academicJournals

Vol. 7(13), pp. 1109-1113, 26 March, 2013
DOI: 10.5897/AJMR12.1623
ISSN 1996-0808 © 2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

The pattern of autoantibodies related to false negative results of serological tests in the diagnosis of human brucellosis

Zeki Yumuk[1]*, Çiğdem Çağlayan[2], Cavit Isık Yavuz[2], Zeynep Ocak[1] and Onur Hamzaoğlu[2]

[1]Department of Medical Microbiology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey.
[2]Department of Public Health, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey.

Accepted 25 March, 2013

For the diagnosis of human brucellosis it is essential to perform diagnostic tests because of the wide variety of clinical manifestations of the disease. Serological tests are widely used for the diagnosis of brucellosis. However, blocking substances limit the use of standard tube agglutination test. Two hundred and forty two (242) serum samples were collected from healthy persons and persons in high-risk occupation. For each sample, the rose Bengal, IgM ELISA, IgG ELISA, serum tube agglutination test (SAT) and anti-nuclear antibody (ANA) tests were performed. Three sera in which rose Bengal or ELISA and ANA tests yielded positive results were having lower level of SAT titer. By an *in vitro* experiment, efficacies of ANA nucleolar pattern to the false-negative reaction in the SAT were supported.

Key words: Human, brucella, serology, autoimmunity, test performance.

INTRODUCTION

Brucellosis remains a worldwide veterinary and medical disease contributing to significant health and economic problems (Dean et al., 2012b). Because the symptoms (fever, headache, fatigue, and arthralgia) of brucellosis are nonspecific, it is generally included in the differential diagnosis of various rheumatic diseases such as systemic lupus erythematosus and rheumatoid arthritis (Mert et al., 2003). The relationship between autoimmunity and brucellosis is not clear and there are very limited publications about this interaction (Kojan et al., 2012). Antinuclear antibodies could be found in the sera of a brucellosis patient during the active stage of the disease (Gotuzzo et al., 1985) and those autoantibodies might be related to false negative results of serological tests for brucellosis (Yumuk et al., 2007).

The laboratory tests are usually required to diagnose brucellosis (Mantur et al., 2007). Culture of brucella microorganisms from body specimens is time consuming and is often unsuccessful particularly in the chronic form (Mantur et al., 2007). Thus the diagnosis of chronic brucellosis depends largely on serologic tests. However, the value of agglutination tests are severely limited by the unacceptable high proportion of false negative results (Araj et al., 1986). Blocking agglutination reactions are accounted for the failure to detect agglutinins in the serum of patients with brucellosis (Hall and Manion, 1953; Huddleson et al., 1945; Young, 1991; Zinneman et al., 1959). Blocking antibodies appear during the subacute stage of infection, tend to persist for many years independently of activity of infection, and are detected by the Coombs antiglobulin method (Brooks et al., 2007).

*Corresponding author. E-mail: yumuk@me.com. Tel: +90-2623037448.

Determination of ANA, a heterogeneous group of autoantibodies against nuclear antigens, is useful for predicting some connective tissue diseases. ANA may occur in both physiologic and pathologic conditions. From the standpoint of laboratory diagnosis, autoantibodies are relatively common in humans without autoimmune disease. If sufficiently sensitive methods are used, autoantibodies may well occur universally as a normal mechanism for purging the body of effete cell products. Such (naturally occurring autoantibodies) are usually present in low titer, have relatively poor affinity for their corresponding antigen and largely belong to the IgM class. Such is not always the case, however; sometimes IgGs with reasonable binding affinities and elevated titers are present even in the absence of disease (Rose, 1996). The initial autoantibody screening test is usually by immunofluorescence on HEp-2 cells. HEp-2 cells allow recognition of over 30 different nuclear and cytoplasmic patterns that are given by upwards of 50 different autoantibodies. The two most common ANA patterns are homogeneous, speckled and nucleolar staining patterns.

Since, it is known that the autoantibodies are one of the reason for the false negative results of agglutination tests (Gotuzzo et al., 1985), there is no data concerning the detail of autoantibody and agglutination tests interactions in the literature. In this study, we aimed to further investigate the autoimmune aspect of false negative results for the serological diagnosis of brucellosis. We found that anti-nucleolar antibodies, kind of a antinuclear antibody pattern, might be the reason more frequently for the false negative results.

MATERIALS AND METHODS

Two hundred and forty two (242) serum samples were collected from healthy persons and persons in high-risk occupation such as veterinarians, abattoir workers and veterinary laboratory personnel, who had contact with infected animals, their carcasses, or their blood. For each sample, the rose Bengal, IgM ELISA, IgG ELISA, serum tube agglutination test (SAT) and antinuclear antibody (ANA) tests were performed.

The rose bengal test with antigens purchased from Chromatest, Linear Chemical, Spain, was performed and interpreted according to routine methods and criteria. This uses as antigen a dense suspension of smooth Brucella cells stained with rose bengal and suspended in an acid buffer. Briefly, the rose bengal test was performed by mixing a drop of serum spotted on a glass plate with an equal volume of the antigen. The result was read after 2 min.

ELISA tests were performed and interpreted according to the manufacturers' instructions (Virotech Brucella ELISA IgG/IgM test kit, Genzyme Virotech, Rüsselsheim, Germany). All sera were diluted 1:100 with dilution buffer. After 30 min of incubation at 37°C, the plate was washed four times with washing solution, and 100 µl of ready to use conjugate was dispensed into each well. After further incubation for 30 min at 37°C, plates were washed four times with washing solution. The assays were developed by adding 100 µl of substrate solution, and the reaction was stopped after 30 min in the dark by the addition of 50 µl of citrate stopping solution to each well. The color intensity was determined at 450/620 nm.

Virotech Units (VE) were calculated according to the following equation: [(absorbance of patients serum) / (absorbance of cut-off control)] x10. The cut-off value, determined with positive and negative sera, was 10 VE. If the measured values were above 9.0 – 11.0 (borderline value), they were considered to be positive.

For serum tube agglutination test (SAT), dilutions of serum were made in 0.5 ml volumes of phenol saline (0.85% NaCl containing 0.5% phenol) in 65x12 mm round-bottomed agglutination tubes. To each dilution was added an equal volume of Brucella abortus S99 antigen (Seromed Company, Istanbul, Turkey) diluted 1:10. Each serum was titrated to at least 1:2560 to avoid errors due to prozone phenomena. Each batch of tests included a positive 1:1280 control and a negative saline control. The tubes were kept at 37°C for 2 days and read in indirect light. A definite agglutination of the suspension was read a positive reaction. If prozone phenomenon was encountered, the higher dilution agglutination was recorded.

For the Coombs test, the SAT was performed as described above except that any tube containing serum that failed to agglutinate were centrifuged at 2000g for 15 min. The supernatant was discarded and the remaining antigen was re-suspended in PBS. This process of centrifugation and re-suspension was repeated three times. After the final wash, the cells were re-suspended in 0.9 ml of suitably diluted goat antihuman globulin (AHG) was added to each tube. After thorough mixing of their contents, the tubes were incubated at 37°C for 24 h and reexamined for agglutination.

Anti-nuclear antibody (ANA) and related patterns were determined by indirect immunofluorescence using HEp-2 cells (Euroimmun, Lubeck, Germany). Briefly, all sera were diluted 1:100 with phosphate-buffered saline; those positive for ANA were diluted further to 1:500 or 1:1000. Titer between these dilutions was estimated from the staining intensities. Microscopy was performed by a microbiologist. For evaluation of fluorescence intensity and pattern, a fluorescence microscope (Zeiss, Germany) at a magnification of X400 was used.

RESULTS

All sera were screened for ANA by indirect immunofluorescence method. Twelve (5.0%) sera were found to be ANA positive with various pattern and titer such as speckled, homogenous or anti-mitochondrial and titer one, three or five, respectively. Coombs test were also performed to the ANA positive samples. In three samples, which were nucleolar pattern positive, Coombs titer was found to be meaningful with 4 to 16 fold than SAT titer (Table 1).

Of 242 sera from study population, seven (2,9%) were found to be positive with rose Bengal test. Two (28.6%) of seven rose Bengal positive sera were found to be ANA, nucleolar pattern positive. In one sample, although RB was negative, ANA was found to be nucleolar pattern positive. The later was also found to be borderline positive with ELISA IgG. While RB was negative, ELISA IgG borderline and ANA nuclear pattern were found to positive in only one sample (Table 2).

In order to determine the efficacy of autoantibodies to the performance of SAT, three sera with a SAT titer of 1:160 and one sera with a SAT titer of 1:1280 were diluted with a 1:1000 titer nucleolar pattern positive serum

Table 1. Positive anti-nuclear antibody samples and corresponding serological test results.

No. of Serum	ANA**		Serologıcal tests				
			ELISA (VE)*		RB	SAT (Titer)	Coombs
	Pattern	Titer	IgG	IgM			
15	Nucleolar	+	1.7	2.4	Pos	1:40	1:640
17	Speckled	+	0.3	0.4	Neg	1:20	1:40
23	Homogenous	+	6.6	1.4	Neg	1:20	1:40
35	Anti-mitochondrial antibodies	++++	1.8	1.2	Neg	1:40	1:40
51	Anti-mitochondrial antibodies	+	0.5	0.3	Neg	1:40	1:80
60	Speckled	+	0.3	0.6	Neg	1:40	1:80
72	Nucleolar	+	9.8	0.7	Neg	1:80	1:320
89	Nucleolar	+++	10.9	2.4	Pos	1:40	1:320
122	Nucleolar	+	0.3	5.1	Neg	1:40	1:80
125	Homogenous	+	0.2	1.1	Neg	1:20	1:40
129	Speckled	+	0.1	1.4	Neg	1:40	1:80
189	Homogenous	+++++	0.7	1.8	Neg	1:20	1:40

*VE: Virotech Units; According to the manufacturer's instructions, Borderline: 9 – 11 VE; Positive: >11 VE; Negative: <9 VE. ** ANA: anti-nuclear antibody; Pos: Positive; Neg: Negative.

Table 2. Brucellosis positive samples with serological tests and corresponding ANA results.

No of Serum	Serologıcal tests				ANA**
	ELISA (VE)*		RB	SAT	
	IgG	IgM			
15	1.7	2.4	Pos	1:40	Nucleolar
29	10.7	2.3	Pos	1:160	Neg
55	24.8	1.7	Pos	1:160	Neg
72	9.9	0.7	Neg	1:40	Nucleolar
74	20.4	24.7	Pos	1:1280	Neg
76	11.8	1.9	Neg	1:40	Neg
86	14.7	1.1	Neg	1:40	Neg
89	10.9	2.4	Pos	1:20	Nucleolar
105	16.3	1.7	Pos	1:80	Neg
182	17.7	2.5	Pos	1:160	Neg

*VE: Virotech Units; According to the manufacturer's instructions, Borderline: 9 – 11 VE; Positive: >11 VE; Negative: <9 VE. ** ANA: anti-nuclear antibody. Pos: Positive; Neg: Negative.

sample, each at a ratio of 1/4. ANA-positive serum were semi-quantitated by diluting the sera to 1:1000 by saline solution. Coombs test were performed to the sera which were found to be negative. The ANA nucleolar pattern positive serum that was used in this study was obtained from a patient who was known to have an autoimmune disease. After dilution of SAT positive samples of various titers with nucleolar pattern positive samples, SAT titers were decreased to the titer of 1:10. Thereafter, Coombs test were revealed 5 to 16 fold of beginning SAT titer (Table 3).

DISCUSSION

In the absence of culture facilities, the diagnosis of brucellosis traditionally relies on serological testing with a variety of agglutination tests such as the Rose Bengal test, the serum agglutination test (SAT), and the antiglobulin or Coombs' test (Franco et al., 2007: Dean et al., 2012a). This is mainly because the greatest incidence of brucellosis is found in underdeveloped countries with poor technical resources, as well as the fact that it tends to occur in rural communities (Ruiz-Mesa et al., 2005).

Table 3. Efficacy of anti-nuclear antibody to the SAT titer in serum sample.

No. of serum	Samples diluted with phenol saline	Samples diluted with ANA positive serum and phenol saline	
	SAT titer	SAT titer	Coombs titer
29	1:160	1:10	1:2560
55	1:160	1:10	1:2400
74	1:1280	1:10	1:6400
182	1:160	1:10	1:2560

In this study, anti-nuclear antibodies (ANA) were considered as a reason for false negative results of the serological tests (Tables 2 and 3). The reason for the adverse effects of ANA on the diagnostic yield of the agglutination tests might be related to structural similarity (molecular mimicry) between microbial and self-antigens (Lernmark, 2001). Besides their structural similarity, auto- and blocking antibodies both appear to be consequence of working immune mechanisms (Hall and Manion, 1953; Huddleson et al., 1945; Young, 1991; Zinneman et al., 1959; Brooks et al., 2007; Rose, 1996; Lernmark, 2001; George and Shoenfeld, 1996; Khan et al., 2008; Van Eenennaam et al., 2002).

Autoantibodies, instead of acting against foreign invaders as normal antibodies do, attack the body's own cells. Anti-nuclear antibodies are a unique group of autoantibodies that have the ability to attack structures in the nucleus of cells. Homogeneous, peripheral, speckled and nucleolar are the most commonly recognized patterns by using the fluorescent antinuclear antibody test. The latter, which is also called Anti-nucleolar pattern is a common finding on routine autoimmune screening (Khan et al., 2008) with prevalence of 1.8 to 3.8% (Khan et al., 2008; Van Eenennaam et al., 2002). Although anti-nucleolar activity was found in patients suffering from systemic lupus erythematosus, rheumatoid arthritis, Sjögren syndrome, polymyositis/dermatomyositis, mixed connective tissue disease and primary Raynaud's phenomenon, for some settings it is usually a non-specific finding (Khan et al., 2008).

In the present study, five percent of the population was found to be ANA positive which is comparable with previous studies. In a large scale study, natural autoantibodies were found in 4 to 13% of healthy individuals (George and Shoenfeld, 1996). The prevalence of anti-nucleolar pattern, in this study, was found to be a little less than the prevalence of the previous studies. This might be related to the differences between the study populations.

According to these study results, establishing a diagnosis of brucellosis and prescribing a suitable therapy based on positive serological test in patients with ANA therefore seems not to be justified. Performing ANA testing in presumptive brucellosis cases will be time consuming and laborious and it requires highly trained personnel. Moreover, in resource-poor countries, especially among persons at high risk for brucellosis, ANA testing would most likely be excluded due to cost and/or availability. Therefore, it is suggested that in the diagnosis of human brucellosis, Coombs test might be evaluated in conjunction with ANA testing.

In conclusion, the roles of autoantibodies at the false negative results of serological tests for brucellosis were supported. Also, as a result of this study anti-nucleolar pattern which is one of the most common pattern of anti-nuclear antibodies are found to adversely affect the serological tests for brucellosis. However, to have definite decisions, extensive studies with larger populations are needed.

REFERENCES

Araj GF, Lulu AR, Mustafa MY, Khateeb MI (1986). Evaluation of ELISA in the diagnosis of acute and chronic brucellosis in human beings. J. Hyg. 97:457-469.

Brooks FG, Carrol KC, Buttel JS, Morse SA (2007). Medical Microbiology. In Haemophilus, Bordetella, Brucella & Francisella., pp. 38-42. USA: McGraw Hill (Lange).

Dean AS, Crump L, Greter H, Hattendorf J, Schelling E, Zinsstag J (2012a). Clinical manifestations of human brucellosis: A systematic review and meta-analysis. PLoS Negl Trop. Dis. 6:e1929.

Dean AS, Crump L, Greter H, Schelling E, Zinsstag J (2012b). Global burden of human brucellosis: A systematic review of disease frequency. PLoS Negl. Trop. Dis. 6:e1865.

Franco MP, Mulder M, Gilman RH, Smits HL (2007). Human brucellosis. Lancet Infect. Dis. 7:775-786.

George J, Shoenfeld Y (1996). Natural autoantibodies. In Autoantibodies., eds. Peter JB, Shoenfeld Y, pp. 534-539. Netherlands: Elsevier Science B. V.

Gotuzzo E, Bocanegra TS, Alarcon GS, Carrillo C, Espinoza LR (1985). Humoral immune abnormalities in human brucellosis. Allergol. Immunopathol. 13:417-424.

Hall WH, Manion RE (1953). Comparison of the coombs test with other methods for brucella agglutinins in human serum. J. Clin. Invest. 32:96-106.

Huddleson IF, Wood EE, Cressman AR, Bennett GR (1945). The bactericidal action of bovine blood for brucella and its possible significance. J. Bacteriol. 50:261-277.

Khan S, Alvi A, Holding S, Kemp ML, Raine D, Dore PC, Sewell WA (2008). The clinical significance of antinucleolar antibodies. J. Clin. Pathol. 61:283-286.

Kojan S, Alothman A, Althani Z, Alshehri A, Mansour N, Khathaami A, Haider A, Al-Jumah M (2012). Granulomatous myositis associated with brucellosis: A case report and literature review. Muscle Nerve 45:290-293.

Lernmark A (2001). Autoimmune diseases: Are markers ready for prediction? J. Clin. Invest. 108:1091-1096.

Mantur BG, Amarnath SK, Shinde RS (2007). Review of clinical and laboratory features of human brucellosis. Indian J. Med. Microbiol. 25:188-202.

Mert A, Ozaras R, Tabak F, Bilir M, Yilmaz M, Kurt C, Ongoren S, Tanriverdi M, Ozturk R (2003). The sensitivity and specificity of brucella agglutination tests. Diagn. Microbiol. Infect. Dis. 46:241-243.

Rose NR (1996). Foreword - the use of autoantibodies. In Autoantibodies, eds. Peter JB, Shoenfeld Y, 27-29. Netherlands: Elsevier Science B. V.

Ruiz-Mesa JD, Sanchez-Gonzalez J, Reguera JM, Martin L, Lopez-Palmero S, Colmenero JD (2005). Rose bengal test: Diagnostic yield and use for the rapid diagnosis of human brucellosis in emergency departments in endemic areas. Clin. Microbiol. Infect. 11:221-225.

Van Eenennaam H, Vogelzangs JH, Bisschops L, Te Boome LC, Seelig HP, Renz M, De Rooij DJ, Brouwer R, Pluk H, Pruijn GJ, Van Venrooij WJ, Van Den Hoogen FH (2002). Autoantibodies against small nucleolar ribonucleoprotein complexes and their clinical associations. Clin. Exp. Immunol. 130:532-540.

Young EJ (1991). Serologic diagnosis of human brucellosis: Analysis of 214 cases by agglutination tests and review of the literature. Rev. Infect. Dis. 13:359-372.

Yumuk Z, Afacan G, Caliskan S, Irvem A, Arslan U (2007). Relevance of autoantibody detection to the rapid diagnosis of brucellosis. Diagn. Microbiol. Infect. Dis. 58:271-273.

Zinneman HH, Glenchur H, Hall WH (1959). The nature of blocking antibodies in human brucellosis. J. Immunol. 83:206-212.