Title page:

Acute febrile illness and Brucellosis in the Western region of KSA

Authors:

Corresponding author:
Ibrahim Hassan Kamal, Ph.D.

Address:
1- Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, KSA.
2- Biochemistry Department, Faculty of Science Ain Shams, University, Cairo, Egypt.

Email: imbc57@yahoo.com

Basim Al Gashgari
Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, KSA.

Email: byg396@yahoo.com

Said Salama Moselhey, Ph.D.

Address:
1- Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, KSA.
2- Biochemistry Department, Faculty of Science Ain Shams, University, Cairo, Egypt.

Email: moselhy6@hotmail.com
Taha Abdulla Kumosani, Ph.D.
Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, KSA.
Biochemistry Unit, King Fahad Medical Research Center (KFMRC), King Abdulaziz University, Jeddah, KSA.

Email: t.kumosani@yahoo.com

Khalid Omar Abulnaja, Ph.D.
Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, KSA.

Email: kabulnaja@yahoo.com
Abstract

**Background:** The etiology and the incidence of Acute Febrile Illness (AFI) caused by various pathogens represent a major public health problem because clinical diagnosis is usually unreliable. Brucellosis is a severe acute febrile disease and it is one of the most frequently reported diseases in Saudi Arabia. Human brucellosis incidence in Saudi Arabia is high, particularly in the Central region, and around the city of Riyadh.

**Methods:** A total of 101 AFI serum samples were collected from two different localities in the Western region of the Kingdom of Saudi Arabia (Northern and Central). All samples were subjected to DNA extraction, and *Brucella* genus specific B4/B5 DNA amplification. Positive B4/B5 patients subjected to multiplex species-specific *Brucella* amplification. **Results:** *Brucella* positive patients were confirmed in (81.9%) in AFI samples collected from the west northern region, while all AFI samples collected from the west central region proved to be non-*Brucella*. Positive B4/B5 patients subjected to multiplex species-specific *Brucella* amplification, which detects that *B. abortus* amplification in (10%) and *B. melitensis* in (8%) of the samples, while (82%) of samples showed double product of both *B. abortus* and *B. melitensis*. *B. suis* was not detected in any sample as expected. **Conclusions:** The current study recommends the necessity of epidemiological data to investigate such AFI patients for further information. It is also recommends the necessity of using two stages PCR step as a species specific diagnosis for human brucellosis.
Background

The etiology and the incidence of Acute Febrile Illness (AFI) caused by various pathogens represent a major public health problem because clinical diagnosis is usually unreliable, and diagnostic tests are often not available in disease endemic areas [1]. Most febrile illnesses symptom-based surveillance frequently results in classification errors, because the causes of febrile illnesses may be clinically indistinguishable. The incidence of febrile illness disadvantaged by problems associated with surveillance sensitivity and specificity. Therefore, achieving highly sensitive and specific surveillance system for accurate incidence and causes of febrile illness should detects cases as close as possible to the population level and must be supported by modern molecular diagnostic tests. Brucellosis is a severe acute febrile disease caused by Gram negative bacteria of the genus *Brucella*. Brucellosis results in a wide range of significant veterinary and public health problems, and economic loss [2]. The human brucellosis eradication has major difficulties and the disease has a serious medical impact worldwide [3]. The clinical symptoms of acute febrile illness always overlap with other etiological pathogens than *Brucella* and may lead to misdiagnosis as well as improper antibiotic treatment regimes.

Brucellosis is one of the most frequently reported diseases in Saudi Arabia [4-9]. Human brucellosis incidence in Saudi Arabia is high, particularly in the Central region, and around the city of Riyadh [9, 10].

Since brucellosis is a zoonotic disease it is transmitted from animals to humans by direct contact with infected animals or consumption of raw animal products such as unpasteurized milk or cheese. Direct contact with infected animals, their secretions or their carcasses could lead to infection through inhalation or accidental skin and mucous
membrane penetration [11, 12]. In Saudi Arabia, brucellosis was recognized as a major health problem and many measures to control the disease were implemented as early as 1983 [13].

Four species of the genus *Brucella* are pathogenic for humans and include *B. melitensis* (from sheep and goats), *B. abortus* (from cattle and other bovidae), *B. suis* (from pigs), and *B. canis* (from dogs) [14].

Taking into consideration that most human Brucellosis diagnostic tests which include medical history, clinical examination, routine hematological and biochemical laboratory tests, bacterial culturing, and serological tests are not sensitive and specific enough, the molecular diagnostic test Polymerase Chain Reaction (PCR) could be considered the most sensitive and specific tool.

Queipo-Ortuno and co-workers [15] found 100% sensitivity and 98.3% specificity using the B4/B5 primer pair amplifying a 223 bp fragment of the *bcsp31* gene, compared with 70% sensitivity for blood culture. PCR identification of *Brucella* strains at the species or biovar level has been described by Redkar *et al.*, [16] who developed a real-time PCR assays for the detection of *B. abortus*, *B. melitensis*, and *B. suis* biovar 1. These PCR assays target the specific integration of IS711 elements within the genome of the respective *Brucella* species or biovar.

In this study we evaluated the two stages PCR assays as a molecular diagnostic tool developed for diagnosis of human Brucellosis using similar DNA targets.
Methods

AFI patients

A total of 101 serum samples were collected from two different localities in the Western region of the Kingdom of Saudi Arabia (Northern and Central regions). These samples were obtained from patients suffering from Acute Febrile Illness (AFI), which was defined as any person at age of one year or older with body temperature ≥ 38°C at the time of collection, or fever of more than two days, and no identified cause of fever such as diarrhea, hepatitis or any respiratory tract infections. Control serum sample were collected from 20 healthy individuals within the same localities as the AFI group. No family history or any occupational exposure to Brucella infection was taken into consideration for the healthy control group. All participants meeting inclusion criteria were asked to participate in this study. Informed, written consent was obtained from adult participants and parents of minors.

All samples were subjected to DNA extraction as previously described by [17] with minor modifications as follows; 1% of sodium dodecyl sulfate (SDS) and 10 mg/ml of proteinase K were added to 300 ul of serum, and incubated for 2 h at 37°C. Proteinase K in the digest was inactivated by heating at 90-95°C for 10-15 min. After phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation, DNA was dissolved in 50 ul of nuclease free water.

Brucella genus specific DNA amplification

The PCR target sequence fragment of 223 bp present on a gene encoding a 31 kDa B. abortus antigen was selected for the first amplification to diagnose the Brucella
positive samples. This sequence encodes an immunogenic membrane protein of 31 kDa of *B. abortus* and it is conserved to all *Brucella* biovars [18]. A pair of 21-nucleotide primers, B4 (5’ TGG CTC GGT TGC CAA TAT CAA 3’) and B5 (5’ CGC GCT TGC CTT TCA GGT CTG 3’) (Bioline, Inc., MA, USA), described by Baily *et al.*, [19] were used in this amplification process. PCR was performed in a 25 ul mixture containing template DNA; PCR buffer (10 mM Tris HCl [pH 8.4], 50 mM KCl, 1.5 mM MgCl2); a 10 pmol of each primer; a 200 uM (each) of dATP, dCTP, dTTP and dGTP (Bioline, Inc., MA, USA), and 1.25 U of *Taq* polymerase (Qiagen, USA). The cycle program consisted of a preheating at 95°C for 5 min; 35 cycles of 90°C for 1 min, 60°C for 30 s, and 72°C for 1 min; and incubation at 72°C for 10 min. Positive control based on DNA from *B. abortus* reference strain was included in all tests, as well as negative control containing all of the components of the reaction mixture except DNA. 20% of each PCR product was visualized on a 1% agarose gel stained with 2 ug/ml of ethidium bromide.

**Multiplex species specific *Brucella* DNA amplification**

All positive B4/B5 samples were subjected to multiplex PCR amplification to define the *Brucella* species might cause the infection. Species-specific DNA segments of *B. abortus*, *B. melitensis* and *B. suis* were targeted for amplification using specific primers derived from the *IS*711 element [20]. The forward primer (5’ CAT GCG CTA TGT CTG GTT AC 3’) spans 803 to 823 nt of *IS*711 and generates a 113 bp PCR product with *B. abortus* reverse primer (5’ GGC TTT TCT ATC ACG GTA TTC 3’), 252 bp PCR product with *B. melitensis* reverse primer (5’ AGT GTT TCG GCT CAG AAT AAT C 3’), and 170 bp product with *B. suis* reverse primer (5’ ACC GGA ACA TGC AAA TGA
Amplification conditions were the same as mentioned before except the annealing temperature (58°C). Positive and negative PCR controls were used in all tests. *B. suis* primers were used as internal negative PCR control. *B. suis* is pathogenic to pigs which are not found in the Kingdom of Saudi Arabia. PCR products were visualized as previously mentioned.

**Results**

A total of 101 AFI patients were enrolled in this study from the Western region of the Kingdom of Saudi Arabia, 61 and 40 from northern and central regions respectively, their characteristics are presented in table 1. Forty four samples (72%) from western region were serologically positive for *Brucella* with fluctuating titers during this study (data not shown). All samples were subjected to *Brucella* genus amplification using B4/B5 primers which amplify a conserved region in all *Brucella* species to detect the presence of *Brucella* DNA as the cause for the Acute Febrile Illness (AFI).

Agarose gel electrophoresis of the B4/B5 conventional PCR amplification gave a product size of 223 bp band indicating the presence of *Brucella* genus in (AFI) patients (Fig.1). The same product size detected with *Brucella* reference strain used as positive PCR control. On the other hand, no amplification was detected in the non *Brucella* (AFI) patient samples same as the negative control subjects and negative PCR control. Conventional PCR study confirmed that 50 samples (81.9%) were diagnosed as *Brucella* positive out of the 61 (AFI) samples collected from the Northern region, while the 11
samples (18%) showed as non-Brucella AFI patients. No human Brucellosis was detected from the 40 samples collected from the central western region.

The 50 Brucella positive (AFI) patients were subjected to the species-specific multiplex PCR. Multiplex PCR products electrophoresis results are shown in figure 2, which revealed the presence of 113 bp and 252 bp bands specific for B. abortus and B. melitensis respectively (Fig. 2). Among these 50 samples, B. abortus amplification was evident in 5 samples (10%) and B. melitensis product was detected in only 4 samples (8%), while the rest of the samples (82%) showed double product of both B. abortus and B. melitensis (Table 2). B. suis amplification product (170 bp) was not detected in any sample. These results confirm the high specificity and sensitivity of these primers for the targeted region in Brucella DNA.

Discussion

Acute febrile illness (AFI) still represents the common clinical syndrome among patients seeking hospital care. Brucellosis is one of the various pathogens causing AFI, and it is a public health problem in many developing countries, including Saudi Arabia, where many people by their traditional lifestyle, drink raw milk or have close animal contact [21]. The true human brucellosis incidence has been estimated to be between 10 and 25 times higher than the number of annual reported cases [22].

The diagnosis of human brucellosis depends mainly on culture [23] and serological tests [24]. Brucella is highly virulent bacteria and may lead to exposure hazards for laboratory personnel, its culture is time consuming and the isolation rate is low which may cause critical diagnostic delays [25]. At the early stage of infection, the
sensitivity of serologic tests is low and false-negative or only weak positive reactions may occur [26]. Due to the limitations of culture techniques and serological tests, various molecular microbiological testing methods particularly with Polymerase Chain Reaction (PCR) have been developed for rapid identification of organisms in clinical samples. PCR techniques proved to be very useful, simple, quick, sensitive, specific, and relatively inexpensive technique that merits its adoption in the clinical laboratories. Several articles have been published dealing with various PCR-based methods for *Brucella* detection.

In this study, two PCR assays were used: the genus-specific PCR assay which targeted the 223 bp sequence of the gene encoding a 31 kDa *Brucella abortus* antigen [19], and the multiplex amplification for the identification of *Brucella* to the species level, called AMOS PCR for *B. abortus, B. melitensis, B. ovis,* and *B. suis* [20]. *Brucella* genus specific B4/B5 PCR detected the presence of 223 bp predicted fragment in 81.9% of AFI serum samples collected from northern region, while it did not detect any *Brucella* cases out of the 40 AFI samples collected from central region. These results indicated that using serum as a clinical sample and the two PCR sequential assays are preferable and sensitive for diagnosis of human brucellosis. Same results were also reported by Elfaki and coworkers [27] who reported the presences of the same PCR fragment (223 bp) in 96% of the sera samples used. The authors [27] investigated 25 patients with symptoms of brucellosis from two reference hospitals in central Saudi Arabia.

*Brucella* species-specific multiplex PCR classified the *Brucella* genus positive samples into single *B. abortus* or *B. melitensis* and double amplification (both *B. abortus* and *B. melitensis*), which represents the majority (81%). *B. suis* failed this amplification
as predicted since the swine are not domestic in Saudi Arabia. Corresponding results were previously recorded in Saudi Arabia [27].

Our results showed to be in accordance with other studies [27, 28] which recommended the use of PCR as the diagnostic tool of choice for human brucellosis. Since all studied AFI samples in this study were collected randomly with very limited case definitions, we could not classify the Brucellosis to acute, chronic or a relapse cases.

The existence of the double product may be attributed to active double infection or to the coexistence of double Brucella species free DNA in tested samples. The free DNA in serum may reflect the degradation of Brucella cells during the bacteremic phase of infection [27].

Conclusions

The current results recommend that epidemiological data is needed to investigate such AFI patients for further information about their geographic distribution, life style, age, gender, occupational exposure, other health conditions (antibiotic treatment) and family history.

In this study a two stage design was used to evaluate the reliability of PCR as the method of choice for Brucella DNA detection in serum samples, which minimize the exposure risks for this virulent bacterium and shorten the diagnostic time. These molecular detection methods could be one of the powerful epidemiological tools for confirmation of the disease, tracing of Brucella spp. transmissions and for identification of infection sources.
Hopefully the species-specific diagnosis will lead to new and improved treatment regimens and provide strategies to effectively cure even the most complex cases of brucellosis often seen in endemic areas.

**Consent statement**

Institutional ethical approval for the study was obtained from the Ethical Committee of King Abdulaziz University.

**Competing interests**

None

**Authors’ contributions**

IH Kamal designed and performed the study experiments and was responsible for writing the manuscript. B Al Gashgari participated in the laboratory experiments. SS Moselhy, TA Kumosani and KO Abulnaja were responsible for data management, and revised the draft carefully for important intellectual content. All authors read and approved the final version of this manuscript.

**Acknowledgements**

The authors express their gratitude and grateful thanks King Abdulaziz University, Jeddah, Saudi Arabia and to the Deanship of Scientific Research (DSR) of KAU for the financial support of this study, grant number 429/079-3.
References


17. Chryssanthou E, Andersson B, Petrini B, Lo¨fdahl S, Tollemar J: Detection of 


Fig. 1, Agarose gel electrophoresis of B4/B5 conventional PCR assay products.

Lanes 1, 2, and 3, represent *Brucella* genus positive samples with amplification product size 223 bp. Lanes 4, 5, 6, 7, 8, and 9, represent negative *Brucella* samples.

Lanes (+ve) and (-ve) contain positive and negative PCR controls.

Lane (M) contains a 100-bp ladder (HyperLadder, Bioline, Inc., MA, USA).

Fig. 2, Agarose gel electrophoresis of species-specific multiplex assay products.

Lane #1 *B. melitensis* only (252 bp).

Lanes 5 and 6, *B. abortus* only (113 bp); lanes 2, 3, 4, 7, 8, and 9, both of *B. abortus* and *B. melitensis* (113 bp and 252 bp respectively).

Lane (+ve) and (-ve) contain positive and negative PCR controls.

Lane (M) contains a 100-bp ladder (HyperLadder, Bioline, Inc., MA, USA).
Table 1. Characteristic features of patients with AFI.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Locality at Western region</td>
</tr>
<tr>
<td>No. of AFI samples collected</td>
<td></td>
</tr>
<tr>
<td>No. of <em>Brucella</em> seropositive samples (data not shown)</td>
<td></td>
</tr>
<tr>
<td>No. of samples subjected to conventional PCR (B4/B5 amplification)</td>
<td></td>
</tr>
<tr>
<td>No. of samples subjected to species-specific PCR</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. *Brucella* DNA amplification using B4/B5 and species-specific multiplex PCR.

<table>
<thead>
<tr>
<th>(AFI) Samples locality (Western region) (number)</th>
<th><em>Brucella</em> genus B4/B5 conventional PCR</th>
<th><em>Brucella</em> species-specific multiplex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-ve amplification</td>
<td>+ve amplification</td>
</tr>
<tr>
<td>Non-<em>Brucella</em> patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Northern region) (61)</td>
<td>11</td>
<td>50</td>
</tr>
<tr>
<td>Brucella patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Central region) (40)</td>
<td>40</td>
<td>------</td>
</tr>
</tbody>
</table>


Fig. 1. Agarose gel electrophoresis of B4/B5 conventional PCR assay products. Lanes 1, 2, and 3, represent *Brucella* genus positive samples with amplification product size 223 bp. Lanes 4, 5, 6, 7, 8, and 9, represent negative *Brucella* samples. Lanes (+ve) and (-ve) contain positive and negative PCR controls. Lane (M) contains a 100-bp ladder (HyperLadder, Bioline, Inc., MA, USA).
Fig. 2. Agarose gel electrophoresis of species-specific multiplex assay products. 
Lane #1 B. melitensis only (252 bp). 
Lanes 5 and 6, B. abortus only (113 bp); lanes 2, 3, 4, 7, 8, and 9, both of B. abortus and B. melitensis (113 bp and 252 bp respectively). 
Lane (+ve) and (-ve) contain positive and negative PCR controls. 
Lane (M) contains a 100-bp ladder (HyperLadder, Bioline, Inc., MA, USA).