

SEROLOGICAL STUDY OF BULGARIAN PATIENTS WITH BRUCELLOSIS

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ABSTRACT

Background: Brucellosis is a widespread zoonotic infection with significant health and socio-economic impacts. This determines the need of a reliable laboratory diagnosis both in endemic areas around the world and in countries where it is rare and under diagnosed. Due to prolonged and hazardous cultivation procedures, the diagnosis is mostly serologically confirmed.

Aim: Serological study of Bulgarian citizens with clinical and/or epidemiological data compatible to brucellosis for the period 2005-2015.

Materials and methods: Based on clinical and epidemiological data obtained via standard questionnaire, 3394 persons suspected for brucellosis along with 106 controls were serologically studied with Rose Bengal slide test, Standard agglutination test, Coombs' test, Brucellacapt and ELISA.

Results: Brucellosis was confirmed in 174 patients. In outbreak related cases the tests listed above were positive in 127 (78.88%), 115 (71.43%), 34 (91.98%), 143 (95.33%) and 104 (96.30%), respectively. A statistically significant positive correlation was found between the Coombs' test and Brucellacapt in the studied 55 sera samples ($r_s = 0,72$; $p < 0,0000$). The profile of anti-*Brucella* antibodies was investigated in 46

patients with different duration of the disease. In 29 (63.0%) of them the initial serum sample was positive for the three classes of antibodies and in 17 (36.9%) the primary testing didn't detect IgM, but detected IgG and IgA.

Conclusion: None of the serological tests alone could ensure an accurate diagnosis of brucellosis. It is necessary to use a diagnostic algorithm with appropriately selected serological tests in which Brucellacapt could successfully replace the Coombs' test for detection of incomplete antibodies in brucellosis with long duration.

Keywords: *brucellosis, serodiagnosis, incomplete antibodies, outbreak*

INTRODUCTION

Brucellosis is one of the most widespread endemic zoonosis with more than 500 000 new human cases per year, but it is still considered a "neglected infection" [1-4]. Most of the EU Member States, especially in Western Europe have brucellosis-free status. They detect annually a small number of imported cases. In 2017, 381 confirmed human brucellosis cases were reported in the EU/EEA. The notification rate was 0,09 cases per 100 000 population, with the highest rates reported in southern European countries (Greece, Italy and Portugal) [5]. On the Balkan Peninsula high incidence is observed in Albania, North Macedonia, Kosovo, and Bosnia-Herzegovina [2, 6-9]. Greece still has the highest incidence rate of human brucellosis among EU countries. The average incidence per 100 000 of population for the period 2007- 2012 was estimated to 1,43 [10].

In Bulgaria, brucellosis is a re-emerging infection. In 2005 an outbreak of human cases resulting after exposure outside the country was detected [11, 12]. Although most of the imported cases in non-endemic European countries are associated with travel to endemic regions or consumption of unpasteurized dairy products [13, 14], brucellosis still remains an occupational hazard [15]. The majority of the reported human cases in Bulgaria in 2005 resulted from an occupational contact

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with infected animals in a neighboring endemic country [11]. Moreover, during the next ten years (2005-2015) as a result of “illegal imports” of infected small ruminants, two autochthonous outbreaks were reported [12, 16, 17].

Aim. To conduct a serological study among Bulgarian citizens with clinical and/or epidemiological data compatible to brucellosis for the period 2005-2015.

MATERIALS AND METHODS

Human sera

A total of 3500 serum samples were tested at the National Reference Laboratory for Special Bacterial Pathogens. They were collected along with a standard questionnaire and divided into three panels based on clinical and epidemiological data. Laboratory criteria adopted by the European Centre for Disease Prevention and Control were applied for confirmation of brucellosis [5]. The first panel (Group 1) contained 2792 serum samples from patients in whom brucellosis was suspected. The second (Group 2) consisted of 602 serum samples from healthy persons who were residents of regions where brucellosis outbreaks were detected. They had occupational contacts with infected animals (veterinarians, farmers, stock breeders) and/or consumed unpasteurized milk and dairy products. The control panel (Group 3) contained 106 sera samples from patients tested for other infections and without any symptoms compatible to brucellosis.

The first serum sample taken from each person was defined as the initial one, and the tests performed as the primary ones. All sera were divided to aliquots and kept at -20°C during the whole period of investigation.

Questionnaire

Clinical and epidemiological data were collected via standard questionnaires designed by us which provided information about risk factors like occupation, nutritious habits, and travel history (https://ncipd.org/images/UserFiles/File/OOI/QUESTIONNAIRE_BRUCELLOSIS.pdf) The symptoms listed were: fever, headache,

night sweats, muscle pain, back and joint pains, weakness, anorexia, loss of weight.

Serological tests

Rose Bengal slide test (RBST) - BioSystems, Spain. The result was considered as a positive one when a visible agglutination appeared until the fourth minute.

Standard agglutination test (SAT) - BulBio, Bulgaria. Sera samples with titers $\geq 1:160$ were considered as positive.

Anti-human globulin test (Coombs' test). SAT was performed according to the manufacturer's instructions, except that any tube negative for agglutination, was further centrifuged at 3000 g for 20 minutes and washed three times with PBS (pH 7,2). The remaining antigen was resuspended in 1 ml of PBS and 0,05 ml of standardized anti-IgG human immunoglobulin (BulBio) was added to each tube. After that, the tube content was mixed and incubated at 37°C for 24 h. The highest serum dilution showing >50% agglutination, was accepted as the agglutinating titer.

Brucellacapt - Vircell, Spain. Sera with titers $\geq 1:320$ were considered as suggestive for brucellosis.

ELISA test for detection of IgM, IgG and IgA class antibodies against *Brucella* - IBL, GmbH, Germany. Serum samples were processed as described by the manufacturer. According to the kit protocols we accept 10 U/ml as a cut-off value for each IgM, IgG, and IgA.

Statistical analysis

Statistical software MEDCALC for evaluation of diagnostic tests was used to calculate sensitivity and specificity. The Spearman Rho test coefficient (r_s) was used to establish the correlation between titers obtained with Coombs' and Brucellacapt tests.

RESULTS

For a period of eleven years a total of 3500 Bulgarian residents were serologically tested for brucellosis. Their distribution by groups was based on the analysis of the completed questionnaires and is presented in Table 1 along with the 6860 tests performed for all groups.

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Table 1. Total number of tested persons and serological tests performed for the period 2005–2015.

No	Outbreak related cases (Group 1)	Sporadic cases (Group 1)	Persons with risk factors (Group 2)	Control group (Group 3)	Total
Persons	2361	431	602	106	3500
Serological tests	5214	1021	647	318	6860

The primary testing for persons with risk factors, but without clinical manifestation (Group 2), as well as the controls (Group 3) did not show any positive results for brucellosis (data not shown).

One hundred and seventy-four (6.23%) of the initial serum samples in Group 1, were positive for brucellosis. Data for the type and number of serological tests performed on them, are shown in Table 2.

Table 2. Serological tests of persons with clinical and/or epidemiological data for brucellosis (Group 1).

Test	Sporadic cases	Outbreak related cases	Total
RBST	431	2361	2792
SAT	285	1259	1544
Coombs' test	nt*	230	230
Brucellacapt	267	739	1006
ELISA	17	306	323
Total	1000	4895	5895

* not tested

Thirteen of the positive responders (7,47%), were persons suspected for brucellosis, but found in the non outbreak period 2009-2014 (Table 3). Due to lack of a proven link with one of the three outbreaks registered in Bulgaria, they were excluded from further discussion.

Table 3. Positive results for brucellosis in sporadic cases (2009-2014).

Test	Total number	Number of positive (%)
RBST	13	13 (100)
SAT	13	9 (69.23)
Brucellacapt	13	13 (100)
ELISA	6	5 (83.33)

The epidemiological data showed that the remaining 161 (92.53%) patients were related to one of the above mentioned outbreaks. The primary testing of each person was performed with at least two of the serological tests described above. The initial sera were positive in RBST, SAT, Coombs, Brucellacapt and ELISA for 127 (78.88%), 115 (71.43%), 34 (91.90%), 143 (95.33%) and 104 (96.30%) of the patients, respectively (Table 4).

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Table 4. Positive results for brucellosis in epidemically related cases from the total number of primary testing for a given method.

No test	2005		2006-2008		2015		2005-2015	
	Total	Positive (%)	Total	Positive (%)	Total	Positive (%)	Total	Positive (%)
RBST	37	8 (21.62)	88	83 (94.31)	36	36 (100)	161	127 (78.88)
SAT	37	11(29.73)	88	71 (80.68)	36	33 (91.66)	161	115(71.43)
Coombs	37	34 (91.89)	nt	nt	nt	nt	37	34 (91.89)
Brucellacapt	26	21 (80.77)	88	87 (98.86)	36	35 (97.22)	150	143 (95.33)
ELISA	37	35 (94.59)	47	45 (95.74)	24	24 (100)	108	104 (96.30)

The immunoglobulin profile of anti-*Brucella* antibodies was studied in 46 of the patients with different duration of the disease. From 2 to 4 (mean 2,36) samples for each person were tested with ELISA IgG, IgM and IgA. Twenty-nine (63.0%) of the initial serum sample were positive for all three immunoglobulin classes and in the remaining 17 (36.9%) the primary testing didn't detect IgM, but detected IgG and IgA. (Table 5)

Table 5. Distribution of anti-*Brucella* antibody classes in 46 patients with confirmed brucellosis.

Immunoglobulin classes	No of persons	No of positive (%)
IgM	46	29 (63,0)
IgG	46	46 (100,0)
IgA	46	46 (100,0)

The correlation between Coombs' and Brucellacapt tests was studied in 55 sera from patients with different duration of the disease. The relation between titers in both tests is summarized in Table 6.

Table 6. Relation between titers in Brucellacapt and Coombs tests.

Coombs' titer*	Brucellacapt									
	20	40	80	160	320	640	1280	2560	5120	Total
20				1						1
40										-
80		1**	1							2
160	2	1	1	2	3		1			10
320	2	3	1	1	2	1				10
640		1	1	6	6	3				17
1280		1		3	1	3	2	1	1	12
2560					1	1	1			3
5120										
Total										55

* inverse titers; ** numbers in bold - titers within a range of ± two dilutions

Thirty-eight sera or 69,1% had similar titers in both reactions varying within two dilutions. We found that there is a statistically significant positive correlation between the two methods. ($r_s = 0,72$; $p < 0,0000$).

DISCUSSION

Brucellosis is a widespread zoonotic infection with significant health and socio-economic impacts. Laboratory confirmation could be obtained by different methods, namely cultivation, detection of nucleic acids or specific antibody response. Due to prolonged and hazardous cultivation procedures, the diagnosis is mainly based on serology.

RBST is a rapid slide agglutination test used as a screening test for human brucellosis. It is highly sensitive due to its ability to detect all three classes of immunoglobulins. The low pH (3.6-3.7) of the *B. abortus* suspension reduces the non-specific antigen-antibody bindings and contributes to the successful detection of incomplete (blocking) antibodies [6, 7]. False negative results are rare [8]. Its specificity varies in a wider range depending on the prevalence of the disease in different regions, and is lower in endemic areas [9]. In serological tests based on the detection of anti-lipoplysaccharide LPS antibodies, such as RBST, the lower specificity could also be due to the presence of cross-reactivity with other Gram-negative bacteria [10]. For these reasons, positive RBST results should be confirmed by other serological tests [6, 8]. In the 161 patients diagnosed with brucellosis, RBST was positive in 127 (78,88%) of them. The percentage varies widely for persons linked to each of the three outbreaks - 21.62%, 94.31% and 100% respectively. The smallest number of positive RBST results was obtained for sera collected during 2005, when imported cases were detected in the country. Most patients were late diagnosed (6-37 months after onset of symptoms) and were with long duration of the disease. Other authors also mention a lower sensitivity of the test in such cases [8,

11]. In contrast, patients linked to the second autochthonous outbreak (2015), were diagnosed up to six months from the onset of disease (acute or sub-acute brucellosis), and they were all positive (100%). These results confirm the established practice of using RBST as a screening test, as well as the need for further testing with other serological tests, especially in cases with long-term evolution of the disease, when the sensitivity of the test is significantly lower.

The standard agglutination test (SAT) is considered a reference method in the serological diagnosis of brucellosis [12]. According to generally accepted criteria, values $\geq 1:160$ are assumed as a diagnostic titer [13, 14]. The sensitivity and specificity of SAT vary widely from 32.5 - 100% and 96.4% - 100%, respectively, and specificity is lower in endemic areas due to the above-mentioned high seroprevalence [15-18]. The results in our study show that 115 or 71.43% of epidemically related individuals had titers $\geq 1:160$ in SAT (Table 4). The sensitivity and specificity of the agglutination reaction at thus accepted limit value are 71,43% and 100%, respectively. Similar data are reported in studies by other authors [19-22], as the percentage of positive SAT results varies depending on the phase of the disease and is higher in active brucellosis.

For the remaining 46 persons in whom brucellosis was confirmed by other methods, SAT titers were $\leq 1:80$ (data not shown).

Brucellosis is a chronic-recurrent disease in the later phase of which the so-called incomplete (blocking) antibodies appear. At this stage of the disease, SAT does not reach diagnostic values or is negative. In order to avoid the "blocking" phenomenon, an anti-human globulin (Coombs') test should be included in the diagnostic scheme [12, 23]. It detects the non-agglutinating antibodies, thus showing higher titers than SAT. We found out that titers in Coombs' reaction exceeding those in SAT by two to five times. These data indicate that the anti-globulin test has a higher sensitivity than the classical

agglutination test in brucellosis cases of longer duration. In our study the sensitivity and the specificity are 91,89 % and 100 %, respectively. However, the method is time consuming and requires experience. Therefore, it is preferable to use Brucellacapt, which is an one-step test based on the detection of both agglutinating and non-agglutinating antibodies of class IgG and IgA in the serum [24]. The sensitivity of Brucellacapt is 95,1% - 97,3%, and its specificity is 97% -99%, as the diagnostic titer ($\geq 1: 320$) correlates with the clinical characteristics of the patients [16, 24-26]. We obtained similar results of 95,33% sensitivity and 100% specificity.

In order to replace the Coombs' test with Brucellacapt we studied the correlation between the two tests in 55 serum samples of patients with different disease duration. Based on our results we found a statistically significant positive correlation between the two methods. Data on good correlation and replaceability of the two tests are also reported by other researchers [16, 23]. Our conclusion is that Brucellacapt could be an alternative to Coombs' for serological testing of patients for brucellosis and should be included in the overall set of tests for serological detection of the various stages of the disease.

The ELISA method has been suggested by many authors as a suitable one for monitoring the course of infection, and distinguishing between acute and chronic phases of the disease [6]. Usually, IgM appears first, followed by IgG and IgA, which also appear soon after the onset of infection [27, 28]. The further profile of anti-*Brucella* antibodies depends on the response of the macroorganism to treatment. In clinical recovery, antibody titers begin to decline gradually after the first month, most rapidly IgM, while IgA and especially IgG persist longer. Ariza et al. [39] report that 12 months after a successful treatment, ELISA titers remain positive in 25%, 69%, and 89% for IgM, IgA, and IgG, respectively. The same authors observe a correlation in SAT and IgM titers, which decline in the first six months after a successful treatment, and the

evolution of IgG titers is similar to those in the Coombs' test. The role of IgA antibodies is not so clear. They also appear early in the course of the infection and persist after successful therapy, but for a shorter period than IgG. Moreover, the three classes of immunoglobulin could be observed in the convalescent period in patients with a good therapeutic outcome and negative blood cultures [29]. It is difficult to predict a relapse of the disease based on serological methods alone, but usually in such cases there is a re-peak of IgA and IgG antibodies, but not IgM which correlates with results from the anti-globulin tests [15, 24, 30, 31]

In order to study the patients' immune response in the course of the disease, we selected 46 persons with different durations of the disease, all of them positive in ELISA. Brucellosis was also confirmed by some of the other serological tests (SAT; Coombs' and/or Brucellacapt), isolation of the etiological agent from blood, and/or detection of *Brucella* DNA in the sample (data not shown). Of the 29 individuals positive for all three classes of immunoglobulin, 27 were with disease duration up to 12 months. In 22 (81,48%) of the latter, ELISA IgM titers declined and became negative within 2-6 months from the start of the follow-up. In the rest 5 patients, antibodies continued to be detected by the end of the follow-up for an year. Similar data for persistence of IgG and IgM immunoglobulins from months to several years in 15-20% of the asymptomatic patients after treatment have been reported by other authors. [32]. We also detected IgM antibodies in two patients with a delayed diagnosis by 36 and 41 months, respectively. Both patients were with a focal infection (orchitis) as the clinical presentation of the disease at the time of diagnosis. Our assumption for these patients is that given the long duration of the disease, it could be a question of memory depleting of immunocompetent cells and re-inducing a primary immune response as a result of antigenic stimuli from the infectious

focus. Undoubtedly, this assumption requires confirmation through serological testing of more similar clinical cases.

CONCLUSION

Culture procedures for isolation of brucellae are the “gold standard” for confirming the disease in humans. However, they are time consuming and highly hazardous, as well as successful in small percentage. For this reason, in most cases the diagnosis is based on serological evidence of infection. It should be always kept in mind, that due to the unspecific clinical presentation of the disease and the complexity of the immune response in brucellosis, it is necessary to use an appropriately selected set of reliable serological tests.

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