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EVALUATION OF SOME DIAGNOSTIC METHODS FOR THE BRUCELLOSIS IN HUMANS – A FIVE YEAR STUDY

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A b s t r a c t: Brucellosis is a worldwide zoonosis with a high degree of morbidity in humans. In Bosnia and Herzegovina a progressive increase of brucellosis among humans is evident. As the clinical picture of human brucellosis is fairly non-specific, a definitive diagnosis requires isolation of the causative organism, or the demonstration of the high levels of specific antibodies, or seroconversion.

Aim: To analyse the diagnostic value of the Rose Bengal test, blood culture and immunoenzymatic test (ELISA IgM and IgG) in patients with brucellosis and to examine the relationship between these diagnostic methods.

Methods: We analysed the diagnostic methods in 525 brucellosis patients from 2004 to 2008. All patients were treated at the Infectious Diseases Clinic, University of Sarajevo Clinics Centre. The disease was diagnosed by positive blood culture results and/or by positive relevant serologic test results (ELISA, Rose-Bengal plate-agglutination test).

Results: In total 162/525 (30.8%) patients had positive blood cultures. The Rose Bengal test was positive in all patients – 525/525 (100.0%). Brucella IgM antibodies with ELISA were positive in 341/525 (64.8%). Early in infection, antibodies of the IgM class predominate. Brucella IgG antibodies with ELISA were positive in 236/525 (56%).

Conclusion: This study clearly showed that only a combination of blood culture, Rose Bengal test and ELISA ensured early and precise diagnosis of human brucellosis. The Rose Bengal test is excellent for screening. Blood culture gave excellent results in patients with primary infections. ELISA(IgM, IgG) is the method of choice for the diagnosis of chronic disease and relapse.

Key words: brucellosis, infection, laboratory diagnosis, serology, blood culture.

Introduction

Brucellosis is a worldwide zoonosis with a high degree of morbidity in humans. According to WHO data about 500,000 cases of this disease are registered in the world every year [1, 2]. Bosnia and Herzegovina did not have any significant problems with brucellosis until 2000, when the first cases of human brucellosis were registered. Since then the prevalence of brucellosis has increased and there is no sign of its contrrol.

In Bosnia and Herzegovina progressive increases of brucellosis among humans is evident. There are several reason why brucellosis and other zoonoses became a frequent occurence in Bosnia and Herzegovina after the war. Some include import of livestock from abroad without taking preventive measures, and even more important is import of cattle across the unguarded borders. Adequate prevention of brucellosis requires a multi-disciplinary approach with close cooperation between veterinarians, microbiologists, infectious disease specialists, epidemiologists and public health professionals.

Brucellosis is a considerable problem in other areas of ex-Yugoslavia. In the former Yugoslavia brucellosis was first diagnosed in Istra in 1947. From 1947 until 1954 Istra became an epidemic area with more than 300 cases. In 1990 the disease was again diagnosed in Istra, resulting from animal importation from the east [3]. In the Republic of Macedonia brucellosis is an endemic disease, primarily in the Bitola region. The disease was first diagnosed in 1980. In the last 25 years about 9,800 human patients were registered [4].

In Serbia brucellosis is distributed in Vojvodina in the north and in the southern parts of Serbia on the border with Macedonia. In Vojvodina the most cases of brucellosis were registered in 2004 with 75 patients [5].

The spread of brucellosis represents a classic example of spreading zoonosis as a result of human and animal population interaction. With the appearance of the epidemic of brucellosis recently, we have become conscious of the danger of spreading this infection in the region and also of the necessity of close collaboration among experts from different specializations with the aim of effective diagnostic, therapy and infection control.

Brucellosis is primarily an animal disease, characterized as an asymptomatic chronic infection. Infection of humans follows the incidence of brucellosis infection in animals. Because of methods of transmission (direct contact, aerosol inhalation, food) it is usually a professional disease of consumers of unpasteurized dairy products, cattle-breeders, farmers, butchers and members of their families, veterinarians and laboratory workers. In humans, brucellosis behaves as a systemic infection with a very heterogeneous clinical spectrum. The disease usually presents as a fever with no apparent origin, although there are focal forms in 20–40% of cases. The diagnosis of brucellosis is based on clinical manifestations, epidemiological, anamnestic immune response date and

laboratory analyses. The clinical picture in human brucellosis can be misleading, and cases in which gastrointestinal, respiratory, dermal, or neurological manifestations predominate. Because unusual cases with atypical lesions continue to be reported, diagnosis must be supported by laboratory tests with a definitive diagnosis by isolation of the causative organism, the demonstration of high levels of specific antibodies, or seroconversion.

The aim of this study was to analyse the diagnostic value of the Rose Bengal test, blood culture and immunoenzymatic test (ELISA IgM and IgG) in patients with brucellosis and to examine the relationship between these diagnostic methods.

Methods

Patient data

The study included 525 brucellosis patients observed between 2004 and 2008. All patients were treated at the Infectious Diseases Clinic, University of Sarajevo Clinics Centre. The average age of patients was 35.9 years of life – range from 0.6 to 70. Most of the cases were between 41 and 50 years of age. the gender structure of patients was: 380 (72.5%) males and 145 (27.5%) females.

All laboratory testing for brucellosis was performed at the Clinical Microbiology Institute, University of Sarajevo Clinics Centre. The disease was diagnosed by positive blood culture results and/or by positive relevant serological test results (ELISA, Rose-Bengal slide-agglutination). The study included only patients in which all three methods were applied.

Blood culture

In total 525 blood cultures were examined. Blood culture was performed by inoculation of 8–10 ml of freshly collected blood into each Plus aerobic/F BACTEC bottle and incubation for up to seven days in the BACTEC 9120 semi-automated system (Becton-Dickinson Diagnostic Instruments Systems, Maryland, USA). Bottles were examined for the presence of growth on a 10-minute cycle by the measurement of CO_2-induced fluorescence emitted by the sensor at the boottom of the culture. Bottles giving a positive growth index were Gram stained and subcultured onto blood agar plates. Brucella isolates were identified by conventional biochemical testing (catalase, oxidase and urease activity; glucose fermentation and production of H_2S). *Brucella spp*. suspected isolates were confirmed by slide agglutination using type-specific antisera (Murex Diagnostics, Dartford, United Kingdom).

Serological tests

For serology, blood samples were centrifuged and the serum stored at –20^{o}C until tested. All sera were evaluated using the Rose-Bengal test and

ELISA (IgM and IgG). The Rose-Bengal plate agglutination (RB test) was performed according to standard procedures. Undiluted serum samples (30μL) were mixed with an equal volume of Rose Bengal Slide Screening Test antigen (bio Merioux, Marcy L Etoile/France) on a white agglutination card. Rose Bengal antigen is a concentrate suspension of *Brucella abortus* 99WS (Weybridge), inactivated by heating and preserved in phenol (0.5%), stained with Rose Bengal stain. Results were rated negative when agglutination was absent and 1+ to 4+ positive according to the strength of the agglutination.

Brucella IgM and IgG enzyme-linked immunosorbent assays (ELISAs) were obtained from Genzyme Virotech GmbH/Germany. The test was performed and evaluated according to the kit procedure. The test result was read automaticaly by a BEP 2000-Behring ELISA processor.

Statistical analysis

For the evaluation of the results, standard statistical methods were used. Statistical analysis was performed by using a Chi-square test. Statistical significance was defined as $p < 0.05$.

In our study we observed the ethical principles outlined in the World Medical Association Declaration of Helsinki.

Results

In this study we evaluated the diagnosis of brucellosis by blood culture, Rose Bengal test, and ELISA IgM and IgG. The study included 525 brucellosis patients observed between 1st January 2004 and 31st July 2008. We examined only one specimen for any type of test in each patient because the purpose of study was to analyse the effectiveness of test methods at different stages of illness. We also examined the relationship between these diagnostic methods.

In total 162/525 (30.8%) patients had positive blood cultures. Positive blood cultures were detected very early, mostly during the first week of infection (5%). In more than 2/3 patients blood cultures were positive in the first month of infection (Figure 1). In the graph the time of diagnosis of brucellosis was marked on the abscisa axis (5th, 7th, 15th day etc.).

Brucella IgM antibodies measured by ELISA were positive in 341/525 (64.8%). Brucella IgG antibodies determined by ELISA were positive in 236/525 (56%). Early in infection, antibodies of the IgM class predominate. The peak level was reached 4 weeks later. After 6 months IgM antibodies were not detectable (Figure 2). IgG antibodies were detected 10 days after IgM antibodies. The peak level was reached after 2 months (Figure 3).

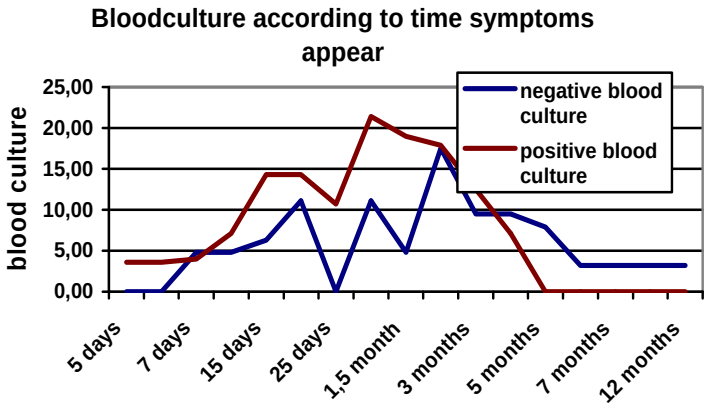

Figure 1 – Results of the examined blood cultures at different stages of illness presented as a percentage of the total of patients

Слика 1– Резултати од испитувањето култури на крв во различни стадиуми на болеста прикажани како проценти од вкупниот број пациенти

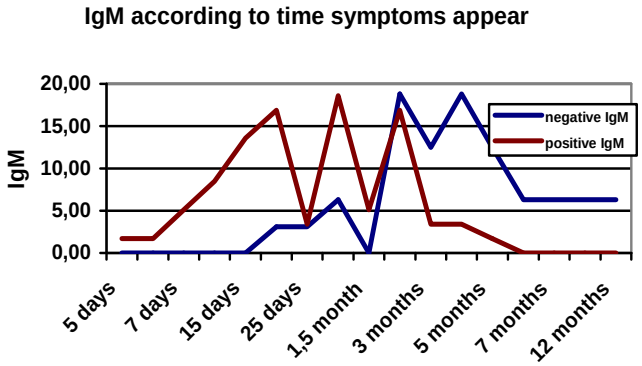

Figure 2 – Results of ELISA IgM as a percentage during different stages of illness
Слика 2 – Резултати од ELISA IgM како проценти во тек на различни стадиуми на болеста

In more than 2/3 of the patients, blood cultures were positive in the first month of the infection. There was a relationship between blood culture and the presence of IgM antibodies. Results of these methods showed a correlation in 59.3% of the patients: in 31.8% of the patients both methods were negative, and in 27.5% both methods were positive. The $\chi 2$ (Chi square test) demonstrates a statistically significant correlation between blood culture and the presence of IgM antibodies ($p = 0,001$).

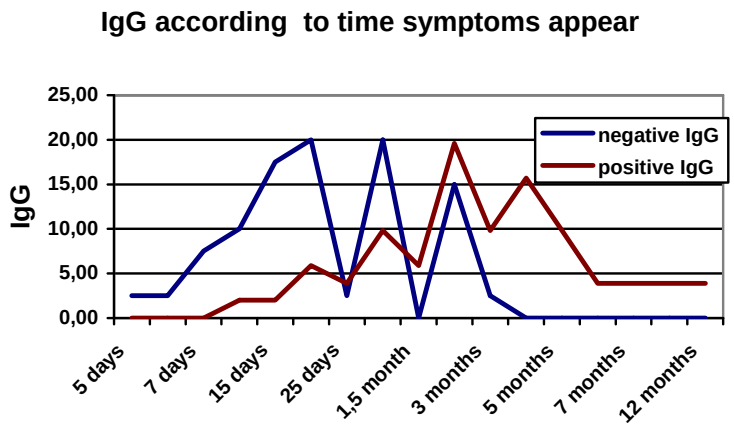

Figure 3 – Results of ELISA IgG as a percentage during different stages of illness
Слика 3 – Резултати од ELISA IgG како проценти во тек на различни стадиуми на болеста

IgG antibodies were detected 10 days after IgM antibodies. The peak level is reached after 2 months.These antibodies were detected longer than 12 months (Figure 4).

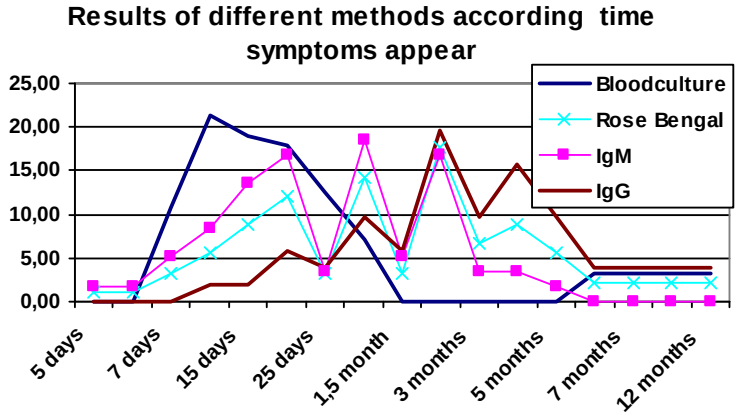

Figure 4 – Review of results in percentages at different stages of illness performed by different diagnostic tests
Слика 4 – Преглед на резултатите во проценти во текот на различни стадиуми на болеста добиени со различни дијагностички тестови

Discussion

Brucellosis represents a prevalent disease in humans and animals in our country. Since the symptoms of brucellosis are non-specific, a clinical diagnosis of the disease is difficult. Therefore, its accurate diagnosis necessitates the use of specific tests, mainly culture and serologic tests.

In this study we evaluated the diagnosis of brucellosis by blood culture, Rose Bengal test, and ELISA IgM and IgG. The study included 525 brucellosis patients observed between 1st January 2004 and 31st July 2008. In total 162/525 (30.8%) patients had positive blood cultures. In more than 2/3 patients blood cultures were positive in the first month of infection. This finding suggested that blood culture is the method of choice for definitive diagnosis in the acute phase of the disease. Blood culture demonstrated a high sensitivity in patients with primary infections but this technique has limitations as a laboratory test in a rural area in which brucellosis is endemic [6–8]. Although blood culture is the "gold standard" in the diagnosis of brucellosis, culture may sometimes be negative due to factors inherent to the growth of the microbe. Isolation of the bacteria is hazardous and brucellosis is one of the most common laboratory-acquired infections [9]. Jordi Serra *et al.* (1995–1998, Spain) analysed the importance of blood culture as a diagnostic method. They described the limitations of this method in endemic areas of brucellosis. In this country patients infected for a prolonged period show induction of immunity. Coincidental seroconversion and septic forms of brucellosis are rare [10].

Despite the important advances made in the diagnostics of human brucellosis following the general introduction of new semi-automated methods for blood culture processing, diagnosis of this disease is still based mostly on the demonstration of specific antibodies by means of different serological techniques. This is mainly because the greatest incidence of brucellosis is found in under-developed countries with poor technical resources.

A large number of different tests have been used for the serological diagnosis of brucellosis, thus demonstrating the lack of a single ideal technique. Rose Bengal is a rapid plate agglutination test that uses a suspension of *Brucella abortus* in an acid buffer. It has a high degree of sensitivity for the diagnosis of infection with *Brucella spp*., irrespective of the stage of the disease. This high sensitivity, together with the fact that the technique is simple and rapid (4 min.), makes the Rose Bengal test ideal for screening patients for human brucellosis. In our study the Rose Bengal test was positive in all patients 525/525 (100.0%). This results were expected because in our study only brucellosis patients were included. Use of the Rose Bengal test as the sole diagnostic tool to establish treatment of brucellosis in endemic areas is not a reliable practice, especially with individuals who are exposed repeatedly to infection, or who have a recent history of the disease. Ruiz-Mesa *et al*. [11] analysed the Rose Bengal test results

of 711 brucellosis patients in a rural region of Spain. The test had a sensitivity value of 92.3% and a specificity value of 75%. The conclusion of this study was that the Rose Bengal test was a useful method for screening but that results must be confirmed by other relevant methods [11]. The Rose Bengal test continues to be the mainstay of laboratory diagnosis, due to its simplicity, low cost, and convenience (> 90% sensitivity) in the diagnosis of acute brucellosis. However, this test suffers from a high false-negative rate in complicated and chronic cases. Because of the limitations of the Rose Bengal test, other assays, especially ELISA, which can determine the classes and subclasses of immunoglobulins in a sensitive and simple manner, can be used as confirmatory tests [12, 13].

In our study Brucella IgM antibodies with ELISA were positive in 341/525 (64.8%). Brucella IgG antibodies with ELISA were positive in 236/525 (56%). Early in infection, antibodies of the IgM class predominate and it is evident that the IgM antibody may be detected during the first week following the entry of the bacterium into the host. The peak level is reached 4 weeks after exposure. There was a relationship between blood cultures and the presence of IgM class antibodies. Results of these methods correlated in 59.3% of the patients: in 31.8% of the patients both methods were negative, and in 27.5% both methods were positive. The $\chi 2$ (Chi square test) demonstrates a statistical significance between blood culture and the presence of IgM antibody ($p = 0.001$).

IgG antibodies were detected 10 days after IgM antibodies in this study. The peak level was reached after 2 months. The appearance of IgG antibodies is delayed although it is found together with IgM 4 weeks after the initial antigenic stimulus; the IgM antibody level always exceeds the IgG antibody level in the acute stage of the disease. In our study, in a few patients in the acute phase of brucellosis with bacteremia, only IgM antibodies were detected. Several studies have shown that ELISA is the test of choice for the diagnosis of complicated and chronic cases, especially when other tests are negative [14–16]. In many studies performed with ELISA, it was determined that IgG positivity and an increase of the antibody titers were of considerable value in detection of relapsed cases and in patients with chronic infections [17, 18].

This study showed that the diagnosis of brucellosis was established in the different phase of the disease. In a few of the patients brucellosis was diagnosed 5 days after the first symptoms appeared, but in most cases patients were ill for a prolonged period before the diagnosis was established. In some of the patients brucellosis was diagnosed as late as 12 months after the first symptoms appeared. Patients performed unsuccessful health checks without a precise diagnosis. In cases where the doctor immediately suspected brucellosis and used corresponding diagnostic methods the diagnosis of brucellosis was made in an early stage of the illness. Early and precise diagnosis of brucellosis and inclusion of adequate antibiotic therapy are of crucial importance to the patients, especially for protection against the development complications and to decrease the occurence of relapses of disease.

As we compared results of the test methods according to the appearance of symptoms, we showed that results were different at different stages of illness. the effectiveness of the test methods was different at different stages of illness and so only a combination of blood culture, Rose Bengal test and ELISA(IgM, IgG) ensured an accurate diagnosis.

Conclusion

In Bosnia and Herzegovina brucellosis is progressively increasing among humans. The diagnosis of human brucellosis may be very difficult. Microbiological methods are very important in the diagnosis of the disease. The present study deals with the usefulness and significance of blood culture and serology tests in the diagnosis of human brucellosis. This study clearly showed that the effectiveness of some diagnostic methods for brucellosis in man are different at different stages of illness, and so only a combination of blood culture, Rose Bengal test and ELISA (IgM, IgG) ensured accurate diagnosis.

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Р е з и м е

ЕВАЛУАЦИЈА НА НЕКОИ ДИЈАГНОСТИЧКИ МЕТОДИ ЗА БРУЦЕЛОЗА КАЈ ЛУЃЕТО – ПЕТГОДИШНА СТУДИЈА

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Вовед: Бруцелозата преставува зооноза присутна во целиот свет со висок степен на морбидитет кај луѓето. Во Босна и Херцеговина евидентирано е прогресивно зголемување на бруцелозата помеѓу луѓето. Поради тоа што клиничката слика на бруцелозата кај човекот е доста неспецифи-

чна, дефинитивната дијагноза бара изолација на предизвикувачот, демонстрирање на високи нивоа на специфични антитела, или сероконверзија.

Цел: Да се анализираат дијагностичките вредности на Rose Bengal тестот, култура од крв и имуноензимски тест (ЕЛИСА IgM и IgG) кај пациенти со бруцелоза и да се утврди корелацијата помеѓу овие дијагностички методи.

Методи: Дијагностичките методи беа анализирани кај 525 пациенти со бруцелоза во периодот од 2004 до 2008 година. Сите пациенти беа третирани на Клиниката за инфективни болести, Клинички центар, Универзитет во Сараево. Заболувањето беше дијагностицирано со позитивни тестови на култура од крв и/или позитивни релевантни серолошки резултати (ELISA, Rose Bengal аглутинациски тест на плочка).

Резултати: Вкупно 162/525 (30,8%) пациенти имаа позитивни култури од крв. Rose Bengal тестот беше позитивен кај сите пациенти 525/525 (100,0%). Бруцела IgM антитела со ELISA беа позитивни кај 341/525 (64,8%). Рано, во тек на инфекцијата, предоминираа антитела од класата IgM. Бруцела IgG антителата со ELISA беа позитивни кај 236/525 (56%).

Заклучок: Оваа студија јасно покажува дека единствено со комбинација на култура од крв, Rose Bengal тест и ELISA се осигурува рана и прецизна дијагноза на бруцелоза кај човекот. Rose Bengal тестот е одличен за скрининг на бруцелозата. Културата од крв даде одлични резултати кај пациенти со примарна инфекција. ELISA (IgM, IgG) е метод на избор за дијагноза на хронична болест и рецидиви.

Клучни зборови: бруцелоза, инфекција, лабораториска дијагноза, серологија, култура од крв.

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