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研究報告の概要

○永続的な微生物:ブルセラDNAは臨床的治癒後何年間も存続する
背景:ブルセラ症の発生率は依然として高いが、病態生理学、診断および治療、特に宿主内でのブルセラ属種の生存能については現在も不明な点が存在している。
方法:定量的リアルタイム・ポリメラーゼ連鎖反応法を用いて、ブルセラ症患者の複数の疾患ステージにおける細菌DNA量をモニターした。39名の急性ブルセラ症患者それぞれから3つ以上の末梢血検体を入手した(診断時1検体、治療終了後1検体、追跡調査時1検体以上)。
結果:大多数の患者(治療終了後では87%、治療終了6ヵ月後77%、治療終了2年以上後70%)は、無症候性であるにもかかわらず、持続的に細菌が検出可能であった。再発を経験した患者3名は、追跡調査中のどの疾患ステージにおいても細菌量に統計的有意差を示さなかった。
結論:適切な治療を行い回復したように見えても、ブルセラ菌DNAは残存する。この知見は、当該疾患の病態生理学に新たな洞察をもたらす。すなわち、ブルセラ菌は除去不可能な持続性の病原体である。

報告企業の意見	今後の対応
ブルセラ症に対して適切な治療を行い回復したように見えても、ブルセラ菌DNAは長期間体内に残存するとの報告である。	日本赤十字社では、全ての輸血用血液製剤について、平成19年1月より保存前白血球除去を実施している。今後も細菌やウイルスの検出や不活化する方策について情報の収集に努める。

使用上の注意記載状況・その他参考事項等

合成血-LR「日赤」
照射合成血-LR「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

MAJOR ARTICLE

An Eternal Microbe: *Brucella* DNA Load Persists for Years after Clinical Cure

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Background. Despite the continuing high incidence of brucellosis, vague aspects of pathophysiology, diagnosis, and treatment continue to exist, particularly with regard to the ability of *Brucella* species to survive inside the host.

Methods. A quantitative real-time polymerase chain reaction assay was used for monitoring bacterial DNA load in brucellosis-affected patients throughout different disease stages. Three or more specimens per patient were obtained (1 at diagnosis, 1 at the end of treatment, and at least 1 during the follow-up period) from 39 patients with acute brucellosis.

Results. The majority of patients (87% at the end of treatment, 77% at 6 months after treatment completion, and 70% at >2 years after treatment) exhibited persistent detectable microbiological load despite being asymptomatic. The 3 patients who experienced relapse did not exhibit any statistically significant difference in their bacterial load at any stage of disease or during follow-up.

Conclusion. *Brucella melitensis* DNA persists despite appropriate treatment and apparent recovery. This finding offers a new insight into the pathophysiology of the disease: *B. melitensis* is a noneradicable, persisting pathogen.

Brucellosis is a zoonosis that is prevalent worldwide [1]. *Brucella* species have recently garnered renewed attention because of their potential for use in biowarfare [2] and their reemergence as a significant cause of travel-related infection [3]. The complex pathophysiology of *Brucella* species [4] is dominated by their ability to manipulate immune response, targeting professional and nonprofessional phagocytes. Therein, *Brucella* species replicate without affecting cellular viability; in fact, the pathogen, by switching off cellular apoptosis, practically renders the cell immortal, thus allowing for its own further survival [5]. This intracellular localization of *Brucella* species in specialized compartments affects both the natural history and the diagnostic and therapeutic principles of brucellosis. The natural history of brucellosis is characterized by a frequently silent, protracted disease evolution. Therapeutically, the disease evolution imposes the need for a prolonged combined treatment that, even when administered in accordance with optimal recommendations, may lead to relapses. Diagnostically, the disease evolution hampers the usefulness of blood cultures and the use of microbiological eradication indexes [6].

Quantification of the microbiological burden may theoretically offer insight into the actual natural history of the disease, and it may allow for the evaluation of when and how the pathogen is eradicated from the human body (the term "microbiological eradication" being questionable for such a disease) [6]. Serological tests are useful for diagnosis [7], but the time required for results after treatment is disappointingly long. In addition, serological test results are usually inadequate in predicting the outcome. The latter may also apply to newer, sophisticated techniques such as ELISA [8]. The development of such novel molecular diagnostic techniques as PCR offered promise—technology preceded clinical application in the context of brucellosis, and even before traditional PCR assays were adequately evaluated clinically [9], novel assays emerged.

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Here, we present the results of the application of a real-time PCR assay for the diagnosis and follow-up of brucellosis in a large number of patients, and we discuss the potential pathophysiological significance of the findings.

MATERIALS AND METHODS

Patients. Peripheral blood specimens (both whole blood and serum) were collected from 39 patients who had received a diagnosis of acute brucellosis and from 50 healthy blood donors (control group). A minimum of 3 samples per patient were obtained (1 at diagnosis, 1 at the end of treatment, and at least 1 during the follow-up period). The control group, matched for age and sex, had no antibodies to *Brucella* species. All patients received a diagnosis of acute brucellosis during the period 2001–2004 in the University Hospital of Ioannina, reference center for the district of Epirus in northwestern Greece, where (like in nearby Greek and foreign regions) brucellosis is endemic [1, 10]. Epidemiological, clinical, and microbiological characteristics of the patient population are presented in table 1.

The diagnosis of acute brucellosis was established according to 1 of the following criteria: (1) isolation of *Brucella* species in blood culture or other clinical samples or (2) the presence of suggestive clinical characteristics together with the demonstration of specific antibodies at high titers, seroconversion, or an increase in antibodies in a serum sample obtained 15–20 days after the first sample was obtained.

The posttreatment phase varied from 2 to 36 months, according to the continuity of patient visits. Three patients experienced relapse during the follow-up period. Of the 39 study patients, 30 were treated with the standard regimen of doxycycline plus rifampin, 7 were treated with doxycycline plus ciprofloxacin, 1 was treated with moxalactamm, and 1 was treated with doxycycline plus streptomycin (the latter for 2 weeks). The duration of treatment was 6 weeks for all but 4 patients. Two patients were treated for 12 weeks (1 with doxycycline plus rifampin and 1 with doxycycline plus ciprofloxacin), and 2 patients were treated for 6 months (1 with doxycycline plus ciprofloxacin and 1 with doxycycline plus rifampin). Analysis of the evolution of the bacterial DNA load was performed at the time of initial diagnosis, at the end of treatment, and during the follow-up period (2, 6, 12–24, and 24–36 months after the end of treatment). The study underwent ethics review and approval.

Bacteriological and serological techniques. Serological tests—including rose Bengal plate (RBP) agglutination, Wright seroagglutination, and ELISA (Serion ELISA Classic Brucella IgG/IgM/IgA; Institut Virion\Serion; detecting IgM, IgG, and IgA antibodies)—were performed on all patient and control specimens; blood cultures were performed for 24 of the initial 39 patients.

The RBP agglutination and the Wright seroagglutination tests were performed in accordance with techniques described elsewhere [11]. The ELISA was performed in accordance with the manufacturer's instructions. Blood cultures were processed with BacT/Alert (bioMérieux) in accordance with standard techniques [12, 13] and were monitored for 10 consecutive

Table 1. Demographic characteristics and clinical and microbiological findings for patients with brucellosis.

Variable	Patients
No. of patients/no. of samples studied	39/130
Demographic characteristic	
Male	30 (77)
Female	9 (23)
Age, mean years (range)	41 (16–78)
Clinical characteristic	
Duration of symptoms, mean days (range)	32 (7–270)
Fever	36 (92.3)
Constitutive symptoms	23 (59)
Osteoarticular complications	16 (41)
Hepato/splenomegaly	7 (18)
Orchiepididymitis	3 (10)
CNS disorders	2 (5)
Diagnostic test result	
Titer ≥1:160, by Wright test	36 (92)
RBP test (from 2+ to 4+)	37 (95)
ELISA test	39 (100)
Proportion (%) of patients with positive blood culture results	13/24 (54)

NOTE. Data are no. (%) of patients, unless otherwise indicated. RBP, rose Bengal plate.

days. If the system failed to detect any growth, the vials were transferred to a conventional incubator for 10 additional days. Blind subcultures were performed on days 10 and 20 on *Brucella* agar (BBL; Becton Dickinson) and were incubated at 37°C in a 5%–10% carbon dioxide atmosphere for 3 days [12, 13]. If growth appeared, the suspected colonies were identified by colonial morphology; Gram staining; oxidase, catalase, and urease tests; and positive agglutination with specific antiserum. Identification and biotyping of *Brucella* species were performed in accordance with standard microbiological procedures [14].

Isolation of DNA from clinical blood specimens and bacteria strains. Peripheral blood samples were collected in EDTA tubes. DNA was extracted from whole blood (200 μL) with the QIAamp DNA Blood Mini Kit (Qiagen) in accordance with the manufacturer's instructions. The kit couples the selective binding properties of silica gels with a microcentrifugation step. The specimens were first lysed with protease in a buffer chosen to optimize the DNA-binding capacities on the QIAamp membrane. The use of the silica column allowed, after DNA coating, efficient washing of the samples to eliminate contaminants. After elution, DNA was stored at −80°C until PCR amplification analysis.

Hybridization probe–based quantitative real-time PCR assay. The real-time PCR assay was based on direct amplification of a 207–base pair DNA sequence of a gene that codes for the synthesis of an immunogenetic 31-kilodalton protein specific for the *Brucella* genus (BCSP31). The primer pair used was that published by Baily et al. [15]. The amplification product was detected by using fluorescence technique hybridization probes labeled with LightCycler Red 640 (detected in channel F2). A control amplification reaction in the third channel (F3) acted as an internal run control. A single-tube duplex LightCycler-PCR (LC-PCR) was performed using the FastStart DNA Hybridization Probes kit (Roche Diagnostics). To each LightCycler glass capillary, we added 20 μL reaction mixture containing 6.6 μL PCR-grade water, 2.5 mmol of magnesium chloride, 4.0 μL reagent mix (containing primers and probes), 2.0 μL FastStart mix, and 5.0 μL template (sample or standard). Primers and probes were designed and provided by TIB MOLBIOL. To detect any ampicon contamination or amplification failure, negative controls that contained 5 μL of PCR water instead of DNA and positive controls that contained DNA of *Brucella melitensis* biovar 1 were included in each real-time PCR run. Cycling conditions consisted of initial denaturation at 95°C for 10 min, followed by 55 cycles at 95°C for 10 s, at 55°C for 8 s, and at 72°C for 15 s in a LightCycler instrument (Roche Diagnostics). Fluorescence curves were analyzed with LightCycler software, version 3.5. After amplification, melting-curve analysis was performed to verify the specificity of PCR products (1 cycle consisted of 95°C for 20 s, 40°C for 20 s, and 85°C for 0 s). The BCSP31-derived product was identified by running the melting curve with a specific melting point of 67.5°C (concentration dependent). A standard curve, comprising 10-fold dilutions of *Brucella* BCSP31 DNA of 10^1–10^7 target equivalents, allowed quantification of unknown samples. In the provided standard row (TIB MOLBIOL), the lowest concentration (10 copies) was amplified in 35–36 cycles; 100, 10,000, and 1,000,000 copies were amplified after 32–33, 25–26, and 17–18 cycles, respectively (with the crossing point calculated by the "second derivative maximum"). With the method "fit points," the crossing point values were 32–33, 28–29, 22–23, and 15–16 cycles, respectively. The detection limit of the method spiked with serial dilution of *B. melitensis* DNA was 10 copies/5 μL DNA extract.

RESULTS

Brucella species was initially isolated in blood cultures from 13 (54%) of the 24 patients with available blood specimens. All strains isolated were identified as *B. melitensis* biotype 2. Of the 13 patients who had positive blood culture results, 12 also had positive results for all 3 serological tests used, whereas 1 had positive results only for 2 serological tests (RBP agglutination and ELISA). The remaining 26 (67%) of the 39 patients with acute brucellosis received their diagnoses on the basis of clinical and serological criteria. The RBP agglutination test result was positive for 37 patients (95%). The Wright seroagglutination test titers were within the diagnostic range (titers, >1:160) in 36 patients (92%). For the assessment of the ELISA results, samples with an optical density of 10% greater than the cutoff optical density were considered to be positive; samples from all 39 patients (100%) were positive. The types of antibodies detected by the ELISA were as follows for the 39 ELISA-positive samples: IgG, 27 (69%); IgA, 34 (87%); and IgM, 31 (80%).

All specimens obtained from 39 patients at initial diagnosis had positive real-time PCR assay results, conferring a sensitivity of 100%. All specimens obtained from the control group were negative for *B. melitensis*, conferring a specificity of 100%.

The evolution of the bacterial DNA load is shown in figure 1. The mean *B. melitensis* DNA load (±SD) for the 39 patients at the time of diagnosis was 803 ± 1236 copies/5 μL DNA extract (range, 26–4570 copies/5 μL DNA extract). At the end of treatment, samples from 34 patients (87%) remained positive for *B. melitensis*, with a mean bacterial DNA load (±SD) of 240 ± 314 copies/5 μL DNA extract (range, 0–1230 copies/5 μL DNA extract). Two months after the end of treatment, samples were collected from 34 patients, and the mean bacterial DNA load (±SD) was 192 ± 236 copies/5 μL DNA extract (range, 0–875 copies/5 μL DNA extract; results for 27 patients remained positive). Six months after the end of treatment, samples were collected from 26 patients, and the mean bacterial DNA load (±SD) was 96 ± 135 copies/5 μL DNA extract

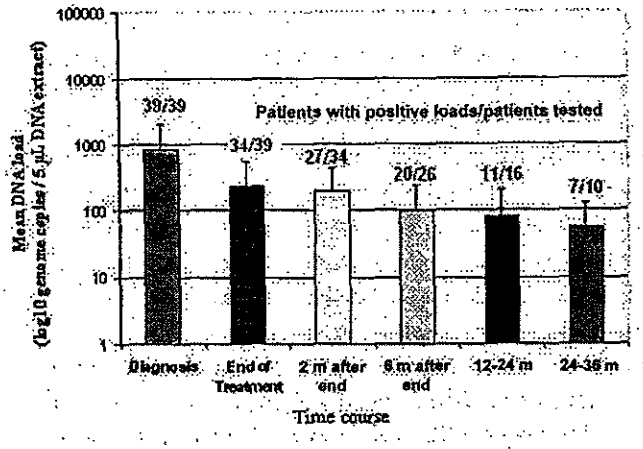

Figure 1. Evolution of *Brucella melitensis* DNA load at initial diagnosis and during the follow-up period. m, Months.

(range, 0–432 copies/5 μL DNA extract; results for 20 patients remained positive). Twelve to 24 months after the end of treatment, samples were collected from 16 patients, and the mean bacterial DNA load (±SD) was 80 ± 126 copies/5 μL DNA extract (range, 0–420 copies/5 μL DNA extract; results for 10 patients remained positive, and 1 patient had negative results for 2 consecutive specimens and then had a positive result but did not experience relapse). Twenty-four to 36 months after the end of treatment, samples were collected from 10 patients, and the mean bacterial DNA load (±SD) was 56 ± 74 copies/5 μL DNA extract (range, 0–220 copies/5 μL DNA extract; 7 patients continued to have positive results). Of 21 patients who were monitored for >1 year after therapy, 13 continued to have positive real-time PCR results but were asymptomatic. One patient had positive real-time PCR results 2 years after infection, although the patient had had negative real-time PCR results at the 1-year follow-up. Serological test results for these patients did not differ significantly between individuals with detectable and undetectable bacterial loads, with a mean Wright agglutination titer of 1:40, the presence of IgG antibodies determined by ELISA in all patients, and the presence of IgA antibodies in a minority of patients. During the follow-up phase of the study, only 3 patients experienced relapse. On relapse, 1 patient had positive blood culture results, whereas the other 2 relapses were diagnosed on the basis of clinical findings. In the 3 patients who experienced relapse, there was no increase in bacterial load during symptom reappearance, compared with their previous follow-up bacterial load measurement, although their titers did not decrease either. There were no statistically significant differences with regard to initial or posttreatment microbiological load between those who did and did not experience relapse. Results of blood cultures, when samples were obtained after treatment completion, were negative for all patients who did not experience relapse.

DISCUSSION

An emerging method for the detection and identification of a variety of infectious agents in the clinical laboratory is real-time PCR [16]. Real-time PCR was developed to improve the sensitivity, specificity, and speed of detecting PCR amplification products [17]. It does not require postamplification handling of PCR products, thereby reducing the risk of laboratory contamination and false-positive results. Moreover, real-time PCR has emerged as a powerful tool for quantification of the microbiological load; this is a concept that is valuable for numerous infectious disorders (e.g., hepatitis and HIV infection); real-time PCR has also blurred the traditional definitions of "eradication." Microbiological eradication (i.e., achievement of negative culture results) has long been used as an end point in clinical trials for several infections. However, these principles do not apply to a potentially chronic intracellular infection such as brucellosis. Therefore, microbiological load could serve as an indirect index of pathogen presence. One has to take into account, however, all of the potential problems of such an application; for example, isolation of DNA particles cannot discriminate between living and "eradicated" microorganisms. Furthermore, microbiological load could theoretically serve as an index of disease burden and thus allow for patient stratification by disease severity, relapse potential, and therapeutic regimens needed.

Our study revealed startling results. A significant number of patients continued to exhibit microbiological load even years after clinical cure and in the absence of any symptom indicative of disease persistence or relapse. We consider our results to be indicative of the long-term presence of viable bacteria in the human body in a cellular reservoir that needs further clarification. Could the results be explained as the outcome of particle shedding by dead bacteria? These dead bacteria could not be the result of antibiotic treatment administered <1 year earlier. Thus, in this case, bacteria indeed persisted for a long period after infection and clinical cure; these bacteria failed to elicit clinical manifestations, possibly because of a robust sustained immune response that eventually eliminates them by switching on the initially cancelled cellular apoptosis [18].

Even if we accept this alternative explanation, the only viable pathophysiological scenario is that, at least in the majority of patients, *Brucella* species persist inside the human body despite apparent clinical cure. The pathogen may replicate at low frequency. It may even cause transient, low-level bacteremia in a manner that can be handled by the body's immune system in such a way as to avoid the evolution of clinical disease. In that case, brucellosis should be considered to be only a chronic infection (much like tuberculosis), the clinical presentation of which depends on the equilibrium between the immune system and the microbial pathogenicity. Certain studies of patients with chronic brucellosis have indeed focused on such a dysregulation

[19]. The level at which this interaction occurs is vague; low-level bacteremia may not be detected easily, and follow-up through cultures of bone marrow specimens, theoretically sound and suggested to be diagnostically superior [20], is not convenient. Moreover, one could hypothesize that, with use of more-sensitive diagnostic techniques, it could be determined that patients with undetectable microbiological loads might actually have microbiological loads.

Contradictory results have emerged from the few relevant studies, with smaller sample numbers, that have recently been reported. Similar findings were elicited in a Spanish study [21] that showed that 4 of 7 individuals who experienced relapse and 3 of 11 who did not experience relapse also exhibited detectable bacterial loads after long-term follow-up. A similar result was also reported from a study in Peru [22] that used plain PCR for follow-up; the majority of the patients had PCR-positive samples even months after treatment completion. These results, however, were not reproduced in another Spanish study [23] that used a slightly different methodology. Two other real-time PCR studies did not evaluate evolution of the microbiological load during disease stages [24, 25].

One could support the hypothesis that patients with detectable microbiological loads were simply inadequately treated and were therefore candidates for relapse. However, the few who experienced relapse did not preferentially belong to the subgroup of patients with continually detected bacterial load. In addition, these patients did not exhibit higher loads or any statistically significant difference than did those of the group that did not experience relapse. Furthermore, relapses usually occur during the first few months after the end of treatment, and 90% of them usually occur during the first follow-up year, a cutoff point surpassed by our patients. No data can be extracted about whether a specific therapeutic regimen was related to long-term detectable microbiological loads because the majority of the patients were treated with the same regimen. In the Spanish study [21], numerous regimens were used, and the small statistical sample did not allow for any conclusions to be drawn.

One might argue that the significance of the study is marred by the fact that all patients with detectable DNA load during the follow-up period had negative results of blood cultures; thus, the PCR results may be considered to be dubious. Yet this fact (i.e., negative blood culture results) underlines the importance of our findings. If these patients had positive results of blood cultures, they would be considered de facto to have experienced relapse (reappearance of positive blood culture results is considered to indicate a relapse even in the absence of symptoms). These patients, however, met absolutely no criteria for relapse or disease in general, despite having detectable DNA load for *Brucella* species. Cases of reappearance of *Brucella* infection years after the initial course of symptoms are not rare, especially in the context of foreign body infection [26].

One might argue that there is selection bias in our results, because bacterial load was evaluated in only 10 patients in the 24–36-month period. However, the other study patients did not experience relapse, as assessed by telephone interview performed by 1 of the authors during this period; thus, the 10 patients for whom bacterial load data were available could be considered to be representative of the entire sample. Furthermore, a similar trend was observed among larger subsets of patients in the various follow-up time frames depicted in figure 1.

Recognizing that brucellosis is a chronic infection means that our understanding of the pathophysiology, diagnosis, and treatment of the disease may be drastically altered. First, one has to understand what suppresses *Brucella* species pathogenicity during the protracted posttreatment period when bacterial load is detectable. Moreover, one has to elucidate which are the critical components of this suppression and whether patients with chronic brucellosis exhibit a defect in these components. Second, one has to define whether the initial bacterial load is related to disease severity, tendency to relapse, or need for enhanced antibiotic treatment. Third, one has to seek other predictors of relapse, because real-time PCR did not exhibit any correlation in the present study. Finally, with regard to therapy, one has to redefine treatment goals. Eradication was never set as an issue in brucellosis, and the "acceptable" percentage of relapses was arbitrary [27]. The future question, however, will be whether to treat aggressively, with monitoring of the bacterial load, or to simply ignore the results and allow the pathogen to parasitize inside the human body.

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Potential conflicts of interest. All authors: no conflicts.

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