MOLECULAR DETECTION OF BRUCELLA INFECTION IN EGYPTIAN PATIENTS

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Abstract:

Background: Brucellosis is a zoonotic disease, endemic in many parts of the world especially the Middle East. It is an important health problem in Egypt. Since the clinical symptoms of human brucellosis are protean and nonspecific, laboratory confirmation by isolation of organism or detection of specific antibodies is necessary for the diagnosis. The objective of this study is to validate a PCR technique for the detection of brucella infection. So, we can differentiate between the true and current infections by this organism from the falsely positive antibodies produced from previous or treated infections.

Methods: This study comprised 97 patients (74 males and 23 females) from different ages experienced the clinical symptoms of brucellosis. This study was carried out at Mansoura University Hospitals. The blood samples collected were subjected to culture, Brucella test and PCR targeting the gene encoding a 31-kDa Brucella abortus (BCSP31).

Results: PCR standardized for the gene encoding a 31-kDa Brucella abortus "BCSP31" gene result in specific amplicon of 223-bp. In positive PCR cases of brucella (total 14), a single band of 223bp corresponding to BCSP31 gene was obtained. The PCR assay was found to be highly specific and the true positive brucellosis cases (n = 6) are positive of brucella test, blood culture and /or PCR. PCR was sensitive than blood culture for diagnosis of brucellosis since the sensitivity and specificity of PCR are 87.5% and 98.73% respectively and the sensitivity and specificity of blood culture are 84.62% and 96.15% respectively. There was a significant correlation between brucella titre and blood culture (p = 0.001) in patients with brucella. Furthermore, there was a significant correlation between brucella titre and PCR (p = 0.001) in patients with brucella.

Conclusion: We recommend using PCR as an alternative to blood culture for diagnosis of acute brucellosis. Further studies are required to compare different techniques. We also recommend performance of a large scale study to test this PCR technique for screening for brucellosis in Egypt.

Key words : Brucellosis, Brucella test, blood culture, PCR.

Introduction

Brucellosis is a zoonotic disease, endemic in many parts of the world especially the Middle East **[1]**. Brucellosis was reported as a top ten zoonosis in terms of impact on human health and economics of impoverished communities and ranking in the top five diseases causing livestock losses worldwide **[11]**.

The disease is endemic in our area. It is a zoonotic disease in which infection is transmitted to humans from domestic animals through direct contact or mostly through consumption of unpasteurized milk and contaminated dairy products **[6]**. Although it has been eradicated in several developed countries, it remains endemic in most areas of the developing world **[7],** particularly in the Middle East **[12]**. It is an important health problem in Egypt and a confirmed cause of 3% of cases of acute febrile illness **[8].**

Clinical management of brucellosis is one of the most challenging obstacles due to a high rate of failure in treatment and subsequent relapse **[2]**. Since the clinical symptoms of human brucellosis are protean and nonspecific, laboratory confirmation by isolation of organism or detection of specific antibodies is necessary for the diagnosis **[6].**

Definitive diagnosis of brucellosis needs comprehensive evaluation of the living conditions of the patient, medical history, clinical examinations, and careful interpretation of laboratory test results

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and radiologic findings **[3]**. Indeed, diagnosis of brucellosis is frequently delayed and often missed especially in the developing countries **[4]**.

Although many serological tests and new automated blood culture techniques have been developed to diagnose brucellosis, there are still significant problems in the diagnosis of the disease **[9].**

There are three approaches for the diagnosis of brucellosis including microbiological, serological, and molecular techniques **[10]**. Blood cultures stand for the "gold standard" of laboratory diagnosis. However, positive blood cultures occur in 10%- 70% of suspected infections, depending on the duration, localization of the infection and the type of Brucella species. In addition, culturing is time-consuming. Serological tests are more practical than culture techniques, while their specificities are low, especially in endemic areas or in people exposed to Brucella because of their profession. False-positive serological tests may also be caused by other illnesses such as tularemia, cholera, yersiniosis and salmonellosis, while false-negative results may occur early in the course of the disease **[6].** Thus, diagnosis relies on the combination of several methods **[5]**.

Currently, molecular biology techniques are being used extensively to identify the causative agents, as these techniques are less time-consuming, have high specificity and sensitivity to detect microorganisms. PCR is very sensitive, highly specific, rapid, and easily amenable for high-throughput screening. It is also more suitable for the detection of slow-growing bacteria such as Brucella. Due to its very high sensitivity, the PCR based method has the capability to detect tiny amounts of bacteria in clinical samples. It has also been demonstrated that the PCR technique is able to detect dead microbes in clinical samples, hence reducing the need for proper sample preservation before analysis. PCR-based protocols for the detection of Brucella spp. in clinical samples have been designed and developed. These methods are based on the amplification of gene BCSP31 which is highly conserved among Brucella spp **[13]**.

The aim of this study is to validate a PCR technique for the detection of brucella infection. So, we can differentiate between the true and current infections by this organism from the falsely positive antibodies produced from previous or treated infections.

For patients with brucellosis, the oligonucleotide primers of 25 bp defined the amplified region of a 224-bp segment of the histidine transport operon of brucella (The gene encoding a 31-kDa Brucella abortus "BCSP31") **[22, 23,24]**.

Subjects and methods

This study comprised 97 patients (74 males and 23 females) from different ages (Mean ± SD; 43.92 ± 10.40) experienced the clinical symptoms of brucellosis. This study was carried out at Mansoura University Hospitals in the period between January 2019 and September 2020. This study was approved by the local ethics committee, and a written consent was taken from patients.

The patients with brucellosis were subjected to: complete history taking with special emphasis on clinical symptoms of brucellosis; including fever and arthritis. Moreover, brucella test was performed as a routine test for diagnosis of the brucellosis. The inclusion criteria included with brucella titre (Abortus and/or melitensis) 1/80, 1/160, 1/320, and 1/640. Meanwhile, the exclusion criteria included brucella titre (Abortus and/or melitensis) less than 1/80, participants were already on antibiotic treatment and those who were diagnosed for other known febrile illness were excluded from the study.

Total leukocytic count, hemoglobin (Hb %), and platelets count were measured in all the studied patients with brucellosis using an electronic cell counter (D-Cell 60 Hematology Analyzer, DIAGON Kft., Hungary). The serum AST, ALT, albumin, bilirubin, creatinine, and fasting blood glucose were measured in all the studied patients with brucellosis using BT3500 automated chemistry analyzer (Biotecnica Instruments,S.p.A. via Licenza, 18 00156 Rome, Lazio, Italy).

For patients with brucellosis, the CROMATEST stained antigens are standardized suspensions of killed bacteria prepared for the detection and semi-quantitation by agglutination in either slide or tube tests of human serum agglutinins, a group of antibodies developed during some febrile infections such as brucellosis, salmonellosis and certain rickettsiosis . The assay is performed by testing the stained antigens –somatic, blue; flagellar, red against unknown samples. The presence or absence of a visible agglutination is usually related with the presence or absence of the corresponding homologous antibody in the sample tested. Results are reported as a semiquantitative test. Positive samples were diluted to know the titre **[14, 15]**.

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All samples were collected in BacT/ALERT bottles (BIOMERIEUX, USA) irrespective of antibiotics administration. Blood samples were subjected to automated blood culture system. Specific identification of all culture positive samples was accomplished by sub-culture on Blood agar, Chocolate agar and MacConkeys agar media (OXOID CO. UK). Inoculated Blood agar and MacConkeys agar plates were incubated aerobically at 37 ^{o}C. The Chocolate agar plates were incubated at 37 ^{o}C under 5-10% CO_2 condition (Candle jar) and examined after 18-24 hours of incubation. Identification of growth was performed by VITEK 2 Compact system (bioMérieux,France) **[16, 17, 18, 19, 20]**.

Extraction of DNA was done using the QIAamp DNA blood Kit (Qiagen) following the instructions published by the manufacturer **[21]**. The cells (maximum 2 x 109 cells) were harvested in a microcentrifuge tube by centrifuging for 10 min at 5000 x g (7500 rpm), and then supernatant was discarded. The pellet was resuspended in 180 µl Buffer ATL. The proteinase K (20 µl) was added and was mixed thoroughly by vortexing, and then was incubated at 56°C, and then was placed in a thermomixer to disperse the samples. Buffer AL (200 µl) was added to the sample, and mix thoroughly by vortexing. Then 200 µl of ethanol (96–100%) was added, and mixed again thoroughly by vortexing. The mixture was pipetted into the DNeasy Mini spin column which was placed in a 2 ml collection tube, and then was centrifuged at ≥ 6000 x g (8000 rpm) for 1 min. The DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), then 500 µl Buffer AW1 was added and centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute. The DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), and then buffer AW2 (500 µl) was added, and was centrifuged for 3 minutes at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a clean 2 ml microcentrifuge tube, and 200 µl Buffer AE was pipetted directly onto the DNeasy membrane and finally was incubated at room temperature for 1 min, and then was centrifuged for 1 min at ≥ 6000 x g (8000 rpm) to elute. For maximum DNA yield, the elution step was repeated once again.

For patients with brucellosis, the oligonucleotide primers of 25 bp defined the amplified region of a 223-bp segment of the histidine transport operon of *brucella* (The gene encoding a 31-kDa Brucella abortus "BCSP31"). The primer sequences for the forward and reverse oligonucleotides, from 5' to 3', were as follows: forward strand, TGGCTCGGTTGCCAATATCAA; reverse strand, CGCGCTTGCCTTTCAGGTCTG **[22, 23, 24]**.

The initial part of PCR optimisation was performed using conventional PCR. Each reaction in a total of 25 µL contained 12.5 µL PCR Master Mix (Promega, USA), 1 µL each of the forward and reverse primers, 5 µL of DNA template and 5.5 µL molecular grade water. The primer concentration used in the reaction was kept constant at 10 µM. Amplification was performed in Stepone thermal cycler. Annealing temperature optimisation was performed at 12 points using the following condition; initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C denaturation for 1 min, annealing at 49.8 °C to 65.1 °C for 1 min and extension, 72 °C for 1 min [25, 26]. PCR products (5 µL) were electrophoresed on 2% agarose gel in a 0.5× TBE buffer at 85 V for 60 min. The gel was stained with GelRed™ (Biotium®, USA). A 50 bp DNA ladder (Fermentas, USA) was included as the molecular weight marker in every electrophoresis run. The PCR products were visualised under ultraviolet (UV) illumination using gel image documentation system (GelDoc 1000 system, Bio-Rad, USA).

Statistical Analysis

Data analysis will be performed using statistical software program (SPSS for Windows, version 21, USA). All the nominal data, regarding clinical presentation and demographic data, as well as brucella titre results were expressed as number (percentage). Chi-square test will be used to assess the distribution of such nominal data between the selected patients. However, for the numerical data, descriptive statistic, including mean ± standard deviation, Median (Range), and 95 % Confidence interval of mean (95 % CI), was presented. Association between bucella titre and other diagnostic methods; blood culture and PCR in patients with brucellosis as well as the association between brucella titre and clinical symptoms in patients with brucellosis was analyzed using Chi-Square test. Correlation between different blood parameters in patients with brucellosis was assessed using Pearson correlation. Finally, ROC curve was done to assess the sensitivity and specificity of blood culture and PCR as a diagnostic method for brucellosis. For all statistical examinations, results were considered significant at P-value ≤ 0.05.

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Results

Table 1. Demographic Data of patients with Brucella

Sex		Chi-square = 26.814 P-value = 0.001
Males No (%)	74 (76.3 %)	
Females No (%)	23 (23.7 %)	
Age (Years)		Chi-square = 25.879 P-value = 0.894
Male (Mean ± SD)	43.92 ± 10.40	
Female (Mean ± SD)	43.74 ± 10.40	
Brucella titre		Chi-square = 84.649 P-value = 0.001
1/80 No (%)	63 (64.9 %)	
1/160 No (%)	16 (16.5 %)	
1/320 No (%)	12 (12.4 %)	
1/640 No (%)	6 (6.2 %)	

Table 1 summarizes the demographic data of patients with brucellosis. Males were 74 (76.3 %) with ages (Mean ± SD) 43.92 ± 10.40 and Females were 23 (23.7 %) with ages (Mean ± SD) 43.74 ± 10.40. For brucella titre, the highest percentage was for titre 1/80. P-values for chi-square test were significant for both sex and brucella titre.

Table 2. Brucella titre in patients with bucellosis

	Brucella titre				**Chi-square**
	1/80 (n = 63)	**1/160 (n = 16)**	**1/320 (n = 12)**	**1/640 (n = 6)**	
Sex					Chi-square = 2.121 P-value = 0.548
Male (n = 74)	46 (62.2 %)	14 (18.9 %)	10 (13.5 %)	4 (5.4 %)	
Female (n = 23)	17 (73.9 %)	2 (8.7 %)	2 (8.7 %)	2 (8.7 %)	
Age	44.24 ± 10.1	42.25 ± 10.8	42.25 ± 12.5	47.67 ± 5.04	Chi-square= 116.856 P-value = 0.264

Table 2 summarizes the brucella titre in patients with brucellosis. There was non-significant (p = 0.548) association between brucella titre and both sex and age.

Table 3. Distribution of clinical symptoms in patients with brucella

	Present	**Not present**	**Total**
Fever	59 (60.8 %)	38 (39.2 %)	97 (100 %)
Arthritis	22 (22.7 %)	75 (77.3 %)	97 (100 %)

Table 3 and figure 4 summarize the distribution of clinical symptoms in patients with brucellosis. The recorded clinical symptoms were fever in 59 (60.8 %) patients, and arthritis in 22 (22.7 %) patients.

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Table 4. TLC, Hemoglobin, and Platelets count in patients with brucella

	Mean ± SD	Median (Range)	95% CI of Mean
TLC (X 10^3)	5.870 ± 0.114	5.6 (4.2 – 7.5)	5.643 – 6.098
Hemoglobin (gm/dL)	14.22 ± 1.06	14.0 (12.9 – 16.0)	14.0 – 14.34
Platelets (X 10^3)	238.5 ± 49.5	231 (144 – 310)	228.6 – 249

Table 4 summarizes the descriptive statistics for TLC, hemoglobin, and platelets count in patients with brucella. Total leukocyte count (X 10^3) was 5.870 ± 0.114, Hemoglobin (gm/dL) percent was 14.22 ± 1.06, and platelets count (X 10^3) was 238.5 ± 49.5.

Table 5. Descriptive Statistics for AST, ALT, Albumin, bilirubin, creatinine, and Fibrinogen levels in patients with brucella

	Mean ± SD	Median (Range)	95% CI of Mean
AST (U/L)	34.32 ± 5.89	32 (15 – 47)	31.44 – 35.20
ALT (U/L)	34.29 ± 5.34	30 (13 – 47)	31.16 – 35.42
Albumin (gm/dL)	4.36 ± 0.72	4.50 (3.0 – 5.80)	4.22 – 4.51
Bilirubin (mg/dL)	0.82 ± 0.15	0.80 (0.50 – 1.10)	0.79 – 0.85
Creatinine (mg/dL)	0.91 ± 0.12	0.90 (0.80 – 1.30)	0.88 – 0.93
Fasting blood glucose (gm/dl)	95.92 ± 12.71	98 (70 – 130)	93.36 – 98.48

Table 5 summarizes the descriptive statistics for AST, ALT, albumin, bilirubin, creatinine, and fibrinogen levels in patients with brucella. AST (U/L) level was 34.32 ± 5.89, ALT (U/L) was 34.29 ± 5.34, albumin (gm/dL) level was 4.36 ± 0.72, bilirubin (mg/dL) level was 0.82 ± 0.15, creatinine (mg/dL) level was 0.91 ± 0.12, and fasting blood glucose (gm/dl) level was 95.92 ± 12.71.

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Table 6. Correlation of different blood parameters in patients with Brucella

	TLC	Hemoglobin	Platelets	AST	ALT	Albumin	Bilirubin	Creatinine	FBG	Brucella titre
TLC		r = 0.135 p = 0.187	r = 0.033 p = 0.745	r = - 0.144 p = 0.160	r = - 0.109 p = 0.286	r = - 0.058 p = 0.575	r = - 0.100 p = 0.328	r = - 0.212* p = 0.037	r = - 0.016 p = 0.873	r = 0.004 p = 0.972
Hemoglobin			r = 0.059 p = 0.566	r = 0.100 p = 0.328	r = 0.037 p = 0.722	r = 0.092 p = 0.372	r = 0.077 p = 0.455	r = - 0.203* p = 0.046	r = - 0.023 p = 0.821	r = - 0.032 p = 0.758
Platelets				r = 0.146 p = 0.153	r = 0.087 p = 0.396	r = - 0.056 p = 0.586	r = - 0.160 p = 0.118	r = - 0.128 p = 0.210	r = 0.009 p = 0.932	r = - 0.049 p = 0.635
AST					r = 0.691 ** p = 0.001	r = 0.044 p = 0.669	r = - 0.102 p = 0.320	r = - 0.139 p = 0.175	r = 0.100 p = 0.331	r = 0.137 p = 0.181
ALT						r = 0.011 p = 0.915	r = - 0.030 p = 0.772	r = - 0.202 * p = 0.047	r = - 0.023 p = 0.822	r = 0.261 ** p = 0.010
Albumin							r = - 0.125 p = 0.223	r = - 0.129 p = 0.206	r = - 0.001 p = 0.995	r = 0.202 * p = 0.048
Bilirubin								r = 0.165 p = 0.106	r = - 0.030 p = 0.773	r = - 0.066 p = 0.523
Creatinine									r = 0.017 p = 0.867	r = - 0.201 p = 0.048
FBG										r = 0.017 p = 0.867
Brucella titre										

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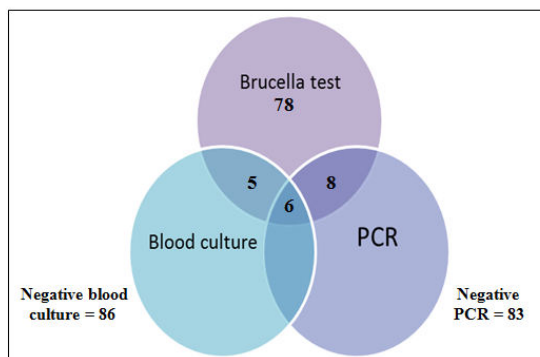

Figure 1. Venn diagram showing number of positive results in each combination of Brucella test, blood culture and PCR (n = 97). The true positive brucellosis cases (n = 6) are positive of brucella test, blood culture and /or PCR.

Table 7. Association between brucella titre and other diagnostic methods; blood culture and PCR in patients with brucella

	Blood culture		**PCR**	
	Negative	**Positive**	**Negative**	**Positive**
Brucella titre				
1/80	59 (68.6 %)	4 (36.4 %)	62 (74.7 %)	1 (7.1 %)
1/160	16 (18.6 %)	0 (0 %)	16 (19.3 %)	0 (0 %)
1/320	9 (10.5 %)	3 (27.3 %)	5 (5 %)	7 (50 %)
1/640	2 (2.3 %)	4 (36.4 %)	0 (0 %)	6 (42.9 %)
Total	86 (100 %)	11 (100 %)	83 (100 %)	14 (100 %)
	Chi-Square value = 24.101 **P-value** = 0.001		**Chi-Square value** = 65.414 **P-value** = 0.001	

Table 7 summerizes the correlation between brucella titre and other diagnostic methods; blood culture and PCR in patients with brucella. There was a significant association between brucella titre and blood culture (p = 0.001) in patients with brucella. Furthermore, there was a significant association between brucella titre and PCR (p = 0.001) in patients with brucella.

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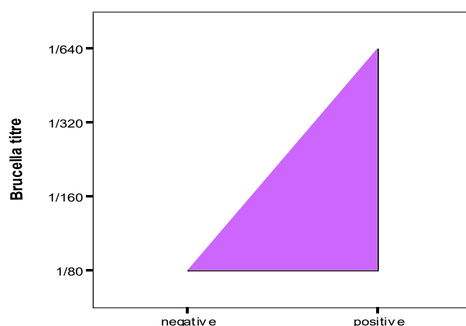

Figure 2. Correlation between brucella titre and blood culture in patients with brucella

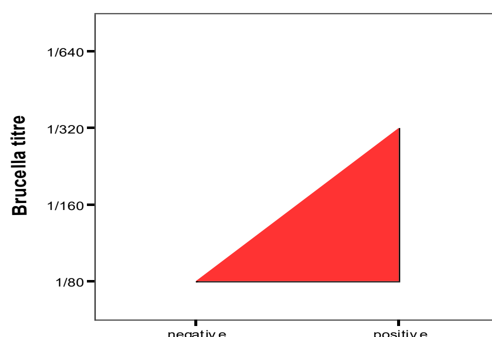

Figure 3. Correlation between brucella titre and PCR in patients with brucella

Table 8. Association between brucella titre and clinical symptoms in patients with brucellosis

	Brucella titre				Total	Chi-square Test
	1/80	**1/160**	**1/320**	**1/640**		
Fever						
Negative	25 (65.8 %)	8 (21.1 %)	2 (5.3 %)	3 (7.9 %)	38 (100 %)	**Chi-Square value** = 3.640
Positive	38 (64.4 %)	8 (13.6 %)	10 (16.9 %)	3 (5.1 %)	59 (100 %)	**P-value** = 0.303
Arthritis						
Negative	48 (64 %)	12 (16 %)	10 (13.3 %)	5 (6.7 %)	75 (100 %)	**Chi-Square value** = 0.466
Positive	15 (68.2 %)	4 (18.2 %)	2 (9.1 %)	1 (4.5 %)	22 (100 %)	**P-value** = 0.926

Table 8 explains the correlation between brucella titre and clinical symptoms in patients with brucellosis. There was a non-significant association between brucella titre and clinical symptoms, including fever, and arthritis in patients with brucellosis.

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Table 9. The validity of Blood culture, and PCR test as a diagnostic tool for Brucellosis

	Blood culture	PCR
AUC	0.012	0.012
Sensitivity (%)	84.62	87.5
Specificity (%)	96.15	98.73
PPV (%)	78.98	86.56
NPV (%)	92.54	94.80
Accuracy (%)	90.67	94.72
Standard error	0.010	0.010
CI 95 %	0.008 – 0.031	0.008 – 0.032
P - value	0.001	0.001

Table 9 showed the validity of Blood culture, and PCR test as a diagnostic tool for Brucellosis. PCR was sensitive than blood culture for diagnosis of Brucellosis.

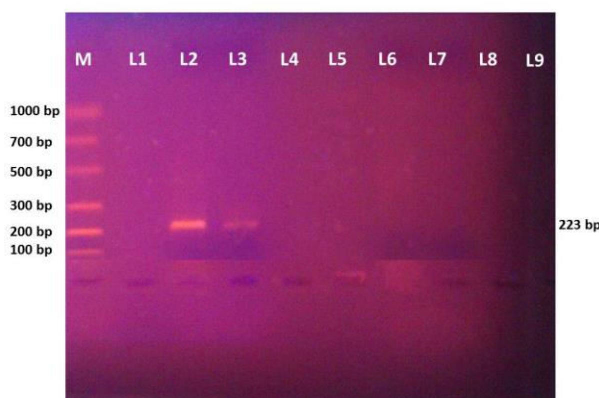

Figure 4. Agarose gel electrophoresis of PCR amplicons after amplification of gene-specific DNA fragments from whole blood sample for patients with brucellosis. Samples were analyzed by electrophoresis through 3% (wtvol) agarose gels. Gels were stained with ethidium bromide and DNA was visualized under UV. This results in amplicons 223 bp.

Discussion

Brucellosis is an important zoonosis. It affects a wide variety of mammals causing significant reproductive failure and enormous economic losses. In humans, it is associated with chronic debilitating infection **[9]**.

In the current study, in patients with brucellosis, Males % were 74 (76.3 %) with ages (Mean ± SD) 43.92 ± 10.40 and Female % were 23 (23.7 %) with ages (Mean ± SD) 43.74 ± 10.40.

All ages are susceptible to brucellosis, with most cases occurring in young and middle-aged people. In addition, there are more cases of males than females in different age groups, which may be related to the fact that men are more involved in livestock production and have more exposure to the source of infection. Humans brucellosis without gender and age differences, infected mainly depending upon the exposure opportunities. Farmers and herdsmen comprise the majority of infected cases, as they are regularly in contact with infected animals. This result was consistent with data from Tongliao

City, Inner Mongolia, most of these were agriculturalists (81.9%) and pastoralists (12.4%), aged 25–59 years (85.4%); the male-to-female ratio was 2.64:1**[43]**.

In the current study, the recorded clinical symptoms were fever in 59 (60.8 %) patients, and arthritis in 22 (22.7 %) patients. Symptoms of brucellosis may show up anytime from a few days to a few months after you've been infected. Signs and symptoms are similar to those of the flu and include: Fever, Chills, Loss of appetite, Sweats, Weakness, Fatigue, Joint, muscle and back pain, and Headache. Brucellosis symptoms may disappear for weeks or months and then return. Some people have chronic brucellosis and experience symptoms for years, even after treatment. Long-term signs and symptoms may include fatigue, recurrent fevers, arthritis, inflammation of the heart (endocarditis) and spondylitis — an inflammatory arthritis that affects the spine and nearby joints **[44, 45]**.

In this study, the total leukocyte count (X 10^3) was 5.870 ± 0.114. However, **Jiao et al., 2015,** found that white blood cells, neutrophils and eosinophils of the Brucellosis patients are decreased significantly compared with the general bacterial infection patients, but their lymphocyte is increased remarkably. The decreased eosinophils and increased lymphocyte percentage indicate that the blood of brucellosis patient changes, at the same time, it is different from the general bacterial infection **[46]**.

In earlier study, **Jiao et al., 2015,** found that after making a further analysis of blood cell classification for 151 brucellosis patients, the patients, whose white blood cells are normal or decreased, account for 90.73% (the patients with normal white blood cells are 67.55% and the patients with the decreased white blood cells are 23.18%); the patients, whose neutrophil proportion is normal or decreased, account for 95.36% (the patients with normal neutrophil proportion are 56.29% and the patients with the decreased neutrophil proportion are 39.07%); The patients, whose lymphocyte percentage is increased, account for 38.41%; it is coincidence with the general views **[46, 47, 48]**. At the same time, they found that the eosinophils proportion and count of brucellosis patients are diseased significantly and there are 28 patients whose eosinophils disappears (18.54%) and 86 patients whose eosinophils is decreased (56.95%). This phenomenon is rarely reported and the specific reasons are unknown. Eosinophils are decreased in typhoid fever, paratyphoid fever, severe tissue injury after surgery and upon application of adrenal cortical hormone or promotion adrenal gland hormone **[46]**.

Spink, 1956, in reviewing 244 patients with brucellosis seen at the University of Minnesota in a 20-yr period, found evidence of hepatic inflammation in 23 patients **[49]**. While splenomegaly has been reported in 10%-20% of patients with acute brucellosis, hepatomegaly is more unusual. None of the 30 patients who were hospitalized with acute brucellosis by Buchanan had evidence of hepatomegaly, hepatitis, or jaundice, and of the 172 cases reported to the Centers for Disease Control in 1978, only one was noted to be accompanied by hepatomegaly **[50]**. In the few reported cases where biopsy of the liver has been done, a granulomatous hepatitis with non-caseating granulomas similar to sarcoidosis has been found in infections with B. abortus and Brucella melitensis. Hepatitis resulting from B. suis is unusual; we are unaware of any biopsy-proved cases **[51, 52]**.

In the existing study, AST (U/L) level was 34.32 ± 5.89, ALT (U/L) was 34.29 ± 5.34, albumin (gm/dL) level was 4.36 ± 0.72, bilirubin (mg/dL) level was 0.82 ± 0.15, creatinine (mg/dL) level was 0.91 ± 0.12, and fasting blood glucose (gm/dl) level was 95.92 ± 12.71.

Williams et al., 1982, stated that while most infections are self-limited or readily controlled by antibiotics, a case of B. abortus hepatitis which was followed with serial biopsy has been shown to progress to micronodular cirrhosis in the absence of alcohol or other toxins **[50]**. Although another similar case has been reported, development of significant sequelae from acute Brucella hepatitis appears to be unusual. Liver involvement is frequent in acute and chronic brucellosis, as an increase in transaminase values and a mild hepatosplenomegaly can occur; sometimes an acute hepatitis develops **[52]**.

Casallas et al., 2018, reported that the epidemiology and patient demographics, clinical presentation, laboratory findings, and diagnostic images, ruled out the possible presence of toxins, viral hepatitis, vascular events, and miscellaneous conditions as a possible cause of acute liver failure, leading to the increasing diagnostic probability of the findings being attributed to brucellosis **[52]**. **La Spada et al., 2008,** in their study found that mean transaminase values were elevated and significantly higher than at recovery and 53% of patients had elevated baseline values of GOT and GPT and there were no significant differences in serum values of albumin and bilirubin before and after therapy **[51]**. **Akritidis et al., 2007,** reported that brucellosis might have been a coincidental bystander in cases of cirrhosis caused by unrecognized chronic viral hepatitis or hereditary liver disease **[54]**. Biochemical parameters such as serum glucose, serum total protein, serum albumin, and creatinine, revealed higher value in infected cattle but, other parameter such as AST, ALT and SD were lower than the reference value **[53].**

The clinical picture of brucellosis alone cannot always lead to diagnosis since the symptoms are nonspecific and often atypical; therefore, diagnosis needs to be supported by laboratory tests.

Although many serological tests and new automated blood culture techniques have been developed to diagnose brucellosis, there are still significant problems in the diagnosis of the disease **[9]**.

The isolation of Brucella spp. is considered as the gold standard technique for the diagnosis of brucellosis. The culture of Brucella is specific and allows definitive identification and typing of the isolates of Brucella spp. that is particularly valuable for epidemiological investigations. Sensitivity of the Brucella spp. isolation is variable depending on the culture method, type of clinical sample, stage of the disease, and history of antibiotic use. The ability to direct isolation and culture of Brucella spp. can vary between acute and chronic manifestations. Although 50% - 80% of acute cases yield positive blood cultures, only 5% of chronic cases are culture-positive. In order to increase the sensitivity, multiple blood sampling should be conducted in the acute phase of brucellosis **[42]**.

The serological diagnosis of brucellosis commonly relies on the confirmation of the rising titters of Brucellaspecific antibodies. This is the indirect proof of infection. Serological assays are used for the primary diagnosis of infection, as well as treatment follow-up. The titer of antibody should decline after an effective treatment. Otherwise, the patient should be examined for the possibility of chronic focal disease or relapse. Furthermore, the significant titers of antibody may persist for several months or even years in patients with the history of brucellosis. False positives in the determination of antiBrucella IgM may be due to the presence of cross-reactions and rheumatoid factor. It may be difficult to distinguish between active infection and simply exposure to the bacteria without clinical relevance in endemic regions by serological methods **[42]**.

PCR was considered as the golden test for diagnosis of brucellosis as suggested by **Maher, 2010,** whereas he stated that this method is more sensitive and specific than culture and serology for diagnosis of brucellosis **[37]**. Also, **Mitka et al., 2007,** stated that PCR is a very useful tool not only for the diagnosis of acute brucellosis but also as a predictive marker for the course of the disease and the post treatment follow-up, which is valuable for the early detection of relapses **[38]**.

As the clinical presentation of human brucellosis is quite nonspecific, it is needed to resort to isolation of the microorganism, by demonstrating high levels of specific antibodies or seroconversion, to make a precise diagnosis. However, all these methods have serious restrictions. Although culture is the 'Gold standard' in microbial diagnosis, culture may sometimes be abolished by factors innate to the microbe itself in spite of strict laboratory measures undertaken. This view was supported by the use of PCR to identify culture negative patients with clinical symptoms of brucellosis **[6]**.

PCR can be performed to amplify and detect Brucella DNA in clinical samples or pure cultures. Several single step PCR assays are developed to amplify and detect specific genomic sequences of the genus, species, or even biotypes of Brucella. Primer pairs used to detect Brucella at the genus-specific level include the primer for sequence encoding BCSP31 **[42]**.

Ghodasara et al., 2010, found that PCR utilizing different gene targets has recently become the most common way of diagnosis for human and animal brucellosis. Even though it is more sensitive, more rapid and less biohazardous than cultural techniques, the isolation of the organism is still accepted as the gold standard. The culture isolation followed by the confirmation by PCR in their study is an another approach of diagnosis, since PCR confirmation can rapidly identify at species level **[55]**.

In the present study, PCR standardized for the gene encoding a 31-kDa Brucella abortus "BCSP31" gene result in specific amplicon of 223-bp. In positive PCR cases of brucella (total 14), a single band of 223bp corresponding to BCSP31 gene was obtained. The PCR assay was found to be highly specific and the true positive brucellosis cases (n = 6) are positive of brucella test, blood culture and /or PCR. PCR was sensitive than blood culture for diagnosis of brucellosis since the sensitivity and specificity of PCR are 87.5% and 98.73% respectively and the sensitivity and specificity of blood culture are 84.62% and 96.15% respectively. The positive and negative predictive values of PCR are 86.56% and 94.80% respectively and the positive and negative predictive values of blood culture are 78.98% and 92.54% respectively. The accuracy (%) of PCR and blood culture are 94.72% and 90.67% respectively. The standard error of PCR and blood culture are 0.010 and 0.010 respectively.

We concluded that PCR was sensitive than blood culture for diagnosis of brucellosis.

These results agree with that shown by **El Kholy et al., 2009,** they used blood culture as a gold standard, founding that their PCR technique showed a sensitivity of 100%. This high sensitivity in culture positive cases suggests that PCR may replace blood culture as the gold standard for acute brucellosis **[27]**. Others who have tried to establish a PCR technique for diagnosis of human brucellosis found the sensitivity was 50% and the specificity 60% **[28].**

El Kholy et al., 2009, found that the PCR positivity in their study increased significantly with increasing seropositivity **[27]**.

Alikhani et al., 2013, found that the sensitivity of blood culture to isolate Brucella was low (14%) and the average incubation time for positive culture was relatively prolonged (six days) **[6]**. In

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other studies, the sensitivity of blood culture varied between 30 to 90%, and incubation time between three to seven days (10, 14-16). The reason for these discrepancies may be related to the discrepancies of disease stage in the population under study, in various reports, because the least rates of positive blood culture are seen in chronic brucellosis and the highest in acute febrile brucellosis **[6]**.

Alikhani et al., 2013, found that the sensitivity of PCR may be affected by the stage and severity of the disease **[6]**. This may explain the lower sensitivity of PCR in their study compared to those of the previous studies. Moreover, they found that the method of PCR may also be important. In a study conducted by **Al-Ajlan et al., 2011**, the comparison of different methods of PCR to diagnose brucellosis revealed that RTPCR was more sensitive than conventional PCR with 77.5% sensitivity in whole blood and 60% in serum **[33]**. **Hedayati et al., 2007,** developed an improved PCR assay for the rapid and specific laboratory diagnosis of acute brucellosis from serum specimens of 30 patients **[34]**. **Adlimoghadam et al., 2008** determined optmization of PCR conditions to detect Brucella in 16 serum samples from patients with brucellosis **[35]**. An important point in the study of **Alikhani et al., 2013,** was the higher sensitivity of PCR of serum compared to those of whole blood **[6]**. **Zerva et al., 2001,** compared serum and whole blood samples to diagnose human brucellosis by PCR. They concluded that serum is the preferred clinical specimen for the molecular diagnosis of brucellosis **[36]**.

Hassanain and Ahmed, 2012, found that comparing to PCR, the sensitivity of serum tube agglutination test (STAT) 1/160 was 100%, but it lacked the specificity, the sensitivity and specificity of STAT 1/320 were 91.7 and 100%, respectively, while the sensitivity and specificity of RBT were 87.5 and 33.3%, respectively **[9]**. **Mantur et al., 2008,** reported that STAT remains the most popular and yet used worldwide diagnostic tool for the diagnosis of brucellosis because it is easy to perform, does not need expensive equipments and training **[39]**. **AL-Garadia et al.**, **2011,** reported that the sensitivity of conventional PCR is 95.89%, while its specificity is 93% **[40]**.

Matar et al, 1996, found that PCR is useful for the rapid detection of brucella DNA directly in blood specimens obtained from persons with brucellosis. The assay proved to be sensitive because it detected Brucella DNA sequences directly in blood specimens from patients with agglutination titers ranging between 1:160 and 1:5,120. The assay is also specific for Brucella species because DNA obtained from patients with acute and chronic brucellosis was amplified, and a 223-bp fragment similar to that from the positive controls was generated. The assay is also rapid because it can provide results to the clinician in less than 24 h **[41]**.

In the present study, there was a significant correlation between brucella titre and blood culture (p = 0.001) in patients with brucella.

Over the last few years, experience on the isolation of Brucella spp. by use of automated blood culture systems has been accumulating at a slow pace. Although the disease is still prevalent in developing countries, the use of modern bacteriologic techniques in these areas is limited, whereas in the developed world, where use of automated blood culture systems is widespread, brucellosis has been successfully eradicated. Medical literature is frequently limited to retrospective reports of single cases or small outbreaks of disease among travelers to areas where the organism is endemic, and prolonged incubation of bottles and blind subcultures of negative media were not always done **[57]**.

Furthermore, in the present study, there was a significant correlation between brucella titre and PCR (p = 0.001) in patients with brucella.

This agrees with **El-Sherbini , 2007,** in Egypt who recommended increasing the seropositivity level of STA to 1/320 **[29]**, while another study from Egypt used a positive agglutination titre > 1/160 as seropositive for brucellosis **[30]**.

The gold standard for diagnosis of Brucellosis remains isolation of Brucella spp. bacteria from samples. However, PCR-based methods that identify nucleic acid fragments from the bacteria are more useful and practical. Most of the new methods for Brucella spp. identification and typing are still being developed and still await validation for use with clinical samples. This is especially true for PCR tests targeting new species of Brucella spp. from marine mammals. Most of these PCR-based methods were developed using Brucella spp. DNA prepared directly from cultured bacteria or extracted from the culture. The quality and purity of Brucella spp. DNA is very important in performing these methods, especially for multiplex PCR methods. Any inhibitor in DNA samples from any sources may limit the use of these methods. False-negative reactions can occur through a number of mechanisms such as specimens that contain EDTA, RNase or DNase, heme, heparin, phenol, polyamines, plant polysaccharides, urine, calcium alginate, and probably a host of other reagents. False-positive reactions resulting from specimen contamination or amplicon carryover also require attention. The sensitivity and specificity of most PCR-based methods are not well established and their real value for use with clinical samples and hence diagnosis has not been validated. There is still a great deal of work required for verification, validation, establishment of standard positive and negative controls, internal and

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inhibition control, reagents, quality assurance, and contamination before any of these methods may be used in routine laboratory testing for brucellosis **[58]**.

In the present study, there was a non-significant correlation between brucella titre and clinical symptoms, including fever, and arthritis in patients with brucellosis.

A study from Saudi Arabia showed that PCR was positive in 80% of symptomatic cases with titre 1/160 and the author recommended that titres > 1/160 should be reported as positive **[31]**. A study from Jordan also considered 1/160 as positive for serodiagnosis and established diagnosis by PCR in 72.7% and 12% by blood culture **[32]**.

Gemechu et al., 2011, reported PCR results were 51.3% accurate for sensitivity of 12.6% and specificity of 100% using STAT as gold standard **[56]**. **Nimri, 2003**, reported 100% sensitivity and specificity **[32]**.

Gemechu et al., 2011, found that early-case reporting is possible by rapid tests like PCR. Thus, PCR is a promising diagnostic tool for routine investigation and surveillance of brucellosis which is the key element for management of prevention and control programmes. Although PCR is going to be the ultimate diagnostic tool for rapid diagnosis of human brucellosis **[56]**.

El Kholy et al., 2009, recommend performing a larger study in all governorates of Egypt to establish a titre for serodiagnosis of brucellosis in Egypt **[27]**.

Conclusion

We recommend using PCR as an alternative to blood culture for diagnosis of acute brucellosis. Further studies are required to compare different techniques. We also recommend performance of a large scale study to test this PCR technique for screening for brucellosis in Egypt.

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