

## Serology *versus* Real Time PCR in the Diagnosis of Human Brucellosis

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With the unwelcome re-emergence of brucellosis in different regions of the world, an accurate and timely diagnosis of this zoonosis has become a daunting challenge. Due to the vague symptoms of the disease, laboratory confirmation is intensely needed to clinch a definite diagnosis. Consequently, reliable laboratory tests can play a pivotal role in proper diagnosis and disease management. Employing standard tube agglutination test (STAT) as the reference method, this study aimed to evaluate the performance of different serological tests as well as quantitative real time PCR (qPCR) in the diagnosis of human brucellosis. Out of 100 serum samples included in this study, 95 samples yielded positive result with STAT. The highest sensitivity (96%) was recovered with Rose Bengal (RB) test, while the sensitivities of ELISA and qPCR were 79% and 65% respectively. Meanwhile, RB test revealed a 100% specificity, while both ELISA and qPCR had specificities of 80% and 40% respectively. The RB test has proven to be a reliable and appropriate screening test for brucellosis. Likewise, ELISA is an attractive option. Meanwhile, STAT, which is accurate, cost-effective, and easy-to-use, remains the most appropriate test for the diagnosis of human brucellosis, particularly in endemic regions. While PCR may be costly and technically demanding for most laboratories, STAT can be very well adopted by laboratories established in low resource settings. It can provide a definite diagnosis of human brucellosis with minimal labor as well as an affordable cost.

**Keywords:** Brucellosis; ELISA; qPCR; STAT; Zoonosis.

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With nearly half a million cases reported yearly to the WHO from 100 countries, brucellosis remains one of the major zoonoses around the world<sup>1</sup>. Despite the continuous progress of brucellosis control measures, the disease still poses an enormous public health problem of grave economic impact. This in turn has necessitated re-evaluating the current tools employed in the diagnosis of brucellosis<sup>2</sup>.

The gold standard for the laboratory diagnosis of brucellosis has been the isolation of *Brucella* spp. from blood, bone marrow or other

tissues by culture. However, isolation of *Brucella* spp. is often hazardous, time-consuming, and of low sensitivity<sup>3</sup>.

In the meantime, serological tests such as the standard tube agglutination test (STAT), Rose Bengal (RB) slide agglutination test, and ELISA have been the most common tests in the laboratory diagnosis of brucellosis. The STAT, which has been a cornerstone in the serological diagnosis of brucellosis, is based on the reaction of a known standardized volume and concentration of whole *Brucella abortus* and *Brucella melitensis* cell suspension with a standardized volume of doubling serum dilutions, usually ranging from 1:20 to 1:1280<sup>4</sup>. Being a non-labor intensive test with a short turn-around time, STAT can be used

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to screen a large number of susceptible populations in endemic areas at a minimal cost<sup>5</sup>.

Meanwhile, RB test is based on the agglutination of serum with a suspension of whole *Brucella abortus* cells stained with Rose Bengal dye and buffered at pH 3.65 to inhibit non-specific agglutinins<sup>4</sup>. This test can be readily and rapidly performed, with a high sensitivity in acute cases<sup>6</sup>.

Likewise, ELISA offers a simple and rapid assay that can reveal total and individual specific immunoglobulins (IgG, IgM, IgA) within 1-2 hours. ELISA is the test of choice for focal lesions and chronic cases, especially when other tests are negative and the case is under high clinical suspicion<sup>4</sup>. This test can detect incomplete antibodies especially in chronic cases<sup>6</sup>.

On the other hand, PCR can provide an additional means to detect and identify *Brucella* spp. While PCR-based tests are undoubtedly promising, they are still hindered with substantial shortcomings, including a high risk of contamination and high expenses. Moreover, infrastructure, equipment, and expertise are lacking, and a better understanding of the clinical significance of the results is still needed<sup>7</sup>.

In the present study, we compared the sensitivity and specificity of RB, ELISA, and quantitative real time PCR (qPCR) in the diagnosis of brucellosis, while employing STAT as the reference method.

## MATERIALS AND METHODS

### Ethical consideration

Before commencement of the study, approval of the protocol was obtained from the Ethics Committee in the Department of Microbiology and Immunology, Cairo University.

An informed consent was taken from all enrolled subjects and a questionnaire was filled out for each patient including various factors such as age, gender, residence (urban or rural), recent ingestion of unpasteurized dairy products, and animal contact.

### Population of study

This cross sectional analytical study was done on 100 serum samples obtained from 100 inpatients clinically suspected of having brucellosis in Imbaba Fever Hospital. Samples were collected

during the period from November 2015 through May 2016.

### Inclusion criteria

Patients with night fever (of 39-40°C) which returns to normal (37°C) during the day for more than 10 days duration, accompanied by night sweating, arthralgia and low back pain, along with a history of animal contact or ingestion of unpasteurized dairy products, were enrolled in this study.

### Exclusion criteria

Patients with fever of another identifiable cause.

### Sample processing

Blood samples were collected from patients and centrifuged at 3000 g for 10 minutes. The serum obtained was divided into two aliquots: one for serological testing and one for qPCR. Both aliquots were collected in cryo-tubes and stored at -80°C until processed. All reagents for serological testing and qPCR were brought to room temperature before use.

### Standard tube agglutination test (STAT)

One milliliter of serially diluted serum sample (from 1:20 to 1:1280) and one drop (50 µl) of the antigen (BioMed-Brucel- *Abortus* / *Melitensis*) were mixed and incubated at 37°C for 24 hours. A positive control (positive serum sample included in the kit) and a negative control (saline) were included in the test<sup>8</sup>.

In the present study, the diagnosis of brucellosis was confirmed in patients who fulfilled the clinical criteria mentioned above, combined with a STAT titre  $\geq 1/160$  °.

### Rose Bengal (RB) plate agglutination test

According to the manufacturer's instructions (BioMed-Rose Bengal), a coarse speckled agglutination after mixing 50 µl of the patient's serum and 50 µl of the antigen (killed *B. abortus*) on a slide and rotating for 4 minutes was considered a positive test<sup>10</sup>.

### Enzyme-linked immunosorbent assay (ELISA)

The ELISA IgG tests were performed and interpreted according to the manufacturer's instructions (Vircell SL, Granada, Spain). A microtiter plate composed of 96 wells coated with LPS antigen of *Brucella abortus* (strain S-99) was used. Positive control, negative control, two cut off sera, and patients' samples were diluted 1:100 with

dilution buffer. Five microliters of the tested serum sample, 5 µl of positive control, 5 µl of cut off control (in duplicate) and 5 µl of negative control were added into the corresponding wells followed by incubation at 37°C for 45 min.

All wells were washed five times with 0.3 ml of washing solution. Anti-human IgG peroxidase conjugate solution (100 µl) was immediately added into each well and the plate was incubated at 37°C for 30 minutes. The substrate solution, tetramethyl benzidine (100 µl) was immediately added into each well followed by incubation at room temperature away from light for 20 minutes. Stopping solution (50 µl) was immediately added into all wells<sup>11</sup>.

The optical density was read at 450/620 nm using STAT FAX 2100 ELISA reader. Antibody index was calculated according to the following equation:

$$\text{Antibody index} = (\text{sample optical density} / \text{cut off serum mean optical density}) \times 10.$$

As per the manufacturer’s instructions, an antibody index less than 9 was considered negative, while an antibody index more than 11 was considered positive. On the other hand, antibody indexes between 9 and 11 were re-tested and confirmed either positive or negative.

**Quantitative Real time Polymerase chain reaction (qPCR)**

Real time PCR was performed at the Molecular Biology Unit, Biochemistry Department, Faculty of Medicine, Cairo University.

DNA extraction from 200 µl of serum was done using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers’ instructions. *Brucella* genus all species, PrimerDesign™ genesig kit and oasig TaqMan 2x qPCR Mastermix were used for DNA detection using Applied Biosystem step one device. Cycling conditions consisted of 50 cycles, starting with Taq polymerase activation at 95°C for 2

minutes, followed by denaturation at 95°C for 10 seconds, then annealing and extension at 60°C for 90 seconds<sup>12</sup>.

Negative control (RNase/DNase free water) and standard were included in all runs. Meanwhile, an internal positive control was used for each sample. The result was considered negative when no amplification occurred or when the cycle threshold (Ct) value exceeded 38 cycles. The bacterial DNA load per milliliter of serum was calculated from the standard curve which included five concentrations of *Brucella* DNA ranging from 2 to 2 X 10<sup>5</sup> copies<sup>13</sup>.

**Statistical Analysis**

In this prospective analytical study, the predictive analytics software (PASW) version 18 was used. Data were statistically described in terms of frequencies (number of cases) and percentages. Chi-Square test of distribution was used to declare the significant difference between group distributions at P<0.05. The sensitivity and specificity of each of RB test, ELISA, and qPCR were calculated.

**RESULTS**

This study was conducted on 100 serum samples collected from 100 inpatients in Imbaba Fever Hospital, during the period from November 2015 through May 2016.

**Serological tests**

Out of the 100 enrolled patients with provisional clinical diagnosis of brucellosis, 95 patients yielded positive result with STAT (titre ≥ 160). Of these 95 patients, 68% had titres of 1/1280 (Figure 1).

Meanwhile, 91 samples yielded positive results with RB test, while 76 samples were positive by ELISA.

Out of the 95 samples positive by STAT, RB was positive in 91 samples (96%) (Table 1),

**Table 1.** Results of RB test compared to STAT

		STAT		Total
		Positive	Negative	
RB	Positive	91	0	91
	Negative	4	5	9
Total		95	5	100

**Table 2.** Results of ELISA compared to STAT

		STAT		Total
		Positive	Negative	
ELISA	Positive	75	1	76
	Negative	20	4	24
Total		95	5	100

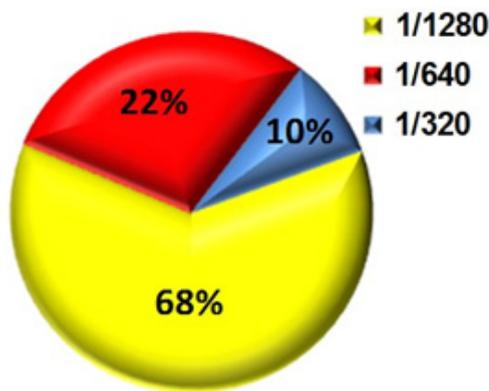
while ELISA was positive in 75 samples (79%) (Table 2).

#### Real-time PCR

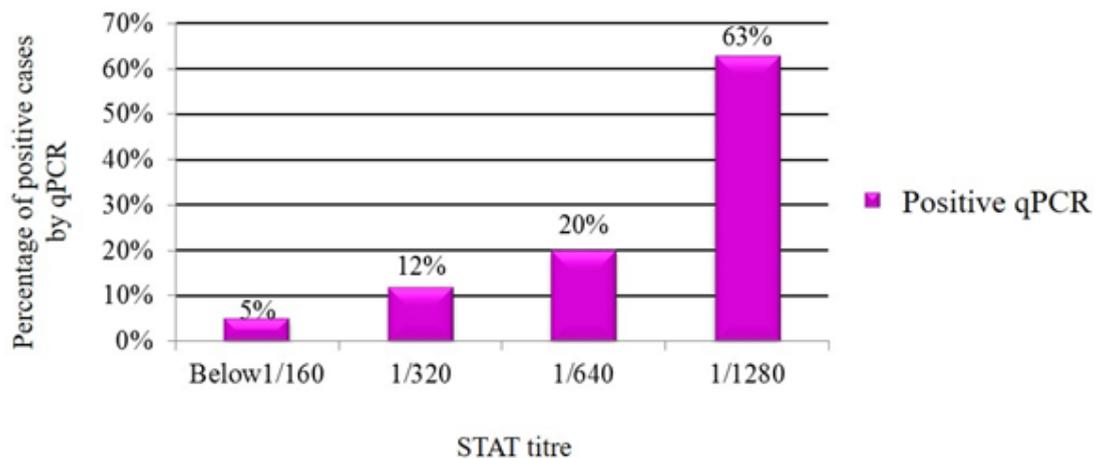
Real time PCR was positive for *Brucella* DNA in 65 out of 100 serum samples. The range of copies detected by PCR was 5-1000 copies/ml serum with a mean of 22 copies/ml serum. Meanwhile, out of the 95 positive samples by STAT, 62 (65%) were positive by qPCR as depicted in table 3.

Of note, qPCR positivity was highest (63%) at STAT titer of 1/1280 (Figure 2).

Table 4 summarizes the sensitivities and specificities of the tests employed in this study. The highest sensitivity (96%) and specificity (100%) were encountered with RB test.



**Fig. 1.** Titres of positive serum samples as detected by STAT results



**Fig. 2.** Relation between STAT titre and qPCR

## DISCUSSION

Brucellosis is an endemic zoonosis in several parts of the world, particularly the Mediterranean basin, the Middle East, as well as central and South America. With the symptoms being nonspecific and often atypical, the clinical picture of brucellosis may masquerade as other clinical entities<sup>14</sup>. This asserts the need for reliable laboratory tests to reach a definite diagnosis.

In this study, out of 95 samples confirmed by clinical diagnosis and positive STAT, 91 (96%) samples were positive by RB. The sensitivity and specificity of RB test were 96% and 100%,

**Table 3.** Results of qPCR compared to STAT

		STAT		Total
		Positive	Negative	
qPCR	Positive	62	3	65
	Negative	33	2	35
Total		95	5	100

**Table 4.** Sensitivities and specificities of RB test, ELISA, and qPCR in the diagnosis of human brucellosis

Test	Sensitivity	Specificity
RB	96%	100%
ELISA	79%	80%
qPCR	65%	40%

respectively. This was in concordance with a previous study<sup>15</sup> which demonstrated that each of the sensitivity and specificity of RB was 100%. Likewise, another study<sup>5</sup> reported the sensitivity and specificity of RB as 100% and 96% respectively, compared to culture as a gold standard. On the other hand, Arabaci & Oldacay<sup>16</sup> stated that the sensitivity and specificity of RB were 48% and 96% respectively.

In the present study, out of the 95 samples positive with STAT, 75 (79%) samples were positive by ELISA. The sensitivity and specificity of ELISA were 79% and 80% respectively. Earlier studies demonstrated results in line with this study. Kalem et al.<sup>17</sup> stated that the sensitivity and specificity of ELISA were 93% and 80% respectively, compared to *Brucella* immune-capture agglutination as a gold standard. On the other hand, Shahrokhbadi et al.<sup>18</sup> reported that considering culture as a gold standard, the sensitivity and specificity of ELISA were 78% and 28% respectively.

Meanwhile, Alshaalan et al.<sup>19</sup> emphasized that while the sensitivity of ELISA ranges from 60 to 98%, it has the advantage of measuring different classes of reactive antibodies including IgG, IgA, and IgM. Hence, it can be used in the diagnosis of brucellosis especially in endemic areas<sup>3</sup>.

In the current study, the range of *Brucella* DNA copies detected by qPCR was 5-1000 copies/ml serum with a mean of 22 copies/ml serum. On the other hand, Sohrabi et al.<sup>20</sup> reported that the range of copies detected by PCR was 64 - 580,000 copies/ml serum, while Tiwari et al.<sup>21</sup> demonstrated that the range of copies of *Brucella* DNA detected by PCR was 10<sup>1</sup>-10<sup>7</sup> copies/ml serum.

In this study, out of the 95 positive samples, only 62 samples (65%) were positive by qPCR. Meanwhile, an earlier study revealed that PCR had a sensitivity and specificity of 64.4% and 77.5%, respectively<sup>22</sup>. In concordance with this finding, Purwar et al.<sup>5</sup> stated that there were brucellosis cases which were not detected by PCR, but had antibody levels above the cut-off value. This may be attributed to the fact that brucellosis is characterized by exacerbations and remissions. During remissions there may not be any bacteremia, and consequently, no DNA breakdown products can be found in serum to be picked up by PCR.

In addition, a study by Queipo-Ortuno et al.<sup>23</sup> attributed the low detection rate of *Brucella* DNA by qPCR to the fact that it was done on serum specimens, while brucellae are intracellular pathogens. Moreover, the differences in PCR results among various studies can be attributed to the lack of uniformity and standardization of PCR protocols including optimal clinical samples, sample volume, extraction method, primers and target sequences, as well as storage conditions<sup>24</sup>. In addition, the low sensitivity of qPCR in our study may be attributed to the use of proteinase K in the extraction kit<sup>25</sup>. Furthermore, Scholz et al.<sup>26</sup> pointed that a small amount of DNA in clinical samples is challenging even for assays with a very low detection limit as PCR.

On the other hand, Gemechu et al.<sup>27</sup> demonstrated that the sensitivity of qPCR was only 12.6%, which was much less than the findings of other researchers, probably because at the time of specimen collection, the bacterial count in the peripheral circulation was below the detection limit of primers used. In the meantime, an extremely low bacterial load is needed to induce brucellosis<sup>28</sup>, thus, the initial bacteremic course may run undetected due to the low number of circulating bacteria.

In this study, qPCR was positive in 5%, 12%, 20% and 63% of samples with STAT titres less than 1/160 (negative), 1/320, 1/640 and 1/1280 respectively. Variable results were obtained in earlier studies. El Kholy et al.<sup>29</sup> demonstrated that PCR was positive in 12%, 24%, 32% and 32% in samples with STAT titres less than 1/160 (negative), 1/320, 1/640 and 1/1280 respectively. Another study by Refaat et al.<sup>30</sup> stated that PCR was positive in 16%, 26%, 19% and 26% in samples with STAT titres less than 1/160 (negative), 1/320, 1/640 and 1/1280 respectively.

In the meantime, qPCR in this study was positive in 3, 5 and 16 samples which were negative by STAT, RB and ELISA respectively. This goes in line with a study by Singh<sup>31</sup> who reported that among eight PCR-positive samples, three samples were negative by STAT.

Of note, serological tests and qPCR in this study were positive in a patient with history of brucellosis who had received two months of brucellosis therapy. Despite successful antibiotic

therapy, *Brucella* DNA often remains detectable in the majority of brucellosis patients throughout treatment and follow-up, as well as years after clinical cure and in the absence of any symptoms.<sup>32</sup>

On the other hand, Mahmood et al.<sup>33</sup> highlighted that although PCR is highly specific (up to 98%); yet, its sensitivity varies from 50 to 100%. In addition, Dias & Dias<sup>34</sup> pointed that the use of PCR implies some limitations including; lack of standardization of extraction methods, as well as costly equipment and reagents. These limitations favor serology as the most useful tool for the laboratory diagnosis of brucellosis as it is reliable, easy to perform, does not need expensive equipment or training, and carries a relatively low risk of laboratory acquired infection.

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