not statistically significant in the performance of Coombs and immunocapture techniques ( $p = 0.697$ , Chisquare Yates corrected = 0.34).

Acute patients showed SAT titres ranging from 1 : 20 to 1 : 10240 with with a mean of 167.2, Coombs titres

Table 2. Diagnostic yield by three different serological methods according to evolution of illness.

Stage of illness	Total No. of patients	No. of patients positive by SAT(n (%))	No. of patients positive by Coombs test(n (%))	No. of patients positive by Immunocapture test(n (%))
Acute (< 8 weeks)	158	11 (6.9)	34 (21.5)	39 (24.6)
Sub-acute (8 - 52 weeks)	11	04 (36.3)	06 (54.5)	06 (54.5)
Chronic (> 52 weeks)	42	06 (14.2)	29 (69)	31 (73.8)
Total	211	21 (9.9)	69 (32.7)	76 (36)

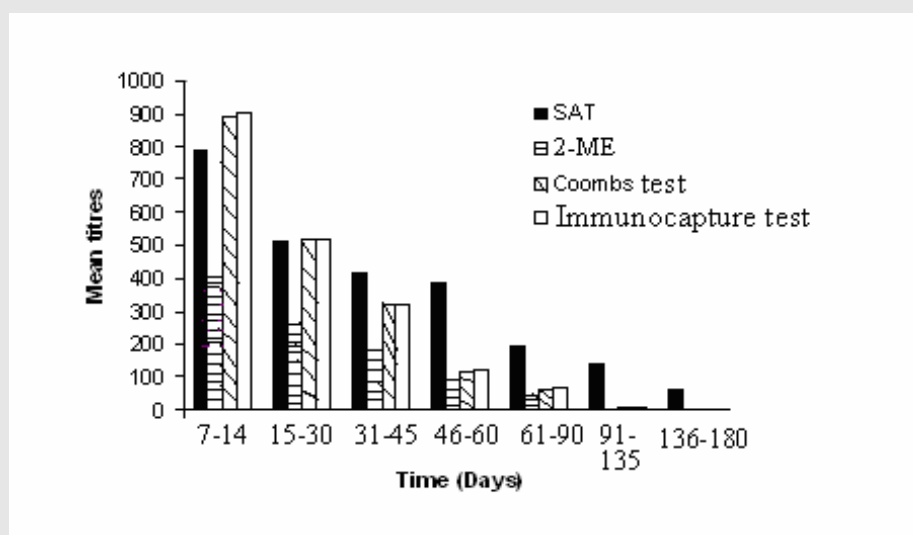


Figure 2. Findings of SAT, 2-ME, Coombs, and immunocapture tests at different follow-up times after diagnosis and treatment in 17 cases. *Brucella* SAT titres remained detectable with diagnostic levels in spite of an effective anti-brucellar therapy and clinical recovery in most cases, but there was a sustained fall in 2-ME, Coombs, and immunocapture titres in 82.3 % (14 / 17) of cases.

ranged from 1 : 20 - 1 : 10240 with a mean of 178.6, and immunocapture recorded titres that ranged between 1 : 40 to 1 : 20480 (mean = 191.6). Sub acute cases recorded SAT titres ranging from 1 : 20 to 1 : 10240 with the mean of 169.6, Coombs titres ranging from 1 : 40 to 1 : 10240 with a mean of 356.8, and immunocapture titres ranging from 1 : 40 - 1 : 10240 with a mean of 602.8. Chronic patients had SAT titres ranging from 1 : 20 - 1 : 5120 with a mean of 153.6, while Coombs titres ranged from 1 : 40 - 1 : 5120 with a mean of 309.6, and immunocapture titres ranged from 1 : 40 - 1 : 20480

(mean = 621.9). A noteworthy finding was the detection of all 76 (100 %) cases by a single method namely immunocapture whereas Coombs test was able to detect 90.7 % of the 76 confirmed cases. Only 3 patients recorded non-significant incomplete antibodies (1 : 20 - 1 : 80) and immunocapture antibodies (1 : 40 - 1 : 80). However, SAT recorded completely negative in 33 and insignificant titres (range 1 : 20 - 1 : 80) in 25 blood specimens and 2-ME completely negative in 31 with non-diagnostic levels (1 : 20 - 1 : 40) in 24. A comparison of sero-diagnostic methods with the duration of il-

ness is shown in Table 2. Coombs and immunocapture tests were found to be more sensitive than SAT in detecting brucellosis in acute ( $p < 0.01$ , Chi-square Yates corrected = 11.76 for Coombs, and  $p < 0.01$ , Chi-square Yates corrected = 15.68 for immunocapture) as well as in chronic forms ( $p < 0.01$ , Chi-square Yates corrected = 15.12 for Coombs and  $p < 0.01$ , Chi-square Yates corrected = 16.9 for immunocapture).

Of the 158 acute cases, Coombs and immunocapture detected 34 (21.5 %) and 39 (24.6 %), respectively, while SAT identified only 11 (6.9 %) cases. Among 42 chronic patients, only 6 (14.2 %) were detected by SAT whereas Coombs and immunocapture identified 29 (69 %) and 31 (73.8 %), respectively.

Analysis of 48 patients for whom paired sera were available showed 16 with both samples negative and 17 cases where both samples were positive by all four serological methods. Blood cultures were positive in 8 of 17 cases positive for serology. In the other 15 cases, initial samples showed insignificant SAT and 2-ME titres along with 3 cases which were also positive using Coombs and immunocapture tests, although at non-diagnostic levels. In twelve of the 15 cases, incomplete antibodies and immunocapture antibodies were observed to diagnostic levels as determined by Coombs and immunocapture agglutination tests while SAT and 2-ME titres remained non-diagnostic in paired serum sample testing. *B. melitensis* biotype 1 was isolated upon blood culture in all of these 12 cases. The remaining three cases showed rising SAT ( $\geq 1 : 160$ ), 2-ME ( $\geq 1 : 80$ ), Coombs, and immunocapture ( $\geq 1 : 320$ ) antibodies between acute and convalescent phase specimens, along with a blood culture positive for *B. melitensis* biotype 1 in one case.

Seventeen patients were reevaluated during follow-up. Fourteen (82.3 %) patients on clinical examination did not show a palpable spleen or liver. In these fourteen patients, SAT titres remained measurable at diagnostic levels, despite elimination of clinical illness (Figure 2). However, a progressive decrease was noted in 2-ME, Coombs, and immunocapture agglutinins in all. In addition they all had negative 2-ME, Coombs, and immunocapture tests in the last follow-up (Figure 2). Three patients experienced a relapse as indicated by a rise in 2-ME, Coombs, and immunocapture antibodies along with positive blood cultures in two and the re-appearance of clinical symptoms and signs.

A total of 10 specimens of blood donors from an area endemic for brucellosis in Belgaum were positive for antibodies. Six blood samples showed SAT titres (1 : 20 - 1 : 80), Coombs titres (1 : 40 - 1 : 80) and immunocapture antibodies (1 : 40 - 1 : 80) along with one specimen which was also positive for 2-ME antibodies (1 : 20). Two blood specimens recorded titres in SAT and Coombs (1 : 20 - 1 : 40). One sample was positive for SAT and 2-ME antibodies (1 : 20). The remaining specimen was positive for SAT (1 : 40), 2-ME (1 : 20), and Coombs antibodies (1 : 40). None of the 6 blood specimens cultured yielded *Brucella*. In a total of 7

blood samples positive for antibodies from blood donors of area non-endemic for brucellosis in Bangalore, 3 samples demonstrated SAT titres (1 : 20 - 1 : 40) alone, and 2 specimens were positive for Coombs (1 : 20 - 1 : 40) and immunocapture (1 : 40). The remaining 2 samples showed SAT (1 : 20 - 1 : 40), Coombs (1 : 20 - 1 : 40), and immunocapture antibodies (1 : 40). No 2-ME agglutinins were recorded. Of 43 non-brucellosis patients, 12 patients were found positive for *Brucella* antibodies. Of the 12, 8 cases were positive for SAT (1 : 20 - 1 : 80), 2-ME (1 : 20 - 1 : 40), Coombs (1 : 40 - 1 : 80), and immunocapture antibodies (1 : 40 - 1 : 80). The remaining 4 cases showed Coombs (1 : 40 - 1 : 80) and immunocapture antibodies (1 : 40 - 1 : 80). None of the 9 blood specimens cultured yielded *Brucella*. Thus, all controls demonstrated either non-diagnostic levels of antibodies or were completely negative by SAT, 2-ME, Coombs, and immunocapture tests (specificity 100 %).

## DISCUSSION

Principal methods of diagnosis include blood culture, RBPT, SAT in conjunction with 2-ME, Coombs, Enzyme-linked immunosorbent assay (ELISA), lateral flow assay (8), and immunocapture (9). SAT is found to be less ideal because it is labor intensive and gives false negative results due to both to the presence of blocking antibodies as well as to collection of samples in the early phases of the disease (10-11). Both males and females were affected equally in this series and this is in accordance with a previous study (12) which has also reported roughly equal incidence of infection among males and females. In our area, working females are often exposed, particularly in milking the cows, and thus having a higher chance of contact and acquiring infection. This could also be attributed to health awareness seen among females in this area.

This study has shown the immunocapture to be a sensitive and specific test for the detection of *B. melitensis* antibodies with sensitivity and specificity of immunocapture in excess of 97 %. A remarkable finding of the study was SAT at low or negative titres ( $\leq 1 : 80$ ) in a considerable number of patients (N = 55) in whom the diagnosis was confirmed with a positive blood culture and / or a positive Coombs and immunocapture. This implies a very serious limitation of traditional agglutination tests for disease diagnosis as prompt therapy is essential for a successful outcome. We would have missed these 55 cases if conventional tests alone had been performed – a noteworthy point. This would have resulted in under-diagnosis and under-reporting which would have reflected in turn on the precise information about incidence and prevalence of brucellosis. This fact reinforces the importance of Coombs and immunocapture techniques in the diagnosis of brucellosis in endemic areas. These results are comparable with several earlier publications (9,13) as those studies also noted the occurrence of a false low or equivocal SAT titre in

patients with brucellosis and consequently the diagnosis was made with the detection of antibodies by Coombs and immunocapture. Orduna *et al* (9) reported SAT titres  $< 1 : 160$  in nearly 35 % of the initial sera from *Brucella* infected patients which, interestingly, were all found positive by Coombs and immunocapture. Casao and associates (13) did a comparative evaluation of SAT, Coombs, and immunocapture for 36 patients with brucellosis. The study concluded the Coombs and immunocapture techniques as more sensitive and specific techniques as opposed to standard serological tests for diagnosing brucellosis.

We pickup a significant number of cases by Coombs and immunocapture - a finding that has relevance to laboratory diagnostic tools. Our results show that immunocapture is close to the Coombs test. This serological picture is very similar to that reported by workers elsewhere in the world (9,13-15). Review of the literature has documented only a few reports with regard to the utility of immunocapture in the diagnosis of brucellosis in humans. Orduna and colleagues (9) described the diagnostic value of the immunocapture in a group of 82 patients with brucellosis and 412 people living in rural areas where brucellosis is prevalent. They reported the sensitivity and specificity for SAT, Coombs, and immunocapture as 65.8 and 100 %, 91.5 and 99.8 %, and 95.1 and 99 %, respectively. Casao *et al* (13) performed another study in Spain and evaluated immunocapture, Coombs, and SAT. They included 26 patients diagnosed as having brucellosis. Ninety-six percent of patients were immunocapture positive on the day of admission. Their study indicated that both immunocapture and Coombs tests have similar performance in the diagnosis of brucellosis in all stages in terms of sensitivity and specificity. Gomez *et al* (14) compared the results of the Coombs and immunocapture in 112 patients of brucellosis compatible with clinical and epidemiological characteristics. Direct correlation was reported in the performance between two diagnostic modalities ( $p < 0.01$ ) with immunocapture detecting higher titres than Coombs. Serra *et al* (16) noted a sensitivity and specificity of 100 and 95 % respectively for immunocapture in a group of 42 patients suffering with brucellosis. A recent publication by Casanova *et al* (15) reported a sensitivity similar for immunocapture and Coombs tests. Antibody levels exhibited by SAT, Coombs, and immunocapture were more or less equal in all of our acutely infected patients. Conversely, Coombs and immunocapture techniques recorded antibody levels two and four times, respectively, than that of SAT in all our chronically infected patients - a finding that is comparable with the results obtained by Orduna and associates (9). Both immunocapture and Coombs tests always recorded higher titres ( $\geq 1 : 640$ ) in patients with long-term brucellosis in a study by Orduna *et al* (9). Sub-acute patients in our series also showed similar antibody patterns to those of chronic cases. However, since the number is small further study would be required for valid conclusions. Antibodies usually begin to appear in the

blood at the end of the first week of the illness, IgM appearing first followed by IgG and both classes peak during the fourth week. Thus, both IgM and IgG antibodies appear promptly after brucellar infection and their concentration rises during the following days. This could be the reason for our cases demonstrating high levels of antibodies as determined by Coombs and immunocapture in sub-acute as well as chronic phase patients. Our results clearly show that immunocapture is superior to SAT in all stages of illness but is not significantly superior to Coombs. In the sub-acute group, the difference between all three modalities of diagnosis was not significantly different.

The immunocapture and Coombs tests were potentially useful in establishing the etiology in 15 initially seronegative cases when the convalescent-phase serum specimens were reevaluated, again, a point worth noting as thirteen of these were culture-positive. It is worthwhile to note that conventional tests proved negative despite the isolation of *B. melitensis* in all twelve patients. However, the serum Coombs and immunocapture tests recorded high levels of antibodies indicating the value of the Coombs and immunocapture methods in the confirmation of the diagnosis. This is a significant finding since precise diagnosis of brucellosis elicits prompt consideration of therapeutic measures and effective case management, especially in endemic areas of the world where the interpretation of the results of conventional tests is difficult. Also, no single titre is diagnostic and paired sera must be sought to demonstrate a rise in the antibody levels or the appearance of detectable levels of antibodies to confirm the etiological agent. In certain studies (17-18), when clinical evidence suggested brucellosis, even values of SAT  $< 1 : 160$  did not rule out the diagnosis. Three cases with non-diagnostic SAT, 2-ME, Coombs, and immunocapture titres revealed rising SAT, 2-ME, Coombs, and immunocapture titres in paired sera of these three cases including a positive blood culture in one case. The data clearly indicated that low titres of SAT, 2-ME, Coombs, and immunocapture tests cannot always be disregarded without follow-up, especially if clinical findings are highly suspicious in endemic areas of the world. This also gives us reason to propose the use of Coombs and immunocapture for the accurate serological confirmation of human brucellosis, an area that needs further clinical studies.

We believe that our three patients had true relapses rather than reinfection since the episodes were noticed in the early months after therapy. 2-ME, Coombs, and immunocapture techniques were all potentially useful in detecting a relapse. Several previous studies (9-10,19) have reported increases in the levels of IgG antibodies in most of the relapsed patients as determined by Coombs, immunocapture tests, and by IgG ELISA.

The *Brucella* antibodies remain high for a long time even after patients with brucellosis recover from the disease. The present study has shown persistence of various levels of SAT antibodies in 14 (82.3 %) clinically

cured patients. These findings emphasize the over diagnosis and diagnostic challenges faced in an area where typhoid, malaria, tuberculosis, and rheumatoid arthritis clinically resemble brucellosis thereby exposing / denying patients access to specific treatment. However, a noteworthy finding of our study was that there was a considerable progressive decrease in 2-ME, Coombs, and immunocapture antibodies demonstrating the 2-ME, Coombs, and immunocapture tests as invaluable tools in the follow-up of brucellosis cases in *Brucella* endemic areas (Figure 2). Although the figures herein presented are not large enough to reach statistical significance, adoption of 2-ME, Coombs, and immunocapture techniques will prove better in assessing the favorable response to treatment thereby avoiding unnecessary continuation of therapy. We previously reported similar findings on the performance of the 2-ME test (18). The 2-ME test has been used by others also to monitor response to therapy (20). Effective clinical cure with anti-brucellar agents could reduce IgG faster than IgM as shown by ELISA (10,21). Gazapo and colleagues (21) reported that the ELISA provided a very useful tool for follow up of brucellosis. However, we advocate the use of the 2-ME test rather than Coombs and immunocapture tests to judge the prognosis as the former is cost-effective without requiring complex equipment or extensive training to perform or to interpret results. This can be readily applied in the developing world, especially to those cases where brucellosis diagnosis has been confirmed by SAT and 2-ME test findings. However, further work should be done to establish the clinical efficacy of these tests.

The strong agreement between Coombs and immunocapture antibodies detection and blood culture, when applied to *Brucella* infected blood samples, suggested that the former methods may be alternatives to cultural isolation for the diagnosis of brucellosis. Thus, both immunocapture and Coombs tests were more sensitive eliminating the ambiguity in the interpretation of the results to diagnose brucellosis. In addition, the immunocapture test has the advantage of being quantitative and is reproducible from assay to assay. It is also capable of being readily applied if a large number of samples is to be tested.

The antibodies (non-diagnostic levels) found in blood donors from the non-endemic Bangalore metropolitan city could be attributed to the floating population of endemic areas from the rest of the regions of Karnataka and neighboring southern states of India.

In conclusion, when results of Coombs and immunocapture tests were compared with those of SAT and 2-ME and even with blood cultures, Coombs and immunocapture findings could be used to diagnose most of our patients reflecting the superiority of both the Coombs and immunocapture tests. The findings that culture-negative patients were positive by immunocapture and Coombs may be useful in settings where antibiotic usage is high and cultures are poor or not available. In addition, these immunocapture agglutination

and Coombs tests may be of great value for patients in whom brucellosis is clinically suspected but standard serological tests are either completely negative or demonstrate low levels (non-diagnostic). The Coombs test is labor intensive and requires more skills. The immunocapture technique is easy to run and interpret and results of the immunocapture test seem to be reproducible. Since an additional 3.3 % of cases were diagnosed exclusively by immunocapture, we recommend this procedure especially in countries where brucellosis is endemic. If clinicians and microbiologists communicate effectively, timely, and often, a correct diagnosis of this difficult to diagnose disease can be efficiently made.

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#### Declaration of Interest:

No conflict of interest declared.

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