

Molecular Detection of *Brucella* spp. DNA in Patients with Manifestations Compatible with Emotional Disorders

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Abstract: *Objectives:* Human brucellosis is a common zoonosis in Greece and an important Public Health issue. Its true incidence is likely to be higher than reported due to underreporting and misdiagnosing of chronic forms which can manifest with depression and other neuropsychiatric symptoms. The aim of this study was to investigate the possible presence of *Brucella* DNA in the blood of patients with emotional disorders who did not have acute brucellosis or a diagnosis of chronic brucellosis.

Methods: From March 2009 to May 2009, inhabitants of a Greek region endemic for brucellosis were examined and grouped as “patients” and “controls” respectively on the basis of the presence or absence of a diagnosis of emotional disorder. A total of 78 whole blood samples were obtained and analyzed by Polymerase Chain Reaction using specific primers.

Results: *Brucella* DNA was detected in 3 (15%) out of 20 patients with emotional disorders, whereas all controls were found to be negative.

Conclusions: The appearance of psychiatric – mainly depressive – symptoms in inhabitants of brucellosis endemic areas should raise a suspicion of chronic brucellosis.

Keywords: Chronic brucellosis, psychiatric disorders, *Brucella* DNA.

INTRODUCTION

Human brucellosis is a zoonosis with a worldwide distribution except in those regions where *Brucella abortus* has been eradicated [1]. The disease remains endemic in the Mediterranean basin, the Middle East, Mexico, Central and South America [2]. In these areas brucellosis represents a significant public health issue and its incidence might reach more than 200 cases per 100 000 population [3]. Due to misdiagnosis and underreporting though, its true incidence remains unknown and might extend to 25 times higher than the official one [4].

In Greece, human brucellosis is endemic and constitutes a serious public health problem especially in rural areas, where animal breeding is a major economic resource [5]. *B. melitensis* has been isolated in the majority of the cases and this reflects the high prevalence of *B. melitensis* infection in sheep and goats [6].

Brucellosis is known for its protean clinical manifestations involving almost all organ systems, which complicate the clinical diagnosis [7]. The diagnostic procedure is difficult when based on clinical data alone, which may be misleading especially in localized, subacute or chronic infections [3]. In endemic areas in particular,

individuals with a high risk of occupational exposure to *Brucella* often present with mild and nonspecific symptoms [6], which may delay or mislead the diagnosis. The innate tendency of the disease towards chronicity is further enhanced by this delay; therefore detailed history and special attention to epidemiological information are considered crucial [8].

The neuropsychiatric manifestations of the disease are reported as part of acute and even more of chronic brucellosis [9, 10]. The clinical spectrum is diverse and includes neuropsychiatric symptoms most of which are compatible with the clinical picture of emotional disorders [10-12]. According to quite recent reports, neurological involvement seems to be more common than previously assumed, may develop at any stage of the disease and can be associated with cognitive and emotional disturbances, which may predominate [13]. The mechanisms implicated in the pathogenesis of the emotional complications of brucellosis, although not fully elucidated yet, include a hypersensitivity reaction to *Brucella* antigens, necrosis of neurons, chronic vasculitis, multiple non caseating granulomas and calcification of the basal ganglia [11, 14-17]. The burden of chronic complaints in patients with chronic brucellosis might also contribute to the appearance of neuropsychiatric symptoms [18, 19]. Finally, brucellosis might trigger [20] or exacerbate [21] neuropsychiatric symptoms in patients with neurasthenic tendencies.

There have been some old studies regarding the emotional disturbances in brucellosis, especially in its

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chronic form [9, 12, 13, 19, 20, 22-25]. Most of these studies examined individuals who were being hospitalized with confirmed or suspected brucellosis and not individuals from the general population without suspicion of the disease. Furthermore, the methods used in those studies were the conventional microbiologic techniques, which have serious limitations in setting diagnosis, especially in the localized and chronic forms of the disease [26]. To the best of our knowledge there have not been any relevant studies using polymerase chain reaction (PCR), which has very high sensitivity and specificity in the diagnosis of all forms of brucellosis in respect of the classical methods [27-32].

The aim of this study was to investigate the possible presence of *Brucella* specific DNA in the blood of inhabitants of a brucellosis endemic area in Greece who had been diagnosed with an emotional disorder or related symptoms, without a suspicion of acute or chronic brucellosis. For this purpose, PCR assays that have recently been successfully applied to the diagnosis of all forms of human brucellosis were used [33].

MATERIALS AND METHODS

Study Population

During the period from March 2009 to May 2009, nine agricultural communities of a region in Central Greece endemic for brucellosis were approached on a weekly basis for the purpose of the present study. The communities were chosen on the basis of lifestyle (occupational exposure to animals, close contact with animals, consumption of raw milk and fresh cheese etc) and therefore had a likely exposure to the causative agent of brucellosis. A total of 78 subjects from the above communities accepted to be involved in the study. None of them had acute brucellosis at the time of the study or had received a diagnosis of chronic brucellosis nor were they being investigated for any suspicion of the disease.

Demographic data, detailed medical histories as well as data from personal medical records were collected and recorded on individual forms. On the basis of their present documented symptomatic status and history the individuals were allocated into three groups.

Group A included twenty (20) individuals with psychiatric manifestations compatible with an emotional illness. Five of them had a history of acute brucellosis, which was considered to be successfully treated 18 to 6 years previously and prior to the development of any psychiatric disorder.

Group B (positive controls) included sixteen (16) individuals without any neuropsychiatric condition, with a past history of acute brucellosis who had subsequently been considered cured.

Group C (negative controls) included forty-two (42) individuals without any history of neither neuropsychiatric disorders nor brucellosis.

Clinical Specimens

A total of 78 peripheral whole blood samples were collected in EDTA containing vials and maintained at -28°C until processing.

DNA Extraction

For DNA extraction a commercial purification system with columns (QIAmp Blood Midi; QIAGEN GmbH, Hilden, Germany) was used according to the manufacturer's instructions.

DNA Amplification

Once the DNA was extracted the amplification process was performed for the detection of *Brucella* DNA. Two different PCR assays, targeting different gene regions of *Brucella* spp. and able to detect *B. melitensis* (the dominant aetiologic agent of brucellosis in Greece), were applied. These assays have recently been evaluated for the diagnosis of acute, chronic, relapsing and focal brucellosis [33] and were applied in this study with slight modifications.

PCR (I) consisted of the amplification of a 223 bp fragment of the gene BCSP31 encoding an immunogenic membrane protein of molecular weight of 31 kDa. This protein is specific for the *Brucella* genus and is conserved in all *Brucella* species. The specific primers used in this amplification process were the B4 and B5 primers. PCR (I) was performed in a total volume of 50 μl , containing 5 μl template DNA, PCR buffer 1x, 2.5 mM MgCl_2 , 200 μM of each deoxyribonucleotide triphosphate (dNTP) (Promega, Madison Wis., U.S.A.), 1.0 μM of each primer and 2 IU of Taq DNA Polymerase (Promega, Madison Wis., U.S.A.). The PCR consisted of an initial 5 min incubation step at 93°C followed by 40 repeated cycles of DNA denaturation at 90°C for 1 min, primer annealing at 60°C for 1 min, extension at 72°C for 1 min, and final incubation at 72°C for 10 min.

PCR (II) consisted of the amplification of a 193 bp fragment of the gene *omp2*, which encodes an outer membrane protein of 36 kDa of *B. abortus* that again is present in all *Brucella* species. The specific primers used were JPF and JFR. PCR (II) was performed in a total volume of 50 μl containing 5 μl template DNA, PCR buffer 1x, 2.5 mM MgCl_2 , 200 μM of each dNTP (Promega, Madison Wis., U.S.A.), 1.0 μM of each primer and 2 IU of Taq DNA Polymerase (Promega, Madison Wis., U.S.A.). The amplification program consisted of an initial 4 min incubation step at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, extension at 72°C for 1 min, and final incubation at 72°C for 10 min.

All specific primers for the PCR assays were supplied by Invitrogen Ltd, Paisley, U.K. and all reactions were performed in a programmable thermocycler (Gene Amp PCR system 9700 Applied Biosystems, Roche Diagnostics).

To guarantee the reliability of the results all positive samples were processed more than once with both PCR assays. In order to detect the presence of any possible PCR inhibitors, certain samples were processed and paired with a positive control sample. All tests included positive controls, which consisted of DNA samples extracted from reference *B. abortus* strains (*B. abortus* biovar 1 strain 544) supplied by the Central Veterinary Laboratory in Weybridge Surrey U.K. as well as negative controls, containing all elements of the reaction mixture except the template DNA.

The amplified products were detected by fluorescence in the presence of 1 µg/ml ethidium bromide after 2% agarose gel electrophoresis and the DNA bands were photographed by Polaroid. A sample was considered positive when DNA with the molecular weight expected for the amplified product was seen.

Sequencing

The identity of the amplicons was further confirmed by sequencing performed by Lark Technologies U.K. The nucleotide sequences of the PCR products were analyzed using Blast software.

Statistical Analysis

The statistical analysis of the results was performed using the statistical software SPSS student version 15.0. Multiple comparisons were conducted with the Pearson chi-square as well as Fisher's exact test when necessary. The level of statistical significance was set to 5% for all the comparisons made.

RESULTS

Of the total 78 whole blood samples processed with PCR three (3) were found to be positive for specific *Brucella* DNA (Fig. 1). The nucleotide sequence of the positive PCR products was determined and showed 99.9% DNA homology to *B. melitensis*.

All three positive samples belonged to individuals from group A (patients' group) and represented 15% of the group. On the other hand, there were no positive PCR results in any of the samples from the other two groups (group B – positive brucellosis control group, group C – negative brucellosis control group). The PCR results as well as the characteristics of the 3 groups examined are shown in Table 1.

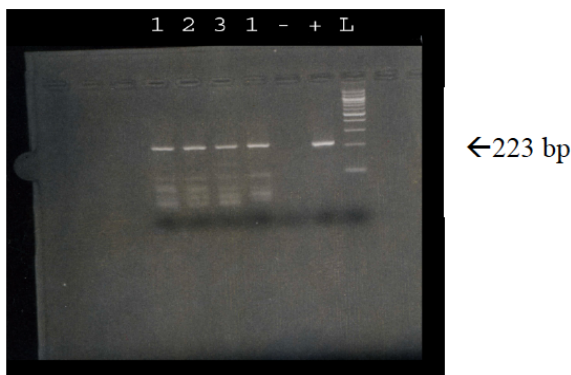


Fig. (1). Agarose gel electrophoresis of PCR (I) products. 1,2,3: positive samples (PCR (I) products derived from patient samples). -: negative control (no DNA added). +: positive control (*B. abortus* biovar 1 strain 544). L: DNA ladder (100 bp).

Of the three positive PCR samples, two belonged to individuals without any brucellosis history and one to an individual with previous *Brucella* infection including one relapse at six months after diagnosis. The PCR results as well as data concerning the brucellosis history of group A individuals are presented in Table 2.

The emotional disturbances of the subjects with psychiatric manifestations who are referred as group A included depression, sleep disturbances, agitation, irritability, anxiety, appetite disturbances and fatigue. The predominant neuropsychiatric manifestations of the three subjects with positive PCR outcome are summarized in Table 3. Two of them were diagnosed with depression while the third one was diagnosed as suffering from significant clinical symptoms without meeting the criteria for a specific Mood Disorder.

Overall, 73% of the 78 subjects investigated had no evident history of past or present brucellosis and 27% of them had a positive history of confirmed past infection. Of the 78 individuals included in the study, 4% were found to be positive for *Brucella* specific DNA. The epidemiological data of the study population are presented in Table 4, while the sex composition of the study groups is shown in Table 5.

Statistical analysis showed that the number of positive samples for *Brucella* specific DNA was significantly higher in group A compared to group B and group C ($P < 0.05$).

As far as the clinical picture (other than neuropsychiatric symptomatology) of the study population is concerned, a significant increase in positive PCR results was observed in individuals presenting with symptoms and signs compatible with the clinical spectrum of brucellosis and its complications, compared to those with no such symptoms.

On the other hand, there were no statistically significant differences between genders as well as between individuals with positive (relapsing or non relapsing) or negative brucellosis history concerning the PCR results.

DISCUSSION

Human brucellosis is a multisystem disease with a broad spectrum of clinical manifestations and complications, which can involve almost every organ system [7]. Neuropsychiatric symptoms, mainly those belonging to the range of manifestations of emotional disorders, have been reported especially in patients with chronic brucellosis [9-13, 16, 19, 20, 22-24] but their pathogenesis has not been fully elucidated. Such atypical manifestations seem to be on the rise [34] and might lead to underdiagnosis or misdiagnosis of the disease.

In the present study positive samples for *Brucella* specific DNA were detected only in the patients' group

Table 1. Characteristics and PCR Results for the Three Groups Examined

| Group | Number of Persons | Characteristics | Number of Positive Samples | Percentage of Positive Samples (%) |
|-----------------------------------|-------------------|--|----------------------------|------------------------------------|
| A (patients) | 20 | Manifestations compatible with emotional disorder | 3 | 15 |
| B (positive brucellosis controls) | 16 | No emotional disturbances, positive history of brucellosis | 0 | 0 |
| C (negative brucellosis controls) | 42 | No emotional disturbances, negative history of brucellosis | 0 | 0 |

Table 2. Brucellosis History and PCR Results of Group A

| Brucellosis History | Number of Individuals | PCR Results | | | |
|---|-----------------------|-------------|---------------------------|----------|---------------------------|
| | | Positive | Percentage (Positive) (%) | Negative | Percentage (Negative) (%) |
| Acute relapsing brucellosis (one relapse) | 2 | 1 | 50 | 1 | 50 |
| Acute non relapsing brucellosis | 3 | 0 | 0 | 3 | 100 |
| Negative brucellosis history | 15 | 2 | 13 | 13 | 87 |

(group A) and this difference was found to be statistically significant. Two of the three PCR positive individuals had no history of acute brucellosis, while the third one had been diagnosed with the disease eighteen years before the study, with one relapse six months after the initial diagnosis, and had been considered cured since. Their dominant neuropsychiatric symptoms were depression, sleep disorders (insomnia) and anxiety, and all the three patients were under psychiatric pharmacological treatment. In brucellosis endemic regions the above symptoms might be a part of the clinical spectrum of chronic brucellosis, especially among persons at a high risk of exposure to the infectious agent (farmers, shepherds etc). This hypothesis appears to be in accordance with a prospective study of 400 brucellosis patients done in Kuwait and where 6% of the patients developed psychiatric complications (with depression and anxiety accounting for 5.5%) predominantly in the chronic stage of the disease [9]. A number of other studies have associated emotional disorders with the chronic form of brucellosis [23, 19, 12, 22], but, due to the limitations of the conventional diagnostic methods used, a definite diagnosis of brucellosis could not be established. Using a highly sensitive and specific *Brucella* PCR assay recently evaluated for the diagnosis of chronic and focal forms of the disease [33], we did not detect statistically significant differences between individuals with positive brucellosis history (relapsing or none relapsing) and those with negative history. The disease tends to evolve to chronicity in those cases that escaped diagnosis, i.e. in cases of inaccurate or unknown history [3, 9]. Moreover, in occupationally exposed individuals like those included in our study, the possibility of a subclinical infection is common, and several authors recommend treatment of asymptomatic individuals presenting with an increased titre of specific antibodies [35]. The moderate value that brucellosis history has in the etiologic diagnosis is outlined also by the results of a study of chronic hepatosplenic suppurative brucellosis in which only six of fifteen patients had a history of acute brucellosis [36].

Table 3. Neuropsychiatric Manifestations of Individuals with Positive PCR Results

| Individuals with Positive PCR Results | Neuropsychiatric Manifestations |
|---------------------------------------|--|
| No 1 | Depression Sleep disturbances Irritability, agitation Anxiety |
| No 2 | Depression Sleep disturbances Anxiety |
| No 3 | Sleep disturbances Anxiety |

In our study we did not find statistically significant differences between genders concerning the PCR results. This contrasts with the vast majority of epidemiological studies that reported a higher incidence of brucellosis in males [4, 5, 37]. The populations studied by us were entirely agricultural and both men and women were equally exposed to animals and their products.

Considering the small number of individuals included and the fact that those positive for *Brucella* DNA were not followed for possible improvement of their neuropsychiatric conditions following subsequent treatment for brucellosis, we cannot definitively conclude that brucellosis is responsible for the neuropsychiatric symptoms observed. However, our data suggest that emotional disturbances of inhabitants of endemic regions should raise the suspicion of brucellosis in the general practitioner. This could contribute to timely diagnosis and reporting of the disease. Given the high failure rates of therapy, perhaps the early recognition of an atypical form of brucellosis might also improve prognosis.

Table 4. The Epidemiological Data of the Study Population

| Epidemiological Data and PCR Results | Number of individuals | Percentage (%) |
|---|-----------------------|----------------|
| Negative brucellosis history and negative PCR | 55 | 70 |
| Positive brucellosis history and negative PCR | 20 | 26 |
| Negative brucellosis history and positive PCR | 2 | 3 |
| Positive brucellosis history and positive PCR | 1 | 1 |
| Total | 78 | 100 |

Table 5. Sex Composition of the Three Groups

| Sex | Group A | Group B | Group C | Positive PCR |
|--------|---------|---------|---------|--------------|
| Male | 6 | 14 | 25 | 1 |
| Female | 14 | 2 | 17 | 2 |

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