Diagnosis of Human Brucellosis by Amplification Reaction of BCSP31 Gene in Egyptian Patients

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ABSTRACT

Background: Human brucellosis is a major public health issue in Egypt. Because the clinical signs of human brucellosis are so varied and vague, scientific confirmation via organism isolation or antibody detection is required to confirm the diagnosis. The aim of this work is measurement of the sensitivity and specificity of PCR for the detection of Brucella abortus BCSP31gene compared with the blood culture methods.

Methods: A total of 97 patients from different ages with symptoms of brucellosis were included in the study. This research study was carried out at Mansoura University Hospitals. The sensitivity and specificity of polymerase chain reaction (PCR) for the detection of BCSP31gene of Brucella abortus compared with blood culture methods.

Results: In positive PCR cases of brucella (total 14), a single band of a 223-bp corresponding to BCSP31gene was obtained. The true positive brucellosis cases (n = 6) are positive of blood culture and PCR. PCR was sensitive than blood culture for diagnosis of brucellosis since the sensitivity and specificity of PCR are 87.5% and 98.73% and the sensitivity and specificity of blood culture are 84.62% and 96.15%. The positive and negative predictive values of PCR are 86.56% and 94.80% and the positive and negative predictive values of blood culture are 78.98% and 92.54%. The accuracy (%) of PCR and blood culture is 94.72% and 90.67%. The PCR assay was shown to be more sensitive and specific.

Conclusion: The PCR showed higher efficiency for rapid diagnosis of human brucellosis, as compared to the blood culture method.

Keywords

Brucellosis, Brucella test, blood culture, PCR.

1. INTRODUCTION

Brucellosis was listed as one of the top ten zoonotic diseases in regards of effect on public health and the economy of disadvantaged communities, as well as one of the top five illnesses responsible for animal deaths around the world. [1].

Despite the fact that it has already been extinguished in several wealthy nations, it is still prevalent in most poor countries [2], especially in the Arab world [3]. It is a significant public health issue in Egypt, accounting for 3% of all occurrences of severe febrile disease [4].

Due to a high likelihood of treatment failure and consequent relapse, clinical care of brucellosis is one of the most difficult challenges [5]. Brucellosis diagnosis is commonly delayed and overlooked, particularly in developing nations [6].

Despite the development of numerous serological tests and innovative automated blood culture tools to identify brucellosis, there are also substantial challenges in the disease's diagnosis [8].

Serological, microbiological and molecular techniques are among the three methods used to diagnose brucellosis [9]. Although blood cultures are the standard method for laboratory confirmation, they are time-consuming. Serology are more convenient than culture systems, however they have lower specificities [7].

Molecular biology approaches for detecting microorganisms are currently less time-consuming, offer great specificity, and sensitivity [29]. PCR is a sensitive, specific, quick, and easy-to-use choice for analysis and screening. It's also better for detecting bacteria that grow slowly, including Brucella. The PCR-based technique may detect very small amounts of bacteria in clinical specimens due to its great sensitivity. Brucella spp. detection techniques based on PCR have been designed and developed for clinical samples. These approaches are based on the amplification of the Brucella spp. gene BCSP31, which would be evolutionarily conserved [10, 29].

The aim of this study is to diagnose brucella infection by the amplification of BCSP31 gene.

2.   SUBJECTS AND METHODS

This study comprised 97 patients from different ages experienced the clinical symptoms of brucellosis (acute and chronic brucellosis). This study was carried out at Mansoura University Hospitals in the period between January 2019 and September 2020. Patients signed a written consent form after the study was approved by the local ethics commission.

The patients with brucellosis were subjected to: complete history taking with special emphasis on clinical symptoms of brucellosis; including fever and arthritis.

Brucella test:

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 [30, 31].

Blood culture examination:

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 oC. The Chocolate agar plates were incubated at 37 oC 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) [11, 12].

Polymerase chain Reaction (PCR):1. Extraction of DNA: Extraction of DNA was done using the QIAamp DNA blood Kit (Qiagen) following the instructions published by the manufacturer [13]. The cells (maximum 2 x 10^9 cells) were harvested in a microcentrifuge tube by centrifugation 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.

2. Amplification and detection: For patients with brucellosis, The amplified area of a 223-bp portion of the brucella histidine transport operon was identified by oligonucleotide primers of 25 bp (BCSP31 gene). The

forward and reverse oligonucleotide primer sequences, from 5' to 3', will be as follows: strand forward, TGGCTCGGTTGCCAATATCAA; reverse strand CGCGCTTGCCTTTCAGGTCTG [14, 15, 16].

3. Conventional PCR optimization: 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 [17, 18]. 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).

A statistical software programmed has been used to analyse the data (SPSS for Windows, version 21, USA). The findings of all statistical tests were deemed significant at P-value ≤ 0.05.

3. RESULTS

On blood samples of 97 patients, PCR was carried out for BCSP31 gene was positive in 14 patients.

The amplified portion of a 223-bp fragment of BCSP31 gene was identified using 25-bp oligonucleotide primers and at the end of the PCR, the amplified product was identified. A total of 83 patients had negative PCR test results.

The PCR for BCSP31 gene of brucella yielded a sensitivity of 87.5 % and yielded a specificity of 98.73 %. The PCR's positive predictive value (PPV) was shown to be 86.56 % with a precision and an accuracy of 94.72 %. The PCR findings were statistically significant (P= 0.001).

Table 1. 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 1 Blood culture and PCR test were shown to be valid diagnostic tools for Brucellosis. For Brucellosis diagnosis, PCR was more sensitive than blood culture.

Table 2. 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 2 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.
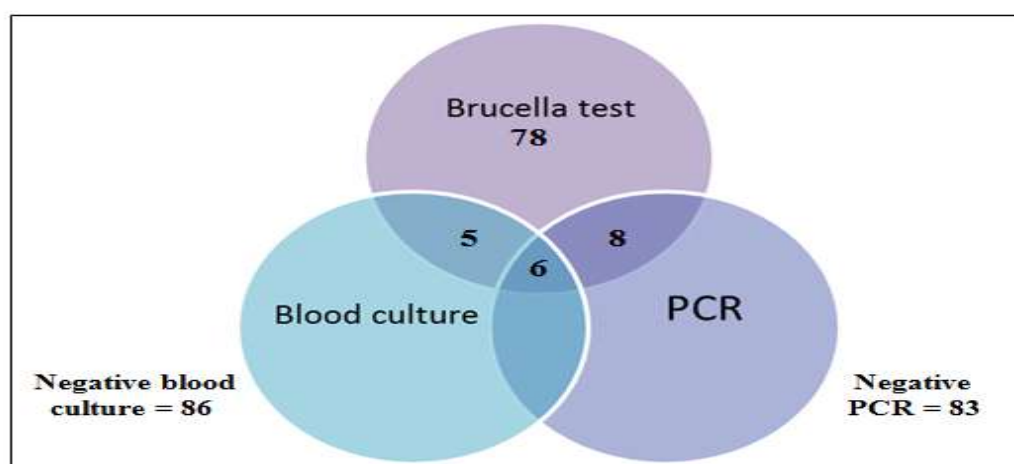

Figure 1. Venn diagram showing number of positive results in each combination of Brucella test, blood culture and PCR (n = 97). The true positive brucellosis cases (n = 6) are positive of brucella test, blood culture and /or PCR.
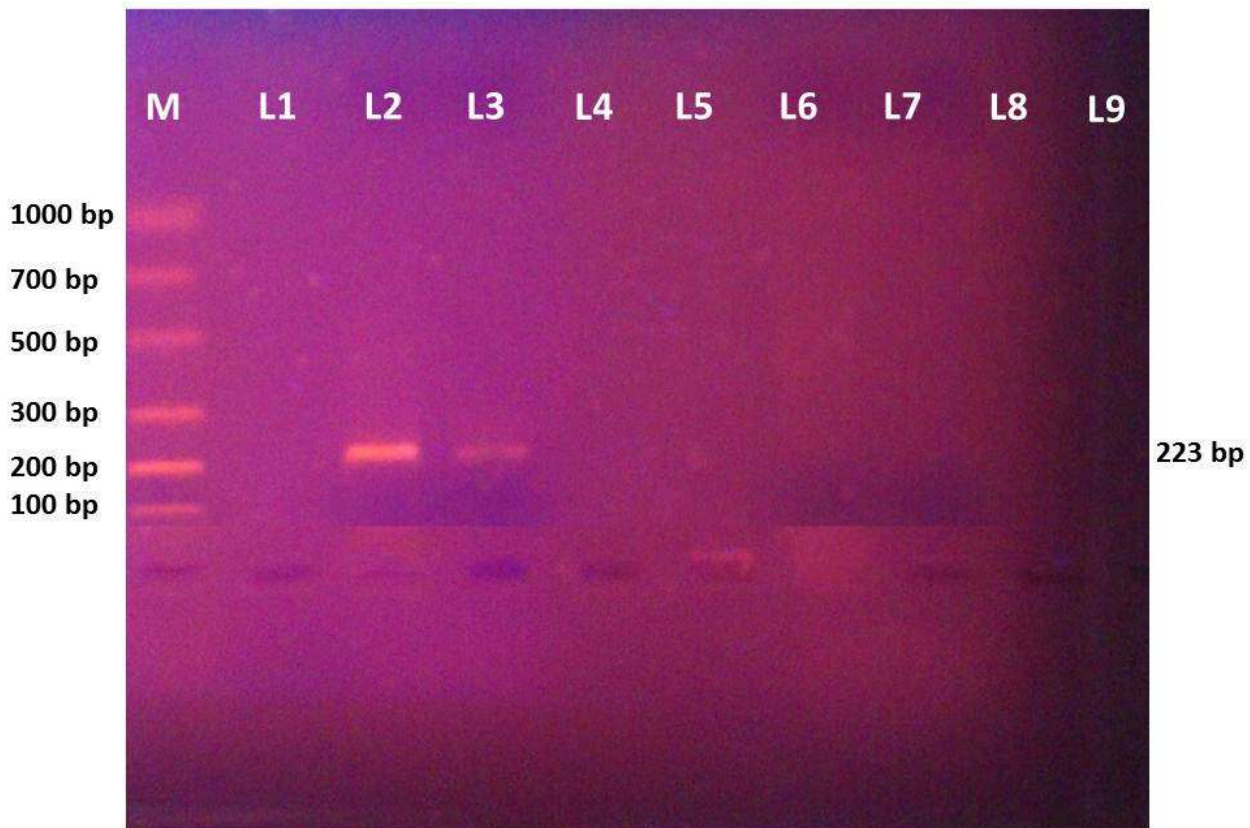
Figure 2. After amplification of gene-specific DNA fragments from a blood sample for brucellosis patients, Standard PCR amplicons electrophoresis on an agarose gel was performed. Electrophoresis on 3 percent (w/ v) agarose gels was used to examine the samples. DNA was seen under UV light after gels were stained with ethidium bromide. This results in amplicons 223 bp.

4. DISCUSSION

Because the symptoms of brucellosis are ambiguous and often atypical, the clinical picture alone cannot usually lead to a diagnosis [8].

Serological assays are utilised for both the initial diagnosis of infection and the monitoring of treatment outcomes. After a successful treatment, the antibody titer should decrease [25].

Despite the fact that culture is the Gold standard' in microbiological identification, culture can sometimes be wiped off by elements inherent to the bacterium itself despite careful laboratory procedures. The use of PCR to detect culture negative patients with brucellosis symptoms backed up this theory [7, 25].

Brucella DNA can be amplified and detected using PCR in samples or cultures. To amplify and detect specific gene sequence of a genus, species, or indeed genotypes of Brucella, numerous single-step PCR techniques have been devised. The primer for the sequence encoding BCSP31 is one of the primer pairs used to detect Brucella at the genus level [25].

PCR was once thought to be the gold standard for brucellosis diagnosis as suggested by [21], For brucellosis diagnosis, he claims that this procedure is more accurate than culture as well as serology. Also, [22] stated that PCR is a significant technique not only for diagnosing acute brucellosis, as well as for predicting the progression of the disease as well as post-treatment follow-up, which is important for detecting relapses early.

[26] revealed that PCR with several gene targets has become the most prevalent method of diagnosing brucellosis in humans and animals. Despite the fact that it is much more sensitive, faster and so less hazardous than cultural methods. Another method of diagnosis used in their study was culture isolation followed by PCR confirmation, because PCR confirmation can quickly identify species at the species level.

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. 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 predictive value of PCR is 86.56% and the positive predictive value of blood culture is 78.98%. The accuracy (%) of PCR and blood culture are 94.72% and 90.67% respectively.

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

These results agree with that shown by [4], they employed blood culture as a standard and discovered that their PCR approach had a 100% sensitivity. Because of its great sensitivity in culture-positive patients, PCR may be able to take the place of blood culture as the gold standard for acute brucellosis diagnosis. [4], found in their investigation that the PCR positives increased dramatically as seropositivity increased.

[7], found that blood culture had a low sensitivity for isolating Brucella, and the average incubation period for positive cultures was considerable and found that the stage and severity of the disease may alter the sensitivity of PCR. This could explain why the PCR sensitivity in their analysis was lower than in earlier investigations. Furthermore, they discovered that the PCR method may be essential. In a study accompanied by [19], when multiple PCR methods for diagnosing brucellosis were compared, it was discovered that PCR was more sensitive. Hedayati et al**,** established a better PCR technique for the quick and specific identification of acute brucellosis in the laboratory using serum samples from 30 patients. A crucial aspect to consider in the research of [7], the sensitivity of serum PCR was higher than that of whole blood PCR [20].

[23], reported that conventional PCR has a sensitivity of 95.89 percent and a specificity of 93 percent. [24], found that PCR can be used to detect brucella DNA directly in blood samples taken from people who have brucellosis. Because DNA from patients with acute and chronic brucellosis was amplified and a 223-bp segment similar with that of the positive controls was created, the test is also exclusive for Brucella species. The assay is extremely quick, delivering data to the clinician in less than 24 hours [24].

Isolation of Brucella spp. from specimens is still the standard method for diagnosing Brucellosis. PCR-based approaches for identifying genomic DNA fragments from bacteria, on the other hand, are more helpful and useful [28].

[27], reported PCR results were 51.3 percent accurate with a sensitivity of 12.6 percent and a specificity of 100 percent, indicating that fast assays like PCR can help with early case reports. Although PCR will be the final diagnostic method for quick human brucellosis diagnosis [4], recommend accomplishment a broader investigation in all Egyptian governorates to develop a titer for brucellosis serodiagnosis.

5. CONCLUSION

The PCR showed higher efficiency for rapid diagnosis of human brucellosis, as compared to the blood culture method.

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