Diagnosis of Human Brucellosis by Immunoblotting of Serum IgG Antibodies

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Abstract

Background: Brucellosis is a neglected zoonotic and contagious infectious disease that has major impact on livestock industry and human health worldwide. The humoral response to brucella infection is mediated by antibodies that were used to identify infection by various serological methods. However, the reactive polypeptides to brucella-specific antibodies were variable and no consensus was established to identify these polypeptides. This study was undertaken to identify the antigenic moieties exhibited by various forms of human brucellosis and their role in clinical diagnosis.

Objectives: To identify immunodominant and differential polypeptides of *B. melitensis* using antisera of patients with confirmed brucellosis due to *B. melitensis*.

Methods: In this study, 27 patients with different clinical forms of brucellosis were enrolled according to our selection criteria. Antisera were collected to evaluate an IgG antibody response by immunoblotting according to standard procedures.

Results: In all tested sera, a differential immunoblot patterns was obtained. Polypeptides of 102, 73, 66, 46, 43, 33, 31, and 27 kDa were dominantly recognized in sera of patients with acute brucellosis. In sera of patients with chronic persistent brucellosis, polypeptides of 81, 73, 66, 46, 43, 33, and 32 kDa were recognized. In contrast, sera of patients with chronic relapsing brucellosis had recognized limited polypeptides of 73, 66, 46, and 43 kDa. After patients' treatment and full recovery, most of the recognized polypeptides were resolved except for polypeptides of 43 and 66 kDa.

Conclusions: These results suggest that an IgG immunoblot patterns may be of great value in the differentiation between acute and chronic forms of human brucellosis. The reactive polypeptides may be of great value in the design of specific serological assay or future vaccine development.

Keywords: Brucellosis; Immunoblotting; Immunodominant Polypeptides

Introduction

Brucellosis is a contagious zoonotic disease that has major impact on livestock industry and human health systems worldwide [1]. The disease remains neglected in most developing countries especially in rural population [2]. In Saudi Arabia, the disease is prevalent in nomads' population and constitutes a major public health threat [3] to the healthcare system. The threat is accentuated by misdiagnosis due to the complexity of protean clinical manifestations [4] exhibited in patients with brucellosis and the resemblance of these signs to malaria, typhoid, and some venereal diseases [5]. In humans, transmission of brucellae is usually acquired through contact with infected animals, including handling of infected carcasses, aborted fetuses, or through ingestion of infected unpasteurized milk or milk products [6]. In rare events, occupational transmission might occur by inhalation of aerosol particles among laboratory workers [7].

The disease presentation varies between animals and humans with consequent economic losses and health debilitation. Human brucellosis is usually presented with undulant fever, osteoarthritis, spondylitis, fatigue, hepatosplenic abscess, and granuloma formation in parenchymatous organs especially the liver and the spleen [8,9]. In some cases, neurological involvement represented by acute meningitis has been encountered in < 5% of patients with brucellosis [10].

The humoral antibody response to brucella infection is well studied in laboratory animals [11,12] as well as in farm animals vaccinated with live attenuated *Brucella* strains [13]. Most of these studies had focused on the role of anti-brucella lipopolysaccharide antibodies in disease and immunity [14]. However, little is known about the role of other brucellar ligands in human infection. This is likely due to limitation of studies reported in human population. However, an early study by our group had documented the persistence of 18 and 43 kDa proteins throughout the course of *Brucella* infection, treatment and remission [15] in a single case of acute human brucellosis. Further, there is no established consensus on the number and size of polypeptides that were provoked at various forms of human infection with brucellae.

In addition to the humoral response, the interplay of cellular cytokines in disease pathogenesis is not well defined in humans infected with brucellae [16]. Most of the available data on cellular responses were derived from the murine model of infection. Several investigators have studied the role of cytokines and other regulatory molecules in the survival of brucellae inside murine macrophages (Mφ). Macrophage-derived cytokines such as IL-1, IL-12 and TNF-α contribute to the control of early *Brucella* infection via IFN-γ pathway [17,18]. Other regulatory molecules produced by Mφ such as oxygen intermediates and nitric oxide have been reported to control the growth of *B. abortus* [19] inside Mφ. Under this stressful environment, the turnover of degraded proteins would be repaired by heat shock proteins that maintain the dormancy of brucellae inside Mφ [20]. In addition to these factors, some intrinsic mediators inherent to brucella cells have been identified as a contributing factor for the survival of brucellae inside host cells. Among these factors lipoproteins [21] and cyclophilins [22] which were demonstrated to promote the survival of brucellae inside the host cells and to subvert the host immunological responses. Thus, brucellae are notorious pathogens that evade the host's cellular responses with consequent tissue retention and relapse of infection may ensue as shown in chronic brucellosis. However, the chronic stealth of brucella infection has been recently analyzed. A study of Durward-Diioia., *et al.* [23] reported that induction of chronic *B. melitensis* infection is caused by CD8+ T cell exhaustion, suppressed IFN-γ production, and delayed memory response. In support of this notion, Machelart., *et al.* [24] had demonstrated a drastic loss of CD169+ MMMs (marginal metallophilic macrophages) and CD209+ MZMs (marginal zone macrophages) in chronic cases of brucella infection. These changes contribute to the immunosuppression and the limited humoral antibody response encountered in chronic brucellosis.

The present study was undertaken to define the reactive polypeptides of *B. melitensis* that were provoked during the course of human infection and to examine their compatibility to differentiate between acute and chronic forms of human brucellosis at the clinical setting.

Material and Methods

Study population and blood specimens

Twenty-seven patients (ages: 28 - 72 years-old; sex: 15 males and 12 females) with symptoms of brucellosis were enrolled in this study according to our selection criteria as previously reported [25]. The patients were classified clinically into acute (7 cases), chronic persistent (10 cases), and chronic relapsing brucellosis (10 cases) as previously described [25]. In all patients, blood was collected twice at the time of disease diagnosis and after completion of treatment regimen. Prior to blood collection, a verbal consent was obtained from each patient as recommended by Hospital' Research Ethics Committee. Briefly, 6 ml of blood was collected from each patient and subsequently aliquoted into designated tubes for microbial culture and serum extraction. All patients were treated with anti-brucella drugs and observed until the resolution of infection and recovery [26].

Laboratory diagnosis of brucellosis

The criteria for the diagnosis of brucellosis were based on identifying one or more of the followings as previously described [15,25,27]: isolation of *Brucella* spp. from blood specimens using the standard BACTEC 9240 System; detection of brucella-specific IgM or IgG antibodies in sera by tube agglutination test with or without 2-ME; and detection of *Brucella* DNA in serum samples.

Reference strains and culture of *Brucella* cells

Reference strains of *Brucella melitensis* and *B. abortus* were provided by the USDA, National Animal Disease Center, Ames, IA, U.S.A. after a mutual agreement. At the USDA lab, *Brucella* cells were grown to logarithmic phase (OD_{600} = 2.8) in tryptic soy broth at 37ºC with vigorous shaking for 48h under biosafety level III environment. The dose count of each inoculum was assessed simultaneously on tryptic soy agar (TSA; FLUKA, U.S.A.) plates. Subsequently, the grown *Brucella* cells were collected by centrifugation at 12000 x rpm for 20 min and the resultant pellet was treated with 70% methanol for 16 hours to render it non-infectious.

Extraction of *Brucella* proteins and fractionation by SDS-PAGE

Brucella cells suspended in 70 % methanol were washed 3 times (3x) in PBS before being processed for protein extraction. Briefly, *Brucella* cells suspended in PBS were dually sonicated with a cell disrupter (Ultrasonics, Inc, Pittsford, NY, U.S.A.) and solubilized with a

detergent buffer for the isolation of mitochondrial, cytosolic and surface protein fractions. The detergent buffer contained 100 mM Tris-HCL, pH 8.0, 200 mM NaCl, 10 mM EDTA, disodium salt, 10% glycerol, 2% Triton X-100, 2% SDS, 1% Sarkosyl, 1 mM dithiothreitol, 5 μg/ml leukopeptin, and 1 μg/ml DNase. The cell lysate was centrifuged at 12,000 rpm for 10 min and the supernatant containing surface, cytosol, and mitochondrial proteins was collected. Subsequently, protein concentration was determined by Bradford method [28] and the protein was treated with 2x sample buffer for SDS-PAGE using Laemmli system [29] on slab gels of 12 % concentration.

Immunoblotting

Brucella polypeptides resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose (NC) membranes using Trans- Blot SD Semi-Dry electrophoretic transfer cell (Bio-Rad Laboratories, Inc, Hercules, CA, U.S.A.). The nitrocellulose blots were subsequently stained with Ponceau red, air- dried, cut into 5-mm strips, and processed for immunoblotting as previously described [15,30].

Results

Diagnosis of brucellosis

Baseline information for all 27 patients diagnosed with brucellosis is shown in table 1. In addition to the clinical symptoms, the diagnosis has been confirmed by microbial isolation, serology, and serum PCR analysis (Table 1). For microbial isolation, blood culture results were positive in 57 % (4/7) of patients with acute brucellosis, and in 22 % (6/27) of all patients (Table 1). No brucella cells were recovered from blood specimens of patients with chronic persistent brucellosis or from negative controls.

For serological analysis, results of STA test and ELISA were presented in table 1. Further, examination of these sera by PCR method had resulted in the amplification of a target sequence of 223 bp in *Brucella* DNA extracted from each serum specimen (Table 1). No target sequence has been detected in DNAs of negative control sera.

Patient no.	STA* titer	ELISA¶ titer	Serum PCR	*Brucella* isolated	Clinical diagnosis
1.	320	12, 500	+	+	Acute brucellosis
2.	160	2,500	+	-	Acute brucellosis
3.	640	12, 500	+	+	Acute brucellosis
4.	320	12, 500	+	+	Acute brucellosis
5.	160	12, 500	+	-	Acute brucellosis
6.	160	12,500	+	+	Acute brucellosis
7.	160	12, 500	+	-	Acute brucellosis
8.	640	62, 500	+	-	Chronic persistent
9.	640	12, 500	+	-	Chronic persistent
10.	320	12, 500	+	-	Chronic persistent
11.	640	12, 500	+	-	Chronic persistent
12.	320	62, 500	+	-	Chronic persistent
13.	320	62, 500	+	-	Chronic persistent
14.	2560	12, 500	+	-	Chronic persistent
15.	320	12, 500	+	-	Chronic persistent
16.	640	12, 500	+	-	Chronic persistent
17.	640	62, 500	+	-	Chronic persistent
18.	320	12, 500	+	+	Chronic relapsing
19.	1280	12, 500	+	+	Chronic relapsing
20.	160	12, 500	+	-	Chronic relapsing
21.	640	12, 500	+	-	Chronic relapsing
22.	160	12, 500	+	-	Chronic relapsing
23.	640	12, 500	+	-	Chronic relapsing
24.	320	12, 500	+	-	Chronic relapsing
25.	160	2, 500	+	-	Chronic relapsing
26.	320	12, 500	+	-	Chronic relapsing
27.	320	12, 500	+	-	Chronic relapsing

***Table 1:** Diagnostic profile of patients infected with Brucella melitensis.*

*: Standard tube agglutination. A titer ≥ 160 was considered positive for brucellosis.

¶: Enzyme linked immunosorbent assay. A titer ≥ 2,500 was positive for brucellosis.

Immunoblot analysis

Analysis of *Brucella*-positive sera by immunoblotting had shown an intense IgG reaction against a number of polypeptides after fractionation of *Soluble Brucella* proteins by SDS-PAGE (Figure 1). In sera of patients with acute brucellosis, polypeptides ranging in size from 18 to 120 kDa were recognized by immunoblotting (Figure 2). A strong serum IgG immunoblot response was seen against polypeptides of 73, 46, 43, and 33 kDa (Figure 2). In contrast, a faint immunoblot response was seen against polypeptides of 102, 66, 27, 22, and 18 kDa in sera of patients with acute brucellosis. The percent reactivity of anti-brucella antibodies in acute sera was 100% to polypeptides of 73, 46, 43 and 33 kDa (Table 2). Polypeptides of 66 kDa were recognized at a rate of 8 % in sera of patients with acute brucellosis. However, the specificity of these antibodies has been demonstrated by immunoadsorption studies. Incubation of *Brucella*-positive sera with whole *B. melitensis* 16M cells resulted in the elimination of reactive antibodies against detectable polypeptides by immunoblotting. The specificity was reconfirmed by treatment of the resolved polypeptides with normal human serum (NHS) or serum from sick control subjects. As expected, no band has been detected (Figure 2).

Figure 1: SDS-PAGE of solubilized brucella proteins extracted from various strains of Brucella melitensis and B. abortus. Proteins were fractionated on 12% polyacrylamide gel by SDS-PAGE. Most proteins were fractionated similarly among all brucella strains. The procedure was conducted as described in Materials and Methods.
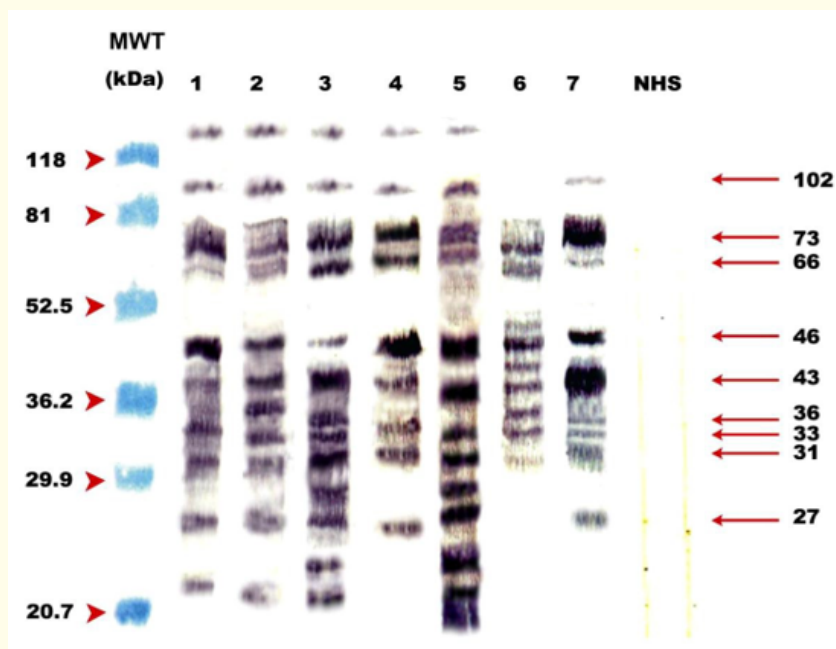

Figure 2: Immunoblot of B. melitensis solubilized proteins with 7 sera from patients with acute brucellosis (lanes 1 - 7) and normal human sera (NHS). The IgG response was monitored by probing the blots with HRP-labeled goat anti-human IgG antibody and developed using TMB kit. MWT = molecular weight marker in kilodalton.

On the other hand, immunoblotting of solubilized *Brucella* proteins with sera from patients with chronic persistent brucellosis showed a strong IgG reactivity to protein bands of 81, 73, 66, 46, 43, 33 and 32 kDa (Figure 3). The binding pattern of anti-brucella IgG antibodies was 100 % to protein bands of 66, 46, and 33 kDa. The percent reactivity of these antibodies to protein bands of 81, 73, and 43 kDa was found to be 50, 80, and 80 %, respectively, in sera of patients with chronic persistent brucellosis (Table 2).
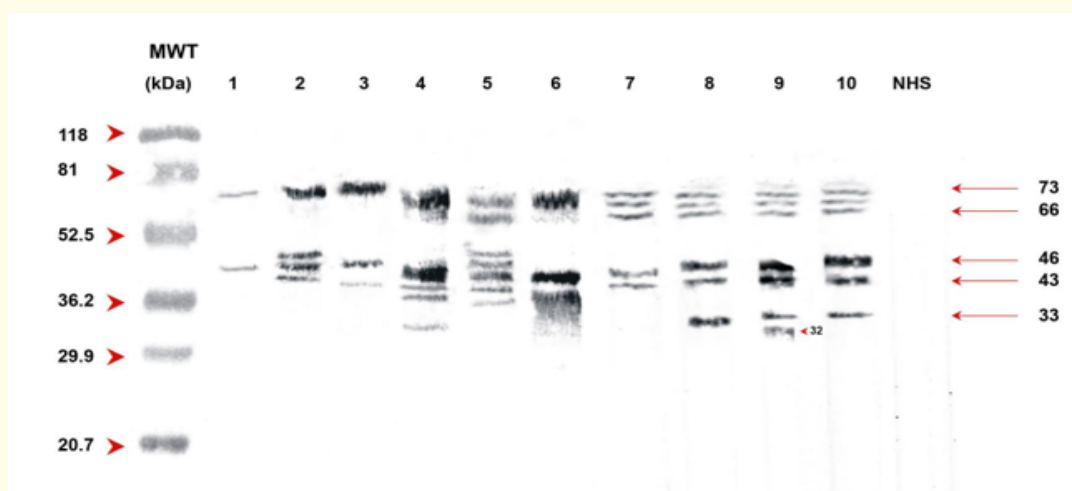
Figure 3: Immunoblot of B. melitensis solubilized proteins with 10 sera from patients with chronic persistent brucellosis (lanes 1 - 10) and normal human sera (NHS). The IgG response was monitored by probing the blots with HRP-labeled goat anti-human IgG antibody and developed using TMB kit. MWT = molecular weight marker in kilodalton (kDa).

In chronic relapsing brucellosis, however, a strong serum IgG immunoblot response was shown against protein bands of 73, 66, 46, 43 and 33 kDa (Figure 4). The percent reactivity of these antibodies was 100% to protein bands of 73 kDa and was 90 % to protein bands of 46 or 43 kDa (Table 2). Thus, in all patients with brucellosis polypeptides of 73, 66, 46, 43 and 33 kDa were predominantly recognized.
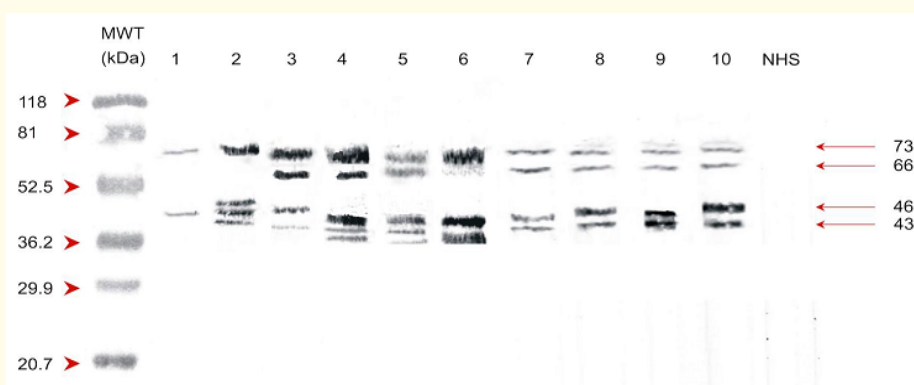

Figure 4: Immunoblot of B. melitensis solubilized proteins with 10 sera from patients with chronic relapsing brucellosis (lanes 1 - 10) and normal human sera (NHS). The IgG response was monitored by probing the blots with HRP-labeled goat anti-human IgG antibody and developed using TMB kit. MWT = molecular weight marker in kilodalton (kDa).

Study group	No. of test sera	No. of sera with reactivity to the following protein bands:									
		102	81	73	66	46	43	33	31	27	22 kDa
Acute brucellosis	7	6	2	7	6	7	7	7	4	5	2
Chronic persistent brucellosis	10	0	5	8	10	10	8	10	1	1	1
Chronic relapsing brucellosis	10	0	0	10	7	9	9	4	1	0	0
Control group	15	0	0	0	0	0	0	0	0	0	0
% Recognition of each band		22	26	93	85	96	89	78	22	22	11

Table 2: Immunoblot analysis of Brucella melitensis solubilized proteins with Brucella- positive sera from patients with known brucellosis.

In all treated and recovered patients, most of the recognized polypeptides were resolved except for polypeptides of 43 and 66 kDa (data not shown). These polypeptides were persistently maintained throughout the course of this study.

Discussion

This study has successfully delineated the reactive polypeptides of brucella proteomes during the course of human infection with *B. melitensis*. Our findings are unique in defining the association between reactive polypeptides of brucella proteomes and the clinical form

of brucellosis in patients spontaneously infected with *B. melitensis*. Serological survey by other investigators [14,31] had run short in setting a rigorous platform to delineate reactive polypeptides in all forms of human brucellosis. The latter is likely attributed to obstacles in recruiting patients for a cohort study and to the limited cases of patients who sought a follow-up and treatment regimen in this region.

Since humoral immunity is equally important for brucellosis [32], our immunoblot data had provided a strong evidence to delineate differences in the frequency of polypeptides recognized during acute phase compared to the chronic form of human brucellosis. On the basis of this study, recognition of small size polypeptides were dominated in acute phase of infection while large size polypeptides were commonly recognized in chronic brucella infection. The appearance of this pattern is likely attributed to an influx and interactions of several proteins [33] triggered during the course of bacteremia encountered in the acute phase compared to the chronic phase of infection. Therefore, in future studies, a complete large-scale immunoproteomics analysis should be conducted on patients' sera before the initiation of western blot experiments against *Brucella* antigens. Such an approach would be much informative to define a beacon of proteomes present in sera than current brucella immunoblot reports described in the literature [15,34,35]. In this study, serum immunoblot results showed polypeptides of 73, 66, 46, 43 and 33 kDa were constantly recognized by anti-brucellar IgG antibodies irrespective of the clinical form of brucellosis. This finding has a strong implication in the design of specific serological assay against human brucellosis. However, the persistence of 43 and 66 kDa after therapy is likely due to protein turnover [36] from cellular responses.

Although immunoblot data are always informative to confirm serodiagnosis, an enormous variations in serum immunoblot data have been reported in animal brucellosis [13,35]. It is not known whether the encountered variation is due to animal breeds, inoculum dose, or to the infectious conditions. In our experience, humans may exhibit similar variation in serum immunoblot responses especially among individuals recruited from endemic areas.

Limitations of the Study

Limitations of this study include the small sample size of enrolled patients; however, this is the largest sampling possible among those diagnosed with brucellosis according to our enrolled criteria. In addition, limited geographical location of the enrolled patients has hindered comparative analysis. Thus, future studies should include diverse regions for appropriate evaluation of brucellosis in Saudi Arabia.

Conclusion

The immunoblot patterns shown in this study may be of great value in the differentiation between acute and chronic forms of brucellosis in clinical setting. The consistent appearance of common polypeptides (73, 66, 46, 43, and 33 kDa) in all forms of human brucellosis indicates that these moieties may contain important epitopes that are useful in the design of specific serological assay or subunit vaccine against human brucellosis.

Acknowledgements

Competing Interests

The authors declare that they have neither competing nor conflict of interests.

Authors Contribution

AAA designed the study, performed the serological assays, wrote and reviewed the manuscript. IS performed the serological assays and reviewed the manuscript. AAA isolated the proteomes and reviewed the manuscript. AAH and TAA were our clinicians who recruited and treated the enrolled patients and reviewed the manuscript. MGE designed the study, run the immunoblot experiments and had reviewed, edited and submitted the manuscript.

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