

Correlation of antigenic expression with progress in antibiotic therapy of acute human brucellosis

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Human brucellosis is a zoonotic disease which is endemic in Saudi Arabia. The aim of this study was to investigate the humoral immune responses and identify the target antigens that persist at different stages in human brucellosis during antibiotic therapy. To do this, an acute case of accidental nosocomial infection was studied experimentally. Blood was collected from the patient at the time of diagnosis, and at weekly intervals during therapy until remission. IgG and IgM immunoblotting was used to characterize specific antigenic determinants, and ELISA antibody titration was performed to quantify the circulating antibodies. Results indicated that protein bands of 12–13.5 kDa bound IgG in the patient's sera but did not bind IgM on immunoblots and are probably not specific for, or important in, early stage infections. However, an 18 kDa band persisted during infection through remission. The pivotal and most important findings were that the number of protein bands seen on immunoblots, the magnitude of ELISA antibody titres and the concomitant changes in the intensity of the polypeptide bands of 42–43 kDa were positively correlated with the stage of infection. High numbers of anti-IgG and -IgM immunoblot bands coupled with high ELISA antibody titres and a concomitant increase in intensity of the 42–43 kDa bands were positively correlated with acute and severe infection. Conversely, a reduction in the number of polypeptide bands as well as a decrease in the intensity, until the complete disappearance of the 42–43 kDa bands, coupled with low (baseline) ELISA antibody titration values indicated successful treatment and remission. The routine use of the methods described here to ascertain the stage of the disease, assess the progress of antimicrobial therapy and monitor cases of relapse in human brucellosis is suggested.

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INTRODUCTION

Brucellosis is an important zoonotic disease. Four main *Brucella* species are capable of causing disease in humans but most human infections are caused by *Brucella melitensis* (Young, 1995). The disease is generally transmitted to humans by ingestion of contaminated dairy and meat products or by direct contact with infected animals. Occupational disease is common in animal herders, abattoir workers and veterinarians but a few cases of *Brucella* infections are acquired through accidental inhalation of contaminated aerosols during travel or in the laboratory (Young, 1995). If not detected and treated, human brucellosis can have deleterious and at times fatal sequelae.

The fact that the wealth of knowledge of *Brucella* pathogenicity was gained from studying animal models means that very little is known or documented for the aetio-immunology of

acute human brucellosis. *Brucella* species consist of pathotypes or biovars and it is important to find out the specific aggressins that play key roles in the infections caused by them. It is also useful to identify candidate proteins that are important in cellular immunity or serve as protective antigens during infection. Such antigens can be further investigated for use as diagnostic markers and probes, or for developing vaccine(s) for immunotherapy. Additionally and most importantly, our ability to identify *Brucella* antigens that are expressed at particular stages during human infections will contribute immensely to our knowledge of the pathogenesis of brucellosis (Oliveira *et al.*, 2002). Due to several reasons, including ethical constraints, it is difficult or impossible to experimentally follow and ascertain the stage of human *Brucella* infections from the onset through therapy until remission.

The aim of this study was to investigate the humoral immune responses and identify the target antigens that persist at different stages during antimicrobial therapy of acute human

Abbreviation: 2-ME, 2-mercaptoethanol.

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brucellosis. Results of antibody titration, ELISA and IgG and IgM immunoblotting from the time of diagnosis through antibiotic therapy to complete remission are presented and some clinical correlates are discussed.

METHODS

Case history. A healthy male research worker complained of feeling feverish but without pyrexia during late afternoon for 10 days. All vital signs were normal and there were no complaints of night sweats, headaches, arthralgia, anorexia, myalgia or back pain. General physical examination was unremarkable and there was no splenomegaly or hepatomegaly. The only positive finding in the patient's history before his symptoms appeared was that he had handled several biological samples, including human and animal sera, and had prepared protein antigens and isolated DNA from two *Brucella* cultures some 20–30 days before the onset of his symptoms.

Laboratory investigations and treatment regimen. Laboratory investigations, which were carried out with patient consent, included a liver function test, renal profile, blood culture and serology. Initially, blood was incubated at 37 °C in the automated Bact/Alert system using both aerobic and anaerobic culture media. Blood and serum samples were also taken at regular intervals for various tests until 21 days after antibiotic treatment ended. For serological tests, the tube agglutination and 2-mercaptoethanol (2-ME) agglutination assays were used to determine the presence of anti-*Brucella* antibodies.

For the identification of *Brucella* species the following biochemical tests were used: oxidase, urea, motility, nitrate, growth on blood agar and agglutination in *Brucella* antiserum. Culture plates were incubated at 37 °C and examined daily for the presence of viable colonies. Colonies that were positively identified from pure isolates as *Brucella* spp. were selected and subcultured. Presumptive and confirmatory identification of the bacteria as *B. melitensis* was carried out using standard methods (Corbel *et al.*, 1979; Chu & Weyant, 2003). Detailed biotype determination was not carried out but a monospecific agglutination test in *Brucella* antiserum was performed. Antibiotic sensitivity testing was done on pure cultures of *Brucella* using the E-Test to determine the MICs of the following antimicrobial agents for the bacteria: ciprofloxacin, imipenem, rifampicin, streptomycin, trimethoprim/sulfamethoxazole and tetracycline. Based on local knowledge, the patient was treated with a combination of 100 mg doxycycline and trimethoprim (160 mg)/sulfamethoxazole (800 mg) twice daily for a period of 6 weeks.

Preparation of *Brucella* antigens. *Brucella* antigens were prepared from pure cultures and used for immunoblotting, ELISA and antibody titration. Briefly, pure cultures of *Brucella*, which were isolated from the patient's blood, were harvested from chocolate or sheep blood agar and suspended (10^{11} organisms ml⁻¹) in PBS (Parsons *et al.*, 1986). The bacteria were washed with PBS and killed with 67% methanol, and the different *Brucella* antigens, namely surface washes, outer-membrane vesicles and cytosolic antigens, were prepared by methods previously described (Parsons *et al.*, 1986; Leith & Morse, 1980; Baldi *et al.*, 1999; Wanke *et al.*, 2002; Estein *et al.*, 2002).

Titration of *Brucella* antibodies. Titration of *Brucella* antibodies in human serum by ELISA was carried out by the indirect method of Voller *et al.* (1976). Briefly, 96-well polystyrene flat-bottomed microtitre ELISA plates were coated with dilutions (100 µl of 50 µg ml⁻¹) of cytosolic antigen in carbonate coating buffer. Plates were kept in a humid chamber at 4 °C overnight and then washed with PBS containing Tween 20. The wells were blocked with PBS containing 3% BSA and then filled with replicate doubling dilutions of the patient's sera collected 3 months before and at weekly intervals after infection. Similarly diluted pooled normal human serum was used as a negative

control. Plates were incubated in a humid chamber at room temperature for 1 h, washed and 100 µl of suitably diluted alkaline phosphatase labelled goat anti-human polyvalent antibody was added to the wells. After further washing, 100 µl *p*-nitrophenol phosphate (1 mg ml⁻¹) in 1 M diethanolamine buffer (pH 9.8) was added to all wells. The reaction was stopped after 20 min by adding 3 M NaOH and the plates were read at 405 nm. The highest dilution that gave a positive result (titre) for each serum sample was recorded and the data were plotted as a histogram.

SDS-PAGE and immunoblotting. SDS-PAGE was performed using the discontinuous method of Laemmli (1970), and immunoblotting experiments were performed with cytosolic *Brucella* antigen as previously reported (Parsons *et al.*, 1986; Towbin *et al.*, 1979). Cytosolic antigen (50 µg ml⁻¹) was electrophoresed using 13% separating and 5% stacking gels (Parsons *et al.*, 1986; Towbin *et al.*, 1979). After electrophoresis the gel was electro-transferred onto nitrocellulose membranes. The membranes were cut into strips (5 mm wide) and these were individually reacted with predetermined dilutions of the patient's pre-infection serum and sera collected at weekly intervals during infection until total remission. The strips were washed after 2 h incubation and probed with alkaline phosphatase conjugated anti-human IgG or IgM for 1 h. After thorough washing, the nitrocellulose strips were reacted with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. The reaction was stopped after optimum colour developed, and the number of immunostaining bands was counted for each strip. The intensity of the bands was noted and the strips were photographed and kept as a permanent record.

RESULTS AND DISCUSSION

Cultures were positive after 4 days and Gram-negative coccobacilli were isolated and identified as *B. melitensis*. The isolate was given the reference number KFSHRC 03-077-02372 and added to the King Faisal Specialist Hospital and Research Centre collection for later studies. Even though detailed biotype determination was not carried out, the isolate was positive in an agglutination test with *Brucella* antiserum, meaning that it belonged to either *B. melitensis* biovar 1 or 3. This information is in agreement with a previous report about biovars that occur in Saudi Arabia (Corbel, 1991).

Results of the first set of titres of *Brucella* tube agglutination and 2-ME titres indicated the presence of infection. These titres rose from relatively high initial values of 1:10 242 and 1:640 to even higher titres of 1:20 480 and 1:1280, respectively, within the first 14 days of diagnosis of the disease and the initiation of antibiotic therapy. Physical examination and detailed haematological findings indicated that the patient did not have any complications of brucellosis. The antibiotic sensitivity testing that was carried out on different isolates from the patient gave the same 'antibiogram' with the following MIC values: trimethoprim/sulfamethoxazole 0.125 µg ml⁻¹; rifampicin 1.5 µg ml⁻¹; streptomycin 0.5 µg ml⁻¹; tetracycline 0.094 µg ml⁻¹; imipenem 0.75 µg ml⁻¹; and ciprofloxacin 0.25 µg ml⁻¹. This indicated that the isolate was resistant to rifampicin.

Results of the antibody titration by ELISA are shown as a histogram in Fig. 1. These results revealed a trend, which is similar to that observed for the tube agglutination and 2-ME titres, the only difference being that ELISA antibody titration

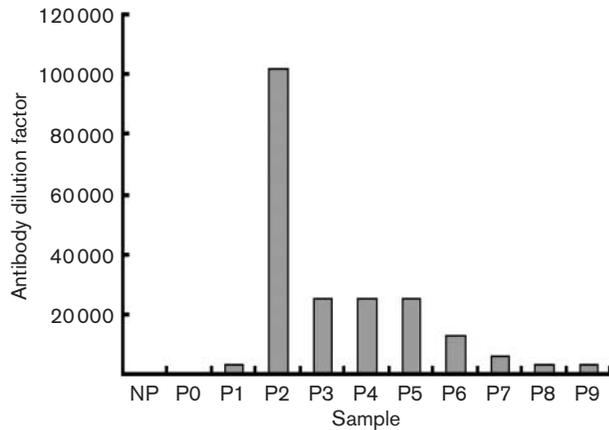


Fig. 1. Histogram of antibody titrations of the patient's sera before infection (P0) and for each of 9 subsequent weeks (P1–P9). NP is the result from a pool of normal human sera. Replicate doubling dilutions of each serum were made and indirect ELISA was used to determine the highest dilutions at which antibody activity could be measured.

values reduced to pre-infection levels whilst *Brucella* agglutination titres did not. The initial ELISA antibody titration value on the day that the diagnosis was made and treatment commenced was 1 : 3200 (barely visible in Fig. 1). This value rose to over 30-fold (1 : 102 400) within a week before falling gradually after 8 weeks to baseline values.

SDS-PAGE of Coomassie brilliant blue stained molecular mass markers, *Brucella* polypeptides (i), and their corresponding IgM (ii) and IgG (iii) immunoblots are presented in Fig. 2(a). The number of immunoreactive bands in these samples on the days that they were taken is plotted as a histogram in Fig. 2(b). The most intense staining, and the highest number of reactive bands, putatively the most important polypeptides in acute human infections caused by *B. melitensis*, were seen within the first 14 days of the onset/diagnosis of the infection (Fig. 2a, lanes 4; Fig. 2b, day 14). In the first week of diagnosis (lanes 3), protein bands of 12–13.5, 18–20, 26, 33.5, 37.2, 41, 42–43, 88.5 and >100 kDa were seen. During the ensuing week, as many as 24 immunoblot bands could be counted (lanes 4). These immunostaining bands were of 12, 13.5, 18, 24, 25.5, 27, 28, 29, 30, 31.5, 34, 37, 38, 42, 43, 49, 51, 58, 62, 67, 76, 86, 95 and >100 kDa. Their IgM (middle panel, ii) and IgG (right panel, iii) antigen spectra demonstrated clearly that the most predominant antigens at the initial stages of human brucellosis were those of 12–13.5, 18, 20, 30, 31.5, 36–38 and 42–43 kDa. After 42 days of treatment the intensities of most of the bands diminished and most of them disappeared.

However, the 42–43 kDa (IgM and IgG) immunoblot bands persisted with diminishing but discernible degrees of intensity throughout the infection and treatment but completely disappeared by week 9. The decrease in their intensity was progressive and positively correlated with decreases in antibody titres and the success of antibiotic therapy as determined by serology and blood cultures. It is clear that

three distinct polypeptide bands of apparent molecular masses 12–13.5, 18 and 42–43 kDa were constantly expressed in sera collected at all stages of infection (lanes 3–10 in panels (ii) and (iii), as denoted by arrows labelled x, y, z on the right). However, the 12–13.5 kDa bands (labelled z) were absent from the IgM immunoblots (Fig. 2a, ii) and the 42–43 kDa band had disappeared by day 63 (lanes 11).

It is widely accepted that the most common presenting symptoms in human brucellosis are arthralgia, fever, night sweats and musculoskeletal complaints. Interestingly, the only symptom that the patient in this report had that was close to or mimicked a brucellosis-like infection was his main complaint of feeling feverish during late afternoons but in this instance there was no pyrexia. Even though the cultures were positive within 4 days and his *Brucella* antibody titres (*Brucella* tube agglutination, 2-ME and antibody titration by ELISA) were extremely high, there were no obvious abnormalities. The elusive attribute of *Brucella* species and the immunocompetence of the patient were demonstrated.

It is known that there is a strong positive association of a high antibody titre with a positive blood culture, and together these are also predictive of a serious *Brucella* infection (Benjamin & Annobil, 1992). Consistent with this notion, the patient's blood culture was positive and this was associated with high antibody titres.

In this study, crude cytoplasmic proteins prepared from sonicated whole killed cells from the *B. melitensis* isolate that caused the infection were used. The decision to use sonicated whole cytoplasmic proteins was based on the results of pilot studies in which proteins from bacterial surface washes, outer-membrane vesicles and whole cell sonicates were compared. SDS-PAGE of these three antigen preparations (results not shown) indicated that bacterial extracts prepared by sonication contained a complete mixture of all the protein bands and some contaminating LPS when compared to surface washes and outer-membrane preparations. The use of such a preparation in the serodiagnosis of human systemic disease as has been demonstrated here has been reported to have the added advantage that it can also be used for the serodiagnosis of human neurobrucellosis, which is an otherwise difficult condition to diagnose (Baldi *et al.*, 1999).

Since the main aim of this study was to find out which antigens are important in human *Brucella* infections so as to be able to assess the stage of the disease and follow the success of antimicrobial therapy, immunoblotting was used to identify these antigens. Apart from the initial surge in antigenic expression, it was clear during antibiotic treatment that the patient's *Brucella* tube agglutination, 2-ME and ELISA titres started reducing, and so did the number and intensities of various immunoreactive protein bands. This correlation was true for both IgM and IgG immunoblot spectra. An IgG immunostaining band of 12–13.5 kDa was present in all serum samples, including the pooled human serum, but was absent in the patient's pre-infection serum. The slight presence of this band in pooled normal human serum from the local population probably means

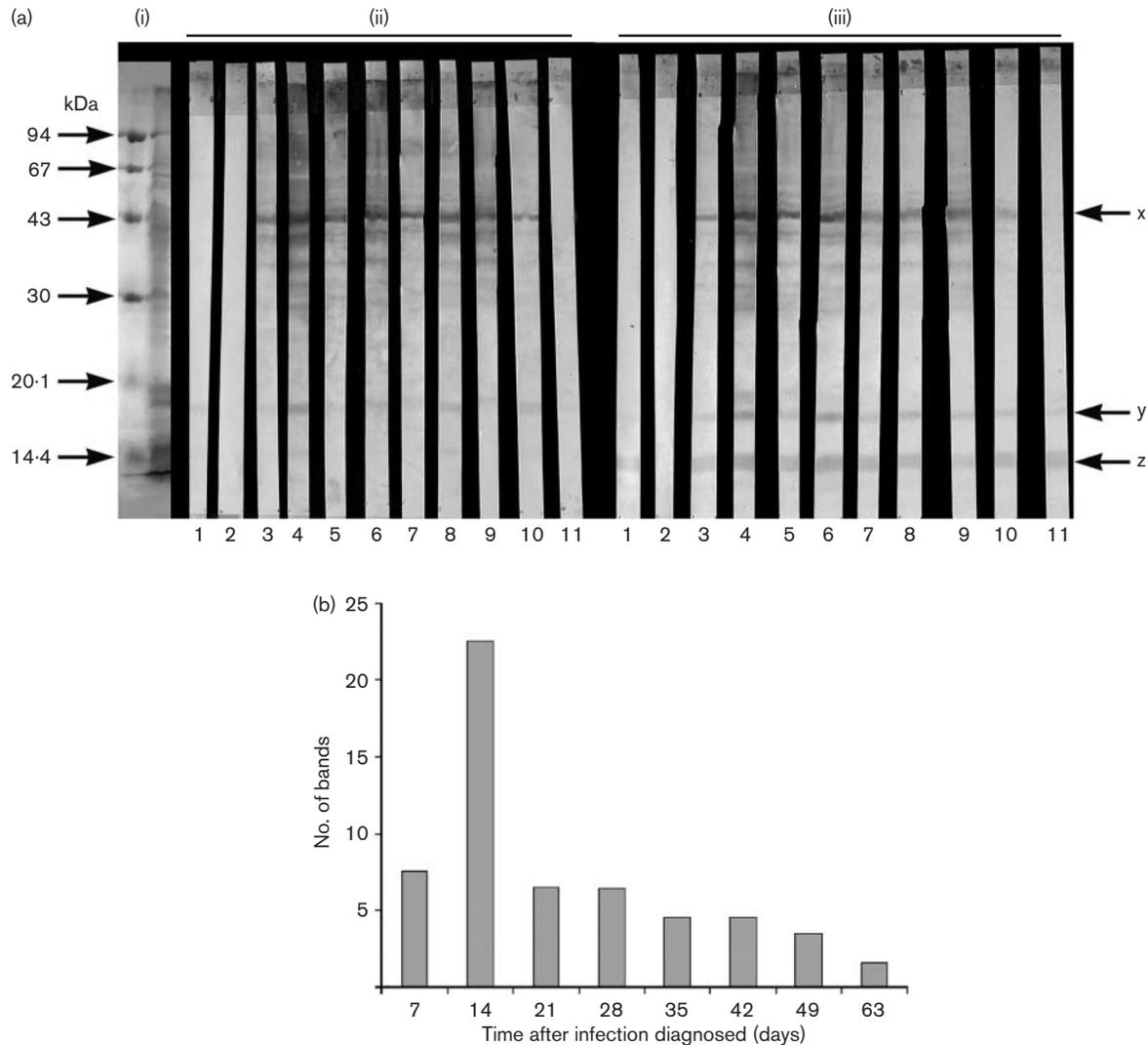


Fig. 2. (a) Antigen spectra in human brucellosis. SDS-PAGE of Coomassie brilliant blue stained molecular mass markers (i) and *Brucella* polypeptides (ii, iii). Arrows in the left margin are Pharmacia low molecular mass markers: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa). Immunoblots of SDS-PAGE separated *Brucella* proteins were transferred onto nitrocellulose membranes. Strips were individually reacted with predetermined dilutions of each of the different sera. IgM (ii) and IgG (iii) immunoreactive bands are shown. Lanes: 1, pooled normal human serum; 2, patient's pre-infection serum; 3–11 sera taken from the patient for 9 weeks from the day that the infection was diagnosed. Note that three of the most recurring common bands have been labelled x, y and z in the right margin. z is absent on the IgM blots. (b) Histogram showing the number of bands counted per strip in (a).

that the band indicates exposure to but not necessarily infection with *Brucella*. Chirhart-Gilleland *et al.* (1998) studied a *Brucella* protein of similar molecular mass (14 kDa) from *Brucella abortus* for its reactivity in naturally infected animals and its T-cell reactivity but were unable to establish its protective capability. It is noteworthy that this 12–13.5 kDa band did not have an equivalent band on the IgM blots, indicating that it is probably not always pathognomonic.

In a study of 144 patients and 62 healthy humans, Leiva *et al.* (1990) identified 21 protein bands that were hetero-

geneous between different patients. In the present study, 24 protein bands were identified in one patient's serum at the peak of infection (day 14) and in comparison most of these bands were similar to those reported by other investigators (Zygmunt *et al.*, 1992; Stabel *et al.*, 1990; Matar *et al.*, 1996), including several of the 21 bands reported by Leiva *et al.* (1990). Leiva *et al.* (1990) reported that nine of these bands were the 'most significant' based on the frequency at which they occurred in different patients. Out of the nine protein bands that they identified, only two bands of 41 and 38 kDa were similar to those identified in the present study of a single patient. The complexity of comparing sera

from heterogeneous sources was evident in their observations, hence emphasizing the need to study single cases of infection as well as large samples.

In the results presented here, a band of 18 kDa was persistent irrespective of the stage of infection and even after remission. A band of similar size has been identified and investigated over several years for its suitability as diagnostic antigen. Goldbaum *et al.* (1999) characterized and described an 18 kDa protein (lumazine) to be of diagnostic value in brucellosis. This protein has been investigated and confirmed to be highly immunogenic and potentially useful for the development of a vaccine for human and animal brucellosis (Goldbaum *et al.*, 1991, 1993, 1999).

Several antigen bands were seen at the initial stages of the infection. This observation is consistent with a well-known microbial pathogenicity phenomenon: once microbial invaders gain entry into the host, the host begins to respond to eliminate the invaders (Smith, 1999). Successful intracellular pathogenic bacteria like *Neisseria* and *Brucella* lodge an 'armoury' to minimize the effectiveness of host defences (Smith, 1999). This is usually accomplished by producing bacterial products, which have been variously referred to as impedins (Glynn, 1972), auxiliary pathogenic factors or aggressins (Smith, 1999), and they enable the survival of these successful pathogens within their hosts (Smith, 1999). It appears that in this instance the armoury of the bacterium was minimized, probably due the host's efficient immune responses, and the expression of these factors started diminishing as antibiotic therapy progressed.

The fact that the 42–43 kDa protein is probably the most important marker for the determination of the presence or absence of infection is exemplified by several references made to this protein by other investigators. Denoel *et al.* (1997) studied proteins in Brucellergene, a commercial delayed-type hypersensitivity allergen from the *B. melitensis* strain B1115, for their ability to induce T-cell proliferation and IFN γ production in peripheral blood. Interestingly, four out of the five proteins that are considered to be the most pathognomic in the present study (namely the 18, 20, 36–38 and 42–43 kDa proteins) also belong to this group of T-cell active proteins. Furthermore, due to the specificity of the 43 kDa polypeptide, a commercially available PCR kit has been developed for routine diagnosis of brucellosis, based on the amplification of a 635 bp fragment of the gene coding for the 43 kDa outer-membrane protein from *B. abortus* strain 19 (Fekete *et al.*, 1990).

These facts clearly demonstrate that this 42–43 kDa protein has already proved useful for its diagnostic value (Denoel *et al.*, 1997; Fekete *et al.*, 1990; Queipo-Ortuno *et al.*, 1997). The present findings not only confirm these published facts, but emphasize in addition that the protein is a useful marker for staging *Brucella* infections as demonstrated in immunoblots here.

Many investigations have been carried out using different techniques and associating an array of proteins with *Brucella*

infections (Oliveira *et al.*, 2002; Wanke *et al.*, 2002; Estein *et al.*, 2002; Fekete *et al.*, 1990; Queipo-Ortuno *et al.*, 1997; Ebani *et al.*, 2000, 2003), but most of these studies have been conducted either on animals or in a variety of infected humans. For human studies the heterogeneity of the selection of patients often makes it difficult to accurately define the temporal relationships between the different proteins produced and the stage of infection.

The present report, although a study of only one patient, is a very important addition to our knowledge of human brucellosis because the infection was diagnosed early and carefully followed until remission, making it possible to accurately trace the course of infection. In agreement with other studies, the relevance of the proteins of 36–38 kDa to acute infections was also clearly demonstrated. About 24 different protein bands were seen on immunoblots during the acute phase (day 14) of infection and most of these bands were similar to several bands that have been described by other investigators (Oliveira *et al.*, 2002; Wanke *et al.*, 2002; Estein *et al.*, 2002; Fekete *et al.*, 1990; Queipo-Ortuno *et al.*, 1997; Ebani *et al.*, 2000, 2003; Debbarh *et al.* 1995; Cloeckart *et al.*, 1995). The most immunodominant antigens reported in the present study were mainly polypeptides of apparent molecular masses of 12–13.5, 18, 25–29, 30–34, 36–38 and 42–43 kDa.

It can be concluded that the presence of higher numbers of IgG and IgM immunoblot protein bands coupled with high ELISA antibody titres and a concomitant increase in intensity of the 42–43 kDa bands, were positively correlated with the severity of disease. Conversely, a reduction in the number of these bands as well as a progressive decrease in the intensity, until the complete disappearance of the 42–43 kDa bands coupled with low (baseline) ELISA antibody titration values indicate the success of therapy up to complete remission of the infection. In addition to the existing standard methods, the routine use of the methods described in this report in following disease aetiology, to determine the effectiveness of antimicrobial therapy and to define total remission of human brucellosis is indicated.

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