ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2016; 2(10): 506-509
www.allresearchjournal.com
Received: 12-08-2016
Accepted: 13-09-2016

Khabibulo Khamdamov
Senior Researcher, Brucellosis Research Laboratory at the Veterinary Research Institute, Samarkand, Uzbekistan

Correspondence
Khabibulo Khamdamov
Senior Researcher, Brucellosis Research Laboratory at the Veterinary Research Institute, Samarkand, Uzbekistan

Development of the first national standard for anti-brucella serum

Khabibulo Khamdamov

Abstract
National anti-Brucella Serum Reference Standard is necessary to ensure that a given antibody assay is capable of measuring antibody activity to a specified level of diagnostic sensitivity. Diagnostic sensitivity relates to the risk of a false negative reaction occurring in an antibody assay when in fact an animal is, or has been, infected. At the same time, plate agglutination test, used for differentiation of the unknown isolates during bacteriology of suspected biomaterials, needs reliable serum capable to reveal and determine antigens of the bacteria. Taking into account the fact that first isolates of brucella cultures can be dissociated to other form than typical "S-form" the first national anti-brucella serum standard against S, R, SR and RS forms of Brucella is developed and tested.

Keywords: Brucellosis, antigen, antibody, sensitivity, serum, Brucella

1. Introduction

1.1 Significance of the problem

Brucellosis is a zoonosis caused by bacteria of the genus *Brucella*, which affect both humans and animals such as cattle, sheep, goats, and swine. This disease is worldwide, with areas of high endemicity such as the Mediterranean, Middle East, Latin America, and Asia. The incidence in humans ranges widely between different regions, with values of up to 200 cases per 100,000 population. Domestic animals brucellosis has a great variety of clinical manifestations, making it difficult to diagnose clinically. Therefore, the diagnosis must be confirmed by isolation of *Brucella*, mostly from culture or by the detection of an immune response to its antigens. The diagnosis of brucellosis based exclusively on *Brucella* isolation presents several drawbacks. The slow growth of *Brucella* in primocultures may delay diagnosis for more than 10 days. Also, culture sensitivity is often low, ranging from 50 to 90% depending on disease stage, *Brucella* species, culture medium, quantity of bacteria, and the culture technique employed. Hence, serological tests play a major role in cases when the disease cannot be detected by microbiological method. However, the interpretation of these tests is often difficult and should be referred to controls - simultaneously conducted tests with standard reagent.

1.2 Worldwide studies on the issue

To eradicate the disease and to qualify for World Organisation for Animal Health (OIE) brucellosis-free or officially free status, it is necessary to conduct serosurveillance. The application of serological testing is also required for the international trade of animals. It is therefore important that serological tests be standardised and harmonised properly so that they provide reliable results.

The first International Standard for anti-Brucella abortus Serum was established in 1952. In 1965, the World Health Organization requested that a second standard be prepared to replace the dwindling stocks of the first. The second OIE International Standard Serum (OIEISS) is currently available and has been applied for the standardisation and harmonisation of some of the tests currently used for the serodiagnosis of domestic animals brucellosis. These include the complement fixation test (CFT) and the Rose Bengal test (RBT). The standard serum was obtained from a cow experimentally infected with *B. abortus strain* 544.

1.3 The purpose of the researches

As it was mentioned above the International Standard for anti-*Brucella abortus* Serum was obtained from a cow experimentally infected with *B. abortus* strain 544. It is therefore not suitable for use in species-specific assays, such as the indirect enzyme-linked immunosorbent assay (iELISA), for species other than cattle. At the same time, plate agglutination test, used for differentiation of the unknown isolates during bacteriology of suspected biomaterials, needs reliable serum capable to reveal antigens of several species of the bacteria genus. Accordingly, the need for a standard prepared with the using S, R, SR and RS forms of different *Brucella* species was identified. Taking into account the fact that first isolates of Brucella cultures can be dissociated to other form than typical "S-form" it was the aim of this project to produce the first National Standard anti-Brucella Serum (ISaBS), and to full evaluate the first national anti- S, R, SR and RS *Brucella* forms serum standard.

2. Materials and Methods

To create a national standard positive anti-*Brucella* serum we used brucellosis strains stored in the brucellosis laboratory museum. To determine the degree of dissociation and for isolation of strains with dissociation R, RS, SR all museum cultures were tested in acriflavine test (0.1% solution). Acriflavine test was a preliminary to primary determine the dissociation of Brucella cultures. Therefore, in order to determine the exact degree of dissociation, we used White-Wilson method of culture dyeing. According to the results of *Brucella* strains tests in acriflavine and White-Wilson methods, from three *Brucella* species - *B. abortus, B. melitensis* and *B. suis* - we have selected 21 strains. R, S, RS and SR cultures suspension of *B. abortus, B. melitensis, B. suis* strains have been inactivated before inoculation to lambs and administered in a volume of 2 ml. For a comparative study of the dynamics of the antibodies formation we tested vaccine strain 19 *B. abortus* (made in Russia, Shchelkovsky Biokombinat) and Rev 1 strain *B. melitensis* (Russia, "Agrovet"), currently used for the vaccination of cattle and small sheep. Firstly, to create ground immunity an antigen was injected subcutaneously, according to the following scheme: Group 1 - vaccine strain 19 *B. abortus*, at a dose of 80 x 10^9 microbe bodies, m.b.; Group 2 - vaccine strain Rev 1 *B. melitensis*, at a dose of 2 x 10^9 m.b.; Group 3 – suspension of S, R, SR, RS form strains at a dose of 200 x 10^9 m.b.; Group 4 - suspension of S, R, SR, RS form strains at a dose of 400 x 10^9 m.b.; Group 5 - vaccine strain 19 *B. abortus*, at a dose of 80 x 10^9 microbe bodies, m.b. with preliminary administration of 2 ml of 2.5% solution of progesterone. On the second inoculation we kept the same dosage of the introducing antigen with the same route – subcutaneously. Third inoculation has been conducted to Groups 3 and 4 by introducing the antigen intravenously on the following dosage: Group 3 – suspension of S, R, SR, RS form strains at a dose of 200 x 10^9 m.b.; Group 4 - suspension of S, R, SR, RS form strains at a dose of 400 x 10^9 m.b. Each group of animals consisted of 1 year male (Group 1-4) and female lambs (Group 5) by five animals in each group. After the antibodies' titers reached the expected threshold, the blood had been separated for serum. It was then pooled and purified with phenol and sterilized by filtration and heating at 58.5 ^{0}C for 30 min. After heating the serum was immediately cooled to 15 ^{0}C and preserved with 0.50% phenol solution.

3. Results

Separated serum samples were studied by Rose Bengal and Tube agglutination test (RBT and TAT) on 7, 15, 25 and 30th day after the first antigen inoculation, on 7 and 15th day after the second inoculation and on 7, 13 and 20 day after third inoculation. The dynamics of titers change after the first and second antigen inoculation is showed on the Table 1.

Table 1: The dynamics of titers change after the first and second inoculation of *Brucella* antigens

Groups	Antigens doses, Subcutaneously	Titers dynamics after the first antigen inoculation								Titers dynamics after the second antigen inoculation			
		On 7th day		On 15th day		On 25th day		On 30th day		On 7th day		On 15th day	
		RBT	TAT	RBT	TAT	RBT	TAT	RBT	TAT	RBT	TAT	RBT	TAT
Group 1	Strain 19 *B. abortus* 80 x 10^9	1:8* ++**	1:2800 ++++	1:8 +	1:2000 ++++	1:8 +	1:400 ++	1:2 +++	1:400 ++	1:16 +	1:800 +++	1:32 +	1:1200 ++
Group 2	Rev 1 *B. melitensis* 2 x 10^9	Native +	1:800 ++	1:4 +	1:400 ++	1:4 ++	1:200 ++	1:2 ++	1:25 ++	1:32 +	1:1600 ++	1:32 +	1:1200 ++
Group 3	Suspension of S, R, SR, RS form 200 x 10^9	1:4 ++	1:1000 +++	1:4 ++	1:1000 ++	1:8 +	1:450 ++	1:2 ++	Negat -	1:16 +	1:800 ++	1:8 +	1:800 ++
Group 4	Suspension of S, R, SR, RS form 400 x 10^9	1:2 ++	1:1600 ++	1:4 +	1:600 ++	1:4 +	1:500 ++	Native ++	1:200 ++	1:4 +	1:800 ++	1:4 +	1:500 ++
Group 5	Strain 19 with preliminary injection of progesterone 2.5%	1:64 ++	1:2800 ++	1:256 +	1:4000 +++	1:64 +	1:1600 +++	1:16 ++	1:800 +++	1:64 +	1:3200 ++	1:32 ++	1:1600 ++

Notes: *Dilution; **Reaction intensity (four + means the highest intensity);

As we can see on the Table 1 on the 30th day after the first antigens inoculation the highest antibodies titers observed at animals sensibilized by strain 19. At the same time, the titers of antibodies amongst these animals have been higher at animals, preliminary received progesterone. It also should be mentioned that this group of animals showed the titer

1:4000 on the 15th day after antigen inoculation. The antibody titer of animals received suspension of S, R, SR, RS forms in dose 200 x 10^9 fell to negative whereas at animals received 400 x 10^9 it was 1:200. After the second sensibilization serum has been tested on 7th and 15th day after antigen inoculation. Again the animals of Group 1 and 5, received strain 19, showed highest level of antibodies, whereas the animals of Group 3 and 4 reached the titers 1:800 and 1:400 respectively. These titers did not match the expected threshold 1:1000 and we conducted the third antigen inoculation to the only Groups 3 and 4 by inoculating the suspension of mixed S, R, SR, RS forms of *B. abortus, B. melitensis and B. suis* strains at a dose of 200 x 10^9 m.b. and 400 x 10^9 m.b. respectively. Results of titers change after the third antigen inoculation are shown on the Table 2.

Table 2: The dynamics of titers change after the third inoculation of *Brucella* antigens

Groups	Antigens doses, Intravenously	Titers dynamics					
		On 7th day		On 13th day		On 20th day	
		RBT	TAT	RBT	TAT	RBT	TAT
Group 3	Suspension of S, R, SR, RS form 200 x 10^9	1:32 ++	1:1200 +++	1:32 +	1:1200 ++	1:18 ++	1:1000 +++
Group 4	Suspension of S, R, SR, RS form 400 x 10^9	1:4 +++	1:600 +++	1:10 ++	1:1000 ++	1:4 +++	1:700 ++

Results of the third antigen inoculation shows that the expected threshold 1:1000 has exceeded on 13th day after intravenously sensibilization of animals. However, on the 20th day these titers have fallen to 1:1000 (at the animals of the second group) and to 1:700. So it was decided to use these animals as serum donors taking blood during the period between 12th and 16th days after intravenously inoculation of antigen with preliminary establishing ground immunity.

The results for the RBT are shows that the highest positive dilution for this test was 1:256 and the lowest dilution was when the serum tested in native form. After the third antigen inoculation RBT was 1:32 and 1:10 respectively which means that the dilution 1:32 should be used as a standard when producing serum against S, R, SR, RS forms of *Brucella*.

4. Discussions

The availability of a national standard serum for standardising diagnostic tests is of importance in guaranteeing quality and providing confidence. Many diagnostic tests are highly effective when performed optimally, whereas suboptimal performance can lead to poor decision-making coupled with over-confidence. An international standard serum for use with serodiagnostic tests for bovine brucellosis has long been available, and this has helped to facilitate effective surveillance and transboundary trade. Although this serum has been, and is, used successfully to standardise tests that are not specific to an animal species (such as the RBT and CFT), tests that are specific to 3 main species of the *Brucella* Genus have not been standardised previously because of the lack of a specific international standard serum. The new serum against three main *Brucella* species: *B. abortus, B. melitensis and B. suis* obtained with the use of S, R, SR, RS forms solves this problem and it may also be used to standardise some tests that are not specific for a particular animal species when they are applied to different samples.

This paper reports three important aspects. First, it provides provenance for the polyvalent serum itself. Secondly, it shows the behaviour of the standard when tested with a range of commonly used brucellosis tests and test reagents. Finally, the minimum acceptable levels of standardization with the use of this serum are met the OIE criteria. The working criteria for the standard serum were defined in accordance with the principles already established for the OIEISS, whereby one standard is used to define positive and negative results, and for the OIEELISASP/WP/NSS as defined in Council Directive 64/432 (annex C) of the European Union (6), where dilutions of the standards are used to determine a limit to sensitivity.

In imperfect diagnostic assays increased sensitivity may result in decreased specificity. This is of particular relevance in the case of brucellosis where infection with Gram-negative bacteria possessing antigens of similar structure to Brucella can cause false-positive results in diagnostic tests. Therefore it has been considered reasonable to limit the analytical sensitivity of the different tests for brucellosis with the objective of minimising false-positive results. Analytical sensitivity is the ability of an assay to detect the presence of small quantities of analyte, and is distinct from diagnostic sensitivity, which is the ability of the assay to identify samples from infected hosts correctly. This has been the main reason for establishing the maximum analytical sensitivity threshold. The drawback of this approach is that the use of such criteria may lead to future complications due to, for example, the introduction of new tests with greatly improved sensitivity and specificity.

5. Conclusions

The use of this standard will provide a very clear, transparent and measurable method of standardising tests. The use of sera calibrated to this standard could also assist in the quality control of reagent production and day-to-day test performance. All this is especially important for effective international trade testing and surveillance programmes. However, it is important to recognise that the defined criteria for the use of this standard represent only the minimum and maximum criteria for analytical sensitivity. Accordingly, their use in no way replaces or abrogates the requirement for all assays to be validated properly for diagnostic performance (i.e. with respect to diagnostic sensitivity and specificity), preferably in accordance with the OIE guidelines.

This standard should be used as a prototype for the production of national or secondary standards. It is hoped that its use will help to improve and regulate the quality of domestic animals serodiagnosis and bacteriology and that this will help in turn to limit the spread of brucellosis.

6. References

1. Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the brucellosis laboratory. Bacteriological methods: Institut National de la Recherche Agronomique, Paris, France.-1988, 13-61.
2. Blasco JM. A critical step for decision makers: control or eradication of Brucella melitensis? In Brucella melitensis in Eurasia and the Middle East. FAO technical meeting in collaboration with WHO and the OIE, May Rome. Animal Production and Health Division, Food and Agriculture Organization of the United Nations, Rome, 2009.
3. Corbel MJ. Recent advances in the study of Brucella antigens and their serological cross-reactions. Vet. Bull., 1985; 55:927-942.
4. Davidson I, Herbert CN, Brinley Morgan WJN. The Second International Standard for anti-Brucella abortus Serum. Bull. WHO, 1969; 40:129-140.
5. European Food Safety Authority/European Centre for Disease Prevention and Control – The Community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. EFSA J. 2009; 223:192.
6. European Union. Commission Decision 2008/984/EC of 10 December 2008 amending Annex C to Council Directive 64/432/EEC and Decision 2004/226/EC as regards diagnostic tests for bovine brucellosis. Off. J Eur. Union, 31/12/2008; L352:38-45.
7. Miller GE. Asymptotic test statistics for coefficients of variation. Communic. Statist. Theor. Meth. 1991; 20:2251-2262.
8. Murray PR, Corbel M. Brucella. In Topley and Wilson's microbiology and microbial infections: bacteriology (S.P. Boriello, P.R. Murray & G. Funke, eds), 10th Ed. Edward Arnold, London, 2007, 1719-1751.
9. Palmer DA, Douglas JT. Analysis of Brucella lipopolysaccharide with specific and cross-reacting monoclonal antibodies. J. clin. Microbiol. 1989; 27(10):2331-2337.
10. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet infect. Dis., 2006; 6:91-99.
11. Refai M. Incidence and control of brucellosis in the Near East region. Vet. Microbiol. 2002; 90:81-110.
12. Robinson A. Brucella melitensis infections: foodborne versus animal contact. In Brucella melitensis in Eurasia and the Middle East. FAO technical meeting in collaboration with WHO and the OIE, May Rome. Animal Production and Health Division, Food and Agriculture Organization of the United Nations, Rome, 2009.
13. World Health Organization (WHO). WHO Technical Report Series No. 68. Sixth report of the WHO Expert Committee on Biological Standardization. WHO, Geneva. 1953, Available at: http://whqlibdoc.who.int/trs/WHO_TRS_68. pdf.
14. World Organisation for Animal Health (OIE). Chapter 1.1.4.: Principles of validation of diagnostic assays for infectious diseases. In Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Ed. OIE, Paris, 2008, 34-45.
15. World Organisation for Animal Health (OIE). Chapter 2.4.3.: Bovine brucellosis. In Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Ed. OIE, Paris, 2008, 624-659.
16. World Organisation for Animal Health (OIE). Chapter 2.7.2.: Caprine and ovine brucellosis (excluding Brucella ovis infection). In Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Ed. OIE, Paris, 2008, 974-982.
17. World Organisation for Animal Health (OIE). OIE Guide 3: International reference antibody standards for antibody assays. In OIE quality standard and guidelines for veterinary laboratories: infectious diseases, 2nd Ed. OIE, Paris, 2008, 45-52.
18. World Organisation for Animal Health (OIE). Chapter 14.1.: Caprine and ovine brucellosis (excluding Brucella ovis). In OIE Terrestrial Animal Health Code, 18th Ed. OIE, Paris, 2009, 679-684.
19. Wright PF. International standards for test methods and reference sera for diagnostic tests for antibody detection. In Veterinary laboratories for infectious diseases (J.E. Pearson, ed.). Rev. sci. tech. Off. int. Epiz. 1998; 17(2):527-533.
20. Wright PF, Tounkara K, Lelenta M, Jeggo MH. International reference standards: antibody standards for the indirect enzyme-linked immunosorbent assay. Rev. sci. tech.Off. int. Epiz. 1997; 16(3):824-832.