Case Report

Specific IgA antibodies in the diagnosis of acute brucellosis

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Abstract

An Egyptian female with night sweats, headache, and back pain was diagnosed with acute brucellosis one week after returning from a North African country. Humoral immune responses to specific immunogenic proteins were investigated before and after treatment. ELISA was performed to detect levels of specific antibody (Ab) titers. Immunoblot analysis of Ab recognizing specific Brucella antigenic bands was also performed. IgA was detected on the day of disease onset. Specific agglutination titer was 1:160; it doubled three days later and treatment was implemented. Blood culture yielded Gram-negative coccobacilli after one month, confirmed as B. melitensis by AMOS-PCR. Immunoblotting revealed IgM Abs against two protein bands of 112 and 130-kDa observed only during the acute stage. On the other hand, the intensity of IgG Abs against 21 and 21.5-kDa protein bands positively correlated with the time of convalescence. Based on our observations we conclude that specific IgA levels may be used as an early diagnostic marker for Brucella and high molecular weight protein bands may be useful in the differentiation between acute and chronic brucellosis.

Key words: brucellosis; IgA antibodies and early diagnostic marker


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Introduction

Brucellosis is one of the most important endemic zoonotic diseases. Four members of the species, Brucella abortus, Brucella melitensis, Brucella suis and Brucella canis, are capable of causing disease in humans. In the Middle East, most infections are caused by B. melitensis, which produces the most severe symptoms with the greatest tissue damage [1]. Brucella is an airborne pathogen categorized as a class B bio-terror agent by the United States Centers for Disease Control and Prevention [2]. Currently, there are no human vaccines against brucellosis. In addition, the treatment of human brucellosis requires the combination of two drugs for at least four to six weeks [3].

Brucellosis may be acquired from direct handling of infected animals or by consuming unpasteurized contaminated milk, dairy products or meat, or through accidental inhalation of contaminated aerosols [1,4]. Brucellosis is common in many resource-limited and transitioning countries such as those located in the Mediterranean basin and Arabian Gulf regions [5-8], Latin America [9], Africa [10], and parts of Asia [11].

Understanding the host’s immune responses during Brucella infection and during the different stages of treatment will not only lead to better understanding of the correlation between the pathogenicity and virulence of the microbe and the elicited immune response, but also may offer the ability to differentiate between chronic and acute brucellosis. In experimental animal models, humoral immunity in brucellosis is primarily due to antibodies (Abs) which react with the O region of the lipopolysaccharide (LPS) [12,13]; however, this is not true in all individuals [14]. Other non-LPS immunogens, such as Brucella spp. outer membrane proteins (OMPs), are important because of their potential use as animal vaccine and diagnostic reagents [15-17]. OMP antigens are categorized according to their electrophoresed molecular weight: group 1 has a relative molecular weight of 88-94 kDa; group 2 antigens have approximately 33 to 43 kDa; and group 3 proteins approximate 25-31kDa [18-19].
The humoral immune response in patients with recent brucellosis with known clinical history is not well studied because of the difficulty of early diagnosis; in addition, there are relatively few documented studies on the immunology of acute human brucellosis. Moreover, there is a need to evaluate serological assays for the follow-up of specific antibody classes from the onset of disease, during therapy, and until cure. Therefore, we aimed to evaluate specific antibody class profiles elicited during the course of Brucella infection, pre- and post-infection, using our patient’s sera obtained at different time points during the course of disease. Another objective was to identify potential Brucella immunogenic fraction(s) which may be used to differentiate between the different stages of infection.

**Case Report**

One week post-return from a seven-day visit to a North African country, a healthy female laboratory worker complained of night sweats, headache, and back pain. The patient was presumptively diagnosed with brucellosis based on the clinical symptoms and laboratory serology. The treatment regimen for the patient consisted of a combination of 100 mg doxycycline and 300 mg rifampicin orally twice daily for a period of six weeks. Treatment started three days after symptom onset; blood culture was performed on blood samples collected on the same day. Using an oligonucleotide primer set that specifically detects B. melitensis, B. abortus, B. suis and B. ovis, PCR (AMOS-PCR) was performed to identify the bacterial strain to the species level [20].

Previous to travelling, the patient’s sera had been collected twice within a two-month period (one sample was collected 10 days prior to travel) and archived as required by the Bloodborne Pathogen Program Policy of the institution for which the patient worked. After illness onset, the archived pre-travel patient serum was screened for specific Brucella Abs and was found to be negative.

As this case provided an opportunity to investigate early responses of the human immune system to Brucella infection, blood samples (5-10 ml) were collected from the patient at the time of disease onset and the presumptive clinical diagnosis (designated as day 0) and at weekly intervals thereafter up to four months post-antimicrobial drug therapy. The following assays were performed.

Standard tube agglutination (STA) was performed using B. melitensis strain ATCC 23456 (SA Scientific, San Antonio, TX, USA) according to the manufacturer’s instructions. Briefly, the bacterial growth was incubated after mixing with a two-fold dilution of the patient’s serum in normal saline, beginning at 1:80 and ranging through to 1:5120 for 48 hours at 37°C.

Enzyme-linked immunosorbent assay (ELISA) was performed using commercially available B. melitensis and B. abortus (USSA 1119-3, SA Scientific, San Antonio, TX, USA). Whole cells were diluted 1:400 in 0.1M carbonate/bicarbonate buffer, pH 9.6 (coating buffer). ELISA was performed as previously described with minor modifications [22]. In this study, we also used the isolated Brucella melitensis strain (HAN-N3) for the capture of systemic-specific Abs. Prior to use, HAN-N3 whole cells were heat-killed at 65°C for 30 minutes. Polystyrene flat-bottomed microtiter plates (96 wells) were coated with 100 µl of 180 x 10^3/ml HAN-N3 whole cells (after heat inactivation) diluted in coating buffer. Microtiter plates were kept in a humid chamber at 4°C overnight followed by washing with PBS containing 0.05% Tween 20 (PBS-T). After blocking the coated wells with 0.1% bovine serum albumin (BSA) in coating buffer for 30 minutes, the patient’s sera, which was obtained at different time points during the study, were tested with two-fold dilution starting from 1:160 to 1:20840 (in duplicate). Positive and negative sera samples were also added in the same ELISA microtiter plate as controls. Plates were incubated at 37°C for 1 hour, in a humid chamber followed by washing with PBS-T. Horseradish peroxidase labeled rabbit anti-human IgA, IgM or IgA antibody (Jackson Immuno-Research Laboratories Inc, West Grove, PA, USA) were added to all wells. The ELISA reaction was developed by adding a chromogen (orthophenylene diamine), and stopped using 1 M sulphuric acid. Absorbance values were read at 492 nm using a Multiskan Ex (Labsystems, Franklin, MA, USA). ELISA-detected Ab titers of 1:640 (diagnostic titer) were considered positive if the optical density (OD) was equal to or greater than 0.3. The end-point titer was determined as the last serum dilution between 1:160 to 1:20,480 (two-fold dilution) with an absorbance value equal to or greater than 0.3.

Enzyme immunoblotting (EIB) was performed according to Kwaasi et al.[4]. Briefly, HAN-N3 Brucella antigen was prepared according to previously described methods [21] and electrically resolved using 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Fractionated protein bands were then electro-transferred to nitrocellulose (NC) membranes followed by blocking in 1% BSA/PBS-0.05 % Tween-
The NC were cut into 3 mm wide strips and allowed to react for 2 hours with pre- and post-patient’s sera collected at different time points during the course of the disease. Tested serum was optimally diluted at 1:1000 in PBS-T and at 1:20 for the detection of IgG or IgA and IgM, respectively. The strips were washed three times with PBS-T and the conjugate, horseradish peroxidase- anti-human IgA, IgM or IgG, was added and incubated for 1 hour. After washing the NC five times in PBS-T, the reaction was developed by adding 4-CN-peroxidase substrate. The reaction was stopped after optimum color developed (20 minutes).

Results

Laboratory investigations revealed that the Brucella STA Ab titer for our study case was 1:160 for sera samples collected on the day of symptom onset and 12 days after return from travelling (day 0) was reported as negative. Three days later (day 3), another serum specimen was tested and a two-fold increase in STA Ab titer was observed. The patient was diagnosed with brucellosis (1:320 is reported as a Brucella-positive result for endemic areas [23]). Drug therapy was implemented on day 3 based on the clinical examination and STA results. After 21 days’ incubation, the patient’s blood culture was positive. Gram-negative cocccobacilli were identified as B. melitensis using AMOS-PCR and the recovered isolate was denoted as HAN-N3.

Levels of systemic Abs against Brucella antigens during the course of disease were evaluated by ELISA using the patient’s sera obtained at different time points post-infection. ELISA was performed using commercially available B. melitensis and B. abortus whole organisms and levels of specific Ab titers against antigens from the two Brucella spp. were similar (data not shown). Therefore, we evaluated the specific Ab level against only B. melitensis antigens. At day 0 when the patient was clinically diagnosed and prior to STA results, a high level of systemic IgA-Ab was detected against commercial B. melitensis antigens (Figure 1A) along with lower levels of IgM than IgG Abs. By day 3 post-onset, nearly a three-fold
increase in serum specific IgA Ab titer was observed. Noteworthy, the IgM Ab response remained lower than the IgG Ab response. Overall, IgA Abs against both *B. melitensis* (Commercial and HAN-N3) isolates were highest at day 6 post-onset followed by a steady decrease until a negative value was reached at day 46. IgM Ab against commercial *B. melitensis* remained detectable at low levels until day 73. IgG Abs against both antigen types were maximally detected between days 6 to 18 but decreased by day 73, followed by a slight increase thereafter (Figures 1A, B and C).

On the other hand, when HAN-N3 *Brucella* isolate was used to capture Abs in the patient’s sera, the specific IgM Ab level was more prominently detected than against commercial *B. melitensis* antigen (Figure 1B). Overall, using the HAN-N3 isolate to capture specific Abs in tested sera obtained during the acute phase of disease allowed detection of the presence of both IgA and IgM Abs along with a lower level of IgG Ab. At day 32 post-therapy, the IgG Ab level was higher than those of IgA or IgM and maintained detectable levels until day 102, while IgA and IgM Abs returned to normal levels at day 49.

Correlation between specific Ab profiles and *Brucella* immunogenic bands revealed that the patient’s sera obtained at different time points, from pre- and post-infection until total remission, were allowed to react with NC strips containing HAN-N3 protein antigens. Immunoblot analysis revealed that the highest number and most intense immunological reactions occurred with bands recognized by IgA Ab and samples collected between days 6 and 18 (Figure 2A; lanes 4-6). Over the course of the disease, protein bands of 96, 72, 69.5, 60, 51, 48, 41, 38, 37, 35 kDa and 5 bands ranging between 34-30 kDa reacted with IgA. An apparent band of approximately 35 kDa persisted until day 46 but by day 73, starting from day 0 which is the collection day, no specific immunologically reactive bands were observed (Figure 2A; lanes 10 and 11, respectively). IgM Ab reactivity was intense against different antigenic bands from day 0 until day 39 (Figure 2B, lanes 3 and 9, respectively). IgM Ab reactivity was observed over several days against bands of 130, 112, 96, 70, 60, 50.5, 48, 41, 38, 35 kDa and 4 bands ranging between 34-30 kDa (Figure 2B). The least number of reactive protein bands was observed with the IgG Ab. The reactive protein bands of 96, 69, 52, 41, 38, 35, 30, 21.5 and 21 kDa (Figure 2C). The greatest intensity observed was from day 10 until day 105 (Figure 2C; lanes 5 and 14, respectively).

The overall reactivity of IgA, IgM and IgG Abs against fractionated HAN-N3 cytosolic protein fractions was concurrent with ELISA-detected serum Ab levels (Figure 2D). Fractionated cytosolic protein bands of approximately 112 and 130 kDa were recognized by only IgM Abs. This result suggests that these two protein immunogens might be useful for differentiating between acute and chronic disease phases. Protein bands of approximately 21.5 and 21 kDa appear to be recognized only by IgG Ab at different time points, specifically days 10, 18, 26, 32 and 105 (where the patient was recognized a being clinically cured). Interestingly, one protein band of approximately 96 kDa persisted during the course of infection including the period of antibiotic therapy and only disappeared at remission. This band was recognized by all antibody classes, IgA (day 0-18), IgM (days 0-46) and IgG (day18 until 105).

**Discussion**

In this report, we detail the history of a previously immunologically naïve case infected with *Brucella* while visiting a North African country. Given the unique circumstances of infection acquisition, we expanded the aims of the study to attempt to understand the humoral immune response, especially specific IgA Ab elicited against *Brucella* infection, at different time points through antimicrobial treatment using the patient’s sera obtained during the acute and remission stages [11], and tried to identify target antigens persisting at different stages of acute human brucellosis. Assessment of these two aims could lead to useful diagnostic tools for the early detection of *Brucella* infection.

Although identification of brucellosis by blood culture is considered the gold standard for laboratory confirmation of disease, antibiotic therapy with a combination of antimicrobial drugs, doxycycline and rifampicin, was started on day 3 of illness (after serodiagnosis) due to the delay in culture growth of up to 21 days. This is in agreement with other studies which mentioned that many cases are missed by culture due to the lack of sensitivity of the method [24], which ranges widely, for acute cases between 70 and 80% [25]; this figure is notably reduced for patients with long illness and focal complications of 30-50% [26].

In this case, blood culture yielded an organism which was subsequently identified as *B. melitensis* using AMOS-PCR. This result is in agreement with reports that *B. melitensis* is particularly common in the Mediterranean Sea countries [5,6,8,27]. *B. melitensis*
Figure 2A: Immunoblot analysis of specific IgA antibodies against cytosolic protein extracted from HAN-N3 at different time points during the course of infection

BIO-RAD prestained SDS-PAGE standards(161-0305)
Lanes 2 to 14 represent serum samples at the time withdrawn from the patient during the course of the disease
Lane 1: Blood sample was collected 17 days before disease onset and the presumptive clinical diagnosis as a baseline sample in Bloodborne Pathogen Program
Lane 2: Blood sample was collected on day of disease onset and the presumptive clinical diagnosis
Lanes 3-14: Blood samples were collected on days 3, 6, 10, 18, 26, 32, 39, 46, 73, 88, 102 and 105 respectively from day of disease onset and the presumptive clinical diagnosis

Figure 2B: Immunoblot analysis of specific IgM antibodies against cytosolic protein extracted from HAN-N3 at different time points during the course of infection

BIO-RAD prestained SDS-PAGE standards(161-0305)
Lanes 2 to 14 represent serum samples at the time withdrawn from the patient during the course of the disease
Lane 1: Blood sample was collected 17 days before disease onset and the presumptive clinical diagnosis as a baseline sample in Bloodborne Pathogen Program
Lane 2: Blood sample was collected on day of disease onset and the presumptive clinical diagnosis
Lanes 3-14: Blood samples were collected on days 3, 6, 10, 18, 26, 32, 39, 46, 73, 88, 102 and 105 respectively from day of disease onset and the presumptive clinical diagnosis
Figure 2C: Immunoblot analysis of specific IgG antibodies against cytosolic protein extracted from HAN-N3 at different time points during the course of infection

BIO-RAD prestained SDS-PAGE standards(161-0305), lanes: 1 to 14 represent serum samples at the time withdrawn from the patient before and during the course of the disease; days -17, 0, 3, 6, 10, 18, 26, 32, 39, 46, 73, 88, 102 and 105, respectively.

Figure 2E: Immunoblot analysis of specific antibodies against cytosolic protein extracted from HAN-N3 at different time points during the course of infection
biovar 1 is the most commonly isolated species from animals of the country visited by our case [28].

The start of laboratory investigations to determine the cause of our patient’s febrile illness was considered as day 0. Our investigations revealed that IgA Ab was the only antibody detected from the patient sera using an available commercial antigen (iter 1:5,000). Within one week, IgA Ab level increased more than 7.5-fold (1: 37,500) over the initial level before decreasing gradually to baseline values. By week 10, no IgA response could be detected. These results indicate that detection of IgA antibody in the absence of or with lower levels of IgG and even IgM might be useful for distinguishing acute from chronic Brucella infection. This assumption is supported by preliminary data of an ongoing study by the same authors that IgA is high in early brucellosis cases caused by B. abortus or B. melitensis (unpublished data).

In this study, there was no significant difference in the reactivity pattern of specific IgA and IgG Abs against antigens representing two different Brucella melitensis strains (Han_N3 and ATCC) at different time points post-infection using ELISA. However, anti-IgM Abs were more prominent against HAN-N3 than they were against the commercially available Brucella antigen in the early stage of the disease. This variation may be due to the differences in preparation methods between the two strains, which did not affect the presence of certain immunogenic epitopes located on the bacterial surface. Our results also suggest that specific IgM Abs are elicited early during Brucella infection as well as IgA, in particular when mucosal immunity is breached. The reactivity of IgA and IgM decreased gradually to baseline values until week 10 when reactivity completely disappeared. This result is in agreement with observations of Kwaasi’s group [4], who demonstrated that the reactivity of IgM and IgG decreased over time until disappearing by week 9. However, our data for IgG Ab level did not agree with that of Kwaasi et al.; the IgG titer against the Brucella strains used in our study remained detectable for a longer period. The pattern of immunoglobulin class responses has been shown to be different in patients with acute brucellosis than those with chronic brucellosis [29]. In this previous study, IgG, IgM and IgA were detected in patients with acute brucellosis while only IgG and IgA classes were observed in cases with chronic brucellosis.

The reactivity pattern of the immunoblotted Brucella protein bands against the three antibody classes, IgA, IgM and IgG, were concurrent with their ELISA results. We identified specific IgA, IgM and IgG Ab that reacted with 20 protein bands during treatment at different time points. These bands consisted of immunogenic bands of approximately 130, 112, 96, 72, 69.5, 60, 50.5, 48, 41, 38, 36.5, 35, 33, 31, 30, 21.5 and 21 kDa. Five of these identified bands were comparable with those reported elsewhere using the serum of an acute accidental nosocomial infected patient at day-14 post-infection [4]. In addition, four protein bands detected in our study appear to have similar Mr to 4/21 protein bands identified from the sera of 144 patients identified in another study [30].

The divergence observed between the different studies may be due to the use of different Brucella strains as well as different methods in the preparation of Brucella protein antigens as discussed above.

This is the first report identifying that IgM obtained from a patient with acute brucellosis that reacted solely with antigenic bands of high molecular weights of 130 and 112 kDa. This result indicates that these two proteins might be useful as immunological markers to differentiate between acute and chronic brucellosis cases. This finding is in contrast to reports by other studies that demonstrate the relevance of proteins of low molecular weights of 38 and 36 kDa (4) and 47, 41, 38 and 33 kDa [30]. In our study, a specific IgG Ab response reacting with two immunogenic bands with low molecular masses of 21.5 and 21 kDa appeared only with IgG during different time points (days 10, 18, 26, 32 and 105), persisting after treatment. While immunological detection of a 96-kDa band persisted from infection through remission, the 96-kDa band appeared on the day of symptom onset. It disappeared at day 18 and day 46 for IgA and IgM respectively, appearing as part of the IgG Ab response from day 18 until day 105.

Overall, we conclude from this report that specific IgA Ab levels may be useful as an early diagnostic marker for brucellosis, especially when the route of infection breaches the mucosal barrier. There is evidence to suggest that the use of heat-killed Brucella antigens in a serodiagnostic assay such as ELISA may be of value to detect specific IgM Abs early in the course of disease, leading to early implementation of specific antibiotic therapy. Further studies are needed to demonstrate that the use of IgM-reactive proteins of 130 and 112 kDa, as well as specific IgA antibody levels may be of value as early diagnostic markers for brucellosis.
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