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Brucellosis, *Brucella abortus* S19, diagnostic agglutinating antiserum, hyperimmunization, immunoglobulins, immunological evaluation, morphological evaluation, rabbit, serological diagnosis

Authors:
[1*]Yusupov Akmal Pulatovich
Alfraganus University, Department of Medicine, PhD (Doctor of Philosophy),
Associate Professor, Tashkent, Uzbekistan.
E-mail: a.yusupov@afu.uz
ORCID:https://orcid.org/0009-0007-4167-5433

[2]Tuychiev Laziz Nadirovich
[a]Department of Infectious and Children's Infectious Diseases, MD, Professor Tashkent State Medical University, Tashkent, Uzbekistan.
[b]Research Institute of the Republican Specialized Scientific and Practical Medical Center of Epidemiology, Microbiology, Infectious and Parasitic Diseases, Tashkent, Uzbekistan.
E-mail: l_tuychiev@mail.ru
ORCID:https://orcid.org/0000-0003-2312-8640

[3]Khudaykulova Gulnara
Head of the Department of Public Health & Healthcare Management, DSc, Professor. Tashkent State Medical University, Tashkent, Uzbekistan.
E-mail: gkhudaykulova@gmail.com
ORCID:https://orcid.org/0009-0003-2238-7373

[4]Rakhmatullaeva Shakhnoza Bakhadirovna
Professor, Department of Infectious and Children's Infectious Diseases, Doctor of Medical Sciences (DSc), Tashkent State Medical University, Tashkent, Uzbekistan.
E-mail: Doctor_shakhnoza@mail.ru
ORCID:https://orcid.org/0000-0001-7257-2081

[5]Muminova Makhbuba Teshayevna
Department of Infectious and Children's Infectious Diseases, PhD, associate professor
Tashkent State Medical University, Tashkent, Uzbekistan.
E-mail: maxbubamuminova515@gmail.com
ORCID:https://orcid.org/0000-0001-8194-8052

[6]Akhmedova Oydin Anvarovna
Assistant, Department of Infectious and Children's Infectious Diseases, Tashkent State Medical University, Tashkent, Uzbekistan.
E-mail: akhmedovaoydin80@gmail.com
ORCID:https://orcid.org/0009-0005-8635-1454

[7]Ikramov Umirzoq Eminjonovich
Assistant, Department of Infectious and Children's Infectious Diseases, Tashkent State Medical University, Tashkent, Uzbekistan.
E-mail: umrzoqikromov1994@gmail.com
ORCID:https://orcid.org/0009-0000-8336-1888

[8]Saidova Sitora
Assistant, Department of Infectious and Children's Infectious Diseases, Tashkent State Medical University, Tashkent, Uzbekistan.
E-mail: s.saidova@tashmeduni.uz
ORCID:https://orcid.org/0009-0007-4882-2854

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Immunological and Morphological Assessment of Diagnostic Agglutinating Sera for Brucellosis

Abstract
The aim of this study was to obtain hyperimmune diagnostic agglutinating antiserum against brucellosis by hyperimmunizing experimental rabbits with the live vaccine strain *Brucella abortus* 19 (BA19) in combination with incomplete Freund's adjuvant, and to evaluate the morphological changes occurring in the liver, spleen, and kidneys during the immunization process. The production of specific antibodies in the obtained antisera was assessed using serological methods, while morphological alterations in the internal organs were investigated by macroscopic and histological examinations. The results demonstrated that hyperimmunization induced the production of high-titer diagnostic hyperimmune antiserum in rabbits. In addition, active immunogenesis was observed in the spleen, whereas predominantly reversible morphological changes developed in the liver and kidneys. These findings provide a scientific basis for the development of high-quality diagnostic agglutinating antisera for the serological diagnosis of brucellosis and contribute to a better understanding of their associated morphological characteristics.

Introduction

Brucellosis is an anthropozoonotic infectious disease caused by Gram-negative coccobacilli of the genus Brucella and remains one of the most significant zoonotic diseases affecting both human and animal health worldwide [1]. According to the World Health Organization (WHO), more than 500,000 new cases of brucellosis are reported annually, with the disease remaining endemic in Central Asia, the Middle East, the Mediterranean region, and Latin America [2,3].

In Uzbekistan, brucellosis continues to represent a major challenge for both the veterinary and public health sectors. The rapid development of livestock farming, close contact between rural populations and domestic animals, and insufficiently developed sanitary and epidemiological surveillance systems in certain regions contribute to the persistent risk of disease transmission [4]. The disease is primarily caused by Brucella melitensis in sheep and goats, Brucella abortus in cattle, Brucella suis in pigs, and Brucella canis in dogs. Human infection occurs mainly through direct contact with infected animals or their products, ingestion of contaminated food of animal origin, and inhalation of infectious aerosols [5,6].

Early and accurate diagnosis of brucellosis is essential for effective epidemiological surveillance, prevention of disease transmission, and timely treatment of infected individuals. Among the currently available diagnostic approaches, serological assays, including the Rose Bengal test, the Wright standard tube agglutination test (SAT), and enzyme-linked immunosorbent assay (ELISA), are the most widely used methods for the laboratory diagnosis of brucellosis [7,8]. The diagnostic performance of these assays largely depends on the quality, specificity, and antibody titer of the diagnostic antisera employed [9].

Diagnostic hyperimmune agglutinating antisera are biological preparations obtained from animals that have been intensively immunized with a specific antigen and contain high concentrations of antibodies directed against a particular microorganism. These antisera constitute one of the principal reagents used in serological diagnostic laboratories. The conventional method for their production is based on the sequential hyperimmunization of laboratory animals, predominantly rabbits, with the target antigen [10]. However, the optimal hyperimmunization protocol—including the number of immunizations, immunization intervals, antigen dose, and adjuvant composition—remains a subject of debate, as different protocols have been recommended by different investigators [11].

The immunomorphological alterations that occur in laboratory animals during hyperimmunization, particularly in the liver, spleen, and kidneys, have not yet been comprehensively characterized. A better understanding of these changes would provide deeper insight into the biological mechanisms underlying hyperimmunization and contribute to the development of scientifically validated criteria for the quality control of diagnostic antisera [12,13].

Accordingly, the present study was designed to achieve the following objectives:

1.To produce high-titer diagnostic hyperimmune agglutinating antiserum by hyperimmunizing experimental rabbits with the live vaccine strain Brucella abortus S19 in combination with incomplete Freund's adjuvant.

2.To evaluate the serological and immunological properties of the obtained hyperimmune antisera.

3.To perform a comprehensive histological assessment of the immunomorphological changes occurring in the liver, spleen, and kidneys of rabbits during hyperimmunization and to determine their relationship with the quality of the diagnostic antisera.

2. Materials and Methods

2.1. Study Design and Ethical Considerations

The study was conducted in accordance with the requirements of the methodological guideline "Methods and Guidelines for the Use of Laboratory Animals in Experimental Microbiological and Immunological Studies," approved by the Ministry of Health of the Republic of Uzbekistan in 2016 (Nuraliev N.A. and Bektimirov A.M.-T.). Prior to the experiment, all rabbits were acclimatized under quarantine conditions for 21 days and maintained under standardized housing and feeding conditions throughout the study.

At the completion of the experiment, the animals were humanely euthanized and their biological remains were disposed of in accordance with the Veterinary and Sanitary Regulations for the Collection, Utilization, and Disposal of Biological Waste, approved by Resolution No. 13 of the State Committee for Veterinary Medicine and Livestock Development of the Republic of Uzbekistan, dated October 14, 2019. Disposal was carried out using a high-temperature cremation facility located in the animal research center vivarium.

2.2. Experimental Animals

The study was conducted using three healthy male rabbits aged 6–12 months and weighing 3.0–3.5 kg. Prior to hyperimmunization, all animals were confirmed to be seronegative for brucellosis by both the Huddleson agglutination test and the Wright standard tube agglutination test (SAT). The rabbits were maintained under standard vivarium conditions at a temperature of 18–22°C, relative humidity of 55–65%, and a 12-h light/12-h dark cycle, with ad libitum access to standard laboratory feed and drinking water throughout the experimental period.

2.3. Antigen and Adjuvant

The live vaccine strain Brucella abortus BA19, manufactured by the Shchyolkovo Biocombinat (Russian Federation), was used for hyperimmunization (batch No. 204; date of manufacture: April 2022; shelf life: 12 months). The vaccine was diluted in sterile physiological saline (0.9% NaCl) to a final concentration of 4×10^9 CFU/mL. During the first and fourth immunizations, the antigen was administered as an emulsion with incomplete Freund's adjuvant, prepared by mixing 6 mL of petroleum oil (vaseline oil) with 2 mL of the Brucella abortus BA19 vaccine. During the second and third immunizations, the live vaccine was administered without adjuvant. The use of incomplete Freund's

adjuvant provides sustained antigen release, thereby enhancing and prolonging the immune response [14].

2.4. Hyperimmunization Protocol

Each rabbit underwent a total of four immunizations at 7-day intervals. During each immunization, a total volume of 1.6 mL of the immunizing preparation was administered at eight injection sites, with 0.2 mL injected per site. The first four injection sites (sites 1–4) were administered subcutaneously, whereas the remaining four sites (sites 5–8) received intramuscular injections. Blood samples were collected one day before each subsequent immunization to monitor the antibody response. Thirteen days after the fourth (final) immunization, whole blood was collected, and hyperimmune antisera were separated for further serological analyses.

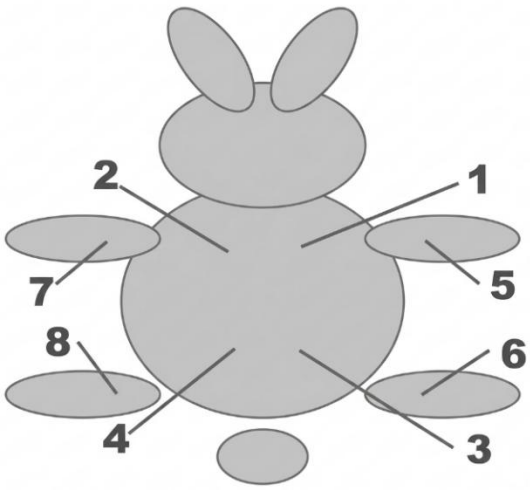

Figure 1. Injection sites used for rabbit hyperimmunization.

2.5. Serum Processing and Storage

The collected blood samples were incubated at 37°C for 30 min to allow clot formation and were subsequently centrifuged at 1,500 × g for 15 min to separate the serum. Serum samples obtained from each rabbit were transferred individually into sterile containers. Sodium merthiolate was added as a preservative to a final dilution of 1:10,000. The sera were then heat-inactivated in a water bath at 56°C for 30 min with continuous gentle mixing. A portion of each serum sample was stored at 2–8°C for serological and immunological analyses, whereas the remaining aliquots were preserved at −20°C for subsequent investigations.

2.6. Serological Analysis

The production of specific antibodies was evaluated in accordance with the requirements of Appendix 12 of Order No. 177 of the Ministry of Health of the Republic of Uzbekistan (May 1, 2015), "Methodological Guidelines for the Laboratory Diagnosis of Brucellosis," using the following serological assays:

Huddleson agglutination test (rapid slide agglutination test): 0.08 mL of the antigen suspension was mixed with serial dilutions of serum on a glass slide and gently rotated on a flat surface for 4 min. Agglutination reactions were graded semi-quantitatively as +, ++, +++, or ++++.

Wright standard tube agglutination test (SAT): Two-fold serial dilutions of serum (ranging from 1:50 to 1:6400) were mixed with the antigen suspension and incubated at 37°C for 24 h. The agglutination results were then evaluated according to the standard interpretation criteria.

2.7. Immunological Analysis

The concentrations of Brucella-specific immunoglobulins of the IgA, IgM, and IgG classes were determined using the Vector-Best enzyme-linked immunosorbent assay (ELISA) kit (Vector-Best, Russian Federation). All analyses were performed strictly in accordance with the manufacturer's instructions. Briefly, serum samples were added to antigen-coated microplate wells and incubated under the recommended conditions. Following incubation, the enzyme conjugate was added, and color development was achieved by the addition of the substrate solution. The optical density (OD) was measured at a wavelength of 450 nm using a microplate reader. Immunoglobulin concentrations were calculated according to the manufacturer's protocol and expressed as ng/mL or mg/mL, as appropriate.

2.8. Biochemical Analysis

Serum total protein concentration was determined by the biuret method, whereas serum albumin concentration was measured using the bromocresol green dye-binding method on a Mindray BA-88A automated biochemical analyzer (Mindray, China). Serum globulin concentration was calculated by subtracting the albumin concentration from the total protein concentration. The albumin-to-globulin (A/G) ratio was subsequently calculated using the standard formula.

2.9. Macromorphological Evaluation

Following the fourth immunization, the experimental rabbits were humanely euthanized. During necropsy, the liver, spleen, and kidneys were subjected to a comprehensive gross examination. Organ weight, size, color, consistency, capsule integrity, and gross morphological changes observed on the cut surface were assessed and compared with those of the control animals. All macroscopic findings were systematically recorded and described.

2.10. Histological Examination

Tissue specimens measuring approximately 0.5 × 0.5 cm were collected from each organ and fixed in 10% neutral buffered formalin (pH 7.4) for 24 h. After fixation, the samples were processed according to the standard paraffin-embedding protocol. Paraffin blocks were prepared, and serial sections 5–7 µm thick were cut using a rotary microtome and mounted onto poly-L-lysine-coated glass slides. For hematoxylin and eosin (H&E) staining, the sections were deparaffinized, rehydrated through a graded series of ethanol solutions, and stained with Mayer's hematoxylin for 5–10 min. The sections were then rinsed in running tap water for 10–15 min to achieve adequate nuclear bluing, followed by counterstaining with 1% eosin for 1–3 min. Subsequently, the sections were dehydrated through graded ethanol solutions, cleared in xylene, and mounted with coverslips using Vitrogel mounting medium. Histological evaluation was performed using an Olympus BX53 light microscope at magnifications of ×40, ×100, ×200, and ×400.

2.11. Statistical Analysis

Statistical analyses were performed using Microsoft Excel 2019 and Biostatistica 6.0 software. Quantitative data are presented as the mean (M) ± standard error of the mean (SEM). Statistical differences between groups were evaluated using Student's t-test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Serological Status Before Hyperimmunization

Prior to the initiation of hyperimmunization, serum samples collected from all three rabbits were examined using the Huddleson agglutination test (rapid slide agglutination test) and the Wright standard tube agglutination test (SAT). No antibodies against Brucella were detected in any of the samples, and all animals tested seronegative for brucellosis. These findings confirmed the absence of pre-existing serological immunity to Brucella in the experimental animals and ensured that the subsequent immune responses were attributable exclusively to the hyperimmunization protocol (table 1).

Table 1. Results of Serological Testing Before Hyperimmunization

Serum Sample	Diagnostic Antigen	Huddleson Agglutination Test (Rapid Slide Agglutination Test)	Wright Standard Tube Agglutination Test (SAT)
0.1.	AG-brucella	Negative	Negative
0.2.	AG-brucella	Negative	Negative
0.3.	AG-brucella	Negative	Negative

3.2. Serological Findings Following Hyperimmunization

The development and kinetics of specific antibody production were monitored throughout the four-stage hyperimmunization protocol in the three experimental rabbits. The Huddleson agglutination test yielded strongly positive reactions in all animals after the first immunization, and this response remained consistently positive throughout the subsequent immunization stages. In contrast, the Wright standard tube agglutination test (SAT) demonstrated a progressive increase in antibody titers during the course of hyperimmunization.

Following the first immunization, antibody titers ranged from 1:200 to 1:400. After the second immunization, the titers increased further, reaching 1:800–1:1600. The highest antibody response was observed after the third immunization, with titers increasing to 1:3200–1:6400, indicating the successful production of high-titer hyperimmune antisera suitable for diagnostic applications. Following the fourth immunization, no further increase in antibody titers was observed. Instead, the titers decreased to 1:800–1:1600. Thirteen days after the final immunization, a further decline in antibody titers to 1:200–1:400 was recorded (Table 2)

Table 2. Serological Findings Following Hyperimmunization

Serum Sample	Diagnostic Antigen	Huddleson Agglutination Test (Rapid Slide Agglutination Test)	Wright Standard Tube Agglutination Test (SAT)
After the first immunization			
1.1.	AG-brucella	Strongly positive	1:200 +++
1.2.	AG-brucella	Strongly positive	1:400 +++
1.3.	AG-brucella	Strongly positive	1:200 ++
After the second immunization			
2.1.	AG-brucella	Strongly positive	1:800 +++
2.2.	AG-brucella	Strongly positive	1:1600 ++++
2.3.	AG-brucella	Strongly positive	1:800 +++
After the third immunization			
3.1.	AG-brucella	Strongly positive	1:3200 ++
3.2.	AG-brucella	Strongly positive	1:6400 ++++

3.3.	AG-brucella	Strongly positive	1:3200 +++
After the fourth immunization			
4.1.	AG-brucella	Strongly positive	1:800 ++
4.2.	AG-brucella	Strongly positive	1:1600 ++
4.3.	AG-brucella	Strongly positive	1:800 ++
Thirteen days after the fourth immunization			
4.1.	AG-brucella	Strongly positive	1:400 ++
4.2.	AG-brucella	Strongly positive	1:400 ++
4.3.	AG-brucella	Strongly positive	1:200 ++

Analysis of the serological response demonstrated that the production of specific antibodies was initiated after the first immunization and progressively increased, reaching its maximum after the third immunization. No substantial increase in antibody titers was observed following the fourth immunization. These findings indicate that a three-dose hyperimmunization regimen is sufficient to induce an optimal humoral immune response in rabbits, whereas administration of a fourth immunization does not provide any additional enhancement of antibody production under the conditions of the present study.

3.3. Dynamics of Biochemical and Immunological Parameters

The analysis of the biochemical and immunological parameters presented in Table 3 clearly demonstrated the progressive development of the humoral immune response in the experimental rabbits during the course of hyperimmunization.

Table 3. Dynamics of Biochemical and Immunological Parameters in Experimental Rabbits

Parameter	Before Immunization	After 1nd Immunization	After 2nd Immunization	After 3nd Immunization	After 4nd Immunization	13 Days After the 4th Imm
Total protein, g/l	59,5±7,83	81,3±14,67 p=0,28	75,43±19,31 p=0,50	154,03±11,97 p=0,007	167,77±12, 64 p=0,005	134,1±5,88 p=0,004
Albumin, g/l	34,67±5,07	41,2±6,74 p=0,49	35,2±6,8 p=0,95	69,3±2,9 p=0,009	82,57±6,08 p=0,009	66,27±2,49 p=0,01
Globulin, g/l	24,53±2,76	40,1±8,73 p=0,18	40,23±12,53 p=0,30	84,73±13,39 p=0,02	85,47±6,46 p=0,003	67,83±3,39 p=0,002
A/G ratio	1,4±0,11	0,91±0,05 p=0,02	0,94±0,17 p=0,1	0,84±0,14 p=0,05	0,96±0,01 p=0,02	0,97±0,01 p=0,03
IgA, mg/ml	0,24±0,001	0,23±0,04 p=0,81	0,26±0,03 p=0,55	0,23±0,03 p=0,76	0,17±0,01 p=0,006	0,16±0,01 p=0,004
IgM, mg/ml	0,37±0,01	1,0±0,08 p=0,004	1,11±0,01 p=0,0001	0,69±0,16 p=0,13	0,47±0,001 p=0,002	0,35±0,21 p=0,93
IgG, mg/ml	2,21±0,25	3,45±0,58 p=0,14	4,7±0,18 p=0,05	5,26±0,27 p=0,003	1,94±0,03 p=0,36	1,6±0,35 p=0,25

Serum total protein concentration increased from Serum total protein concentration increased from 59.5 ± 7.83 g/L before immunization to 154.03 ± 11.97 g/L after the third immunization and further to 167.77 ± 12.64 g/L after the fourth immunization ($P < 0.05$). Similarly, serum albumin concentration increased from 34.67 ± 5.07 g/L at baseline to 69.3 ± 2.9 g/L after the third immunization and 82.57 ± 6.08 g/L after the fourth immunization ($P < 0.01$). An even more pronounced increase was observed in serum globulin concentration, which rose from 24.53 ± 2.76 g/L before immunization to 84.73 ± 13.39 g/L after the third immunization ($P = 0.02$), indicating a marked enhancement of immunoglobulin production during hyperimmunization.

The albumin-to-globulin (A/G) ratio decreased from 1.40 ± 0.11 before immunization to 0.84 ± 0.14 after the third immunization ($P = 0.05$). This reduction was primarily attributable to the substantial increase in the globulin fraction, particularly immunoglobulins, reflecting enhanced antibody synthesis.

The kinetics of IgM exhibited the characteristic pattern of a primary humoral immune response. Serum IgM concentration increased from 0.37 ± 0.01 mg/mL before immunization to 1.00 ± 0.08 mg/mL after the first immunization ($P = 0.004$) and reached a maximum value of 1.11 ± 0.01 mg/mL following the second immunization ($P = 0.0001$). Thereafter, IgM levels gradually declined following the third and fourth immunizations and returned to values close to baseline by the end of the observation period (0.35 ± 0.21 mg/mL, $P = 0.93$), consistent with the transition from the primary to the secondary immune response.

In contrast, serum IgG concentration showed a progressive increase, rising from 2.21 ± 0.25 mg/mL before immunization to 5.26 ± 0.27 mg/mL after the third immunization ($P = 0.003$), confirming the

predominance of the secondary (anamnestic) humoral immune response. Following the fourth immunization, IgG concentration decreased to 1.94 ± 0.03 mg/mL (P = 0.36), suggesting that the additional antigen administration did not further enhance antibody production under the conditions of the present study.

No significant changes in serum IgA concentration were observed during the initial stages of hyperimmunization. However, a significant decrease was detected after the fourth immunization (0.17 ± 0.01 mg/mL, P = 0.006) and at the end of the observation period (0.16 ± 0.01 mg/mL, P = 0.004). These findings indicate that the systemic humoral immune response predominated over IgA-mediated mucosal immunity during the course of hyperimmunization.

3.4. Macromorphological Changes

Macroscopic alterations were observed in all three examined organs during necropsy following the four-round hyperimmunization protocol. The spleen was markedly enlarged in all rabbits, with its average size increasing by approximately 1.2- to 1.4-fold compared with that of the control animals. The splenic capsule appeared tense, the parenchyma was slightly firmer in consistency, and the white pulp was clearly distinguishable on the cut surface. The liver exhibited a moderate increase in size (approximately 10–15%) and appeared slightly darker in color. The liver capsule remained smooth and intact, whereas the cut surface showed mild diffuse cloudiness compared with normal tissue. No appreciable changes in kidney size were observed. However, small reddish subcapsular foci consistent with hyperemia were detected beneath the renal capsule.

3.5. Histological Changes in the Spleen

Histological examination of the spleen demonstrated preservation of the overall histoarchitecture. However, pronounced immunomorphological remodeling associated with the antigen-induced immune response was evident.

The white pulp exhibited marked lymphoid follicular hyperplasia, characterized by an increased number and enlarged size of lymphoid follicles. Well-developed active germinal centers were observed in most follicles. These germinal centers contained numerous blast cells and frequent mitotic figures, indicating active lymphocyte proliferation. The periarteriolar lymphoid sheaths showed a marked increase in the numbers of B lymphocytes, T lymphocytes, and macrophages. In addition, accumulations of dendritic cells and plasma cells were observed around the follicles, representing the morphological basis of active antibody production.

Moderate hyperemia was observed in the red pulp. The venous sinuses were dilated and contained numerous lymphocytes within their lumina. Hemosiderin-laden macrophages (siderophages) and occasional megakaryocytes were also identified, suggesting enhanced erythrophagocytosis associated with the degradation of senescent erythrocytes. Proliferative changes were evident in the endothelial cells of the penicillar arteries. Focal accumulations of neutrophils and epithelioid macrophages, together with minimal areas of necrosis, were observed in several regions (Figure 2).

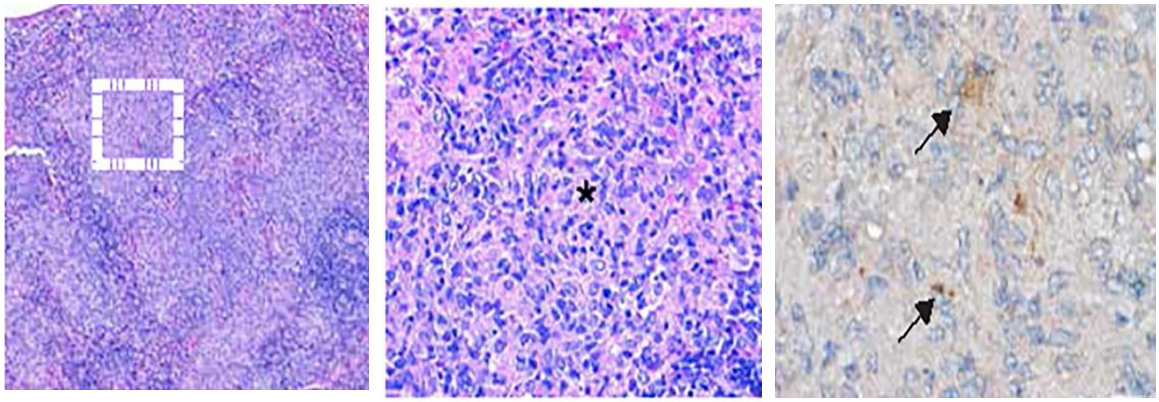

Figure 2. Histological section of the rabbit spleen 28 days after vaccination with the live Brucella abortus BA19 strain, showing focal accumulations of epithelioid macrophages and neutrophils with minimal areas of necrosis. Hematoxylin and eosin (H&E) staining. Original magnifications: ×4, ×20, and ×40.

The reticular stroma was moderately thickened, reflecting increased immunomorphological activity. The observed histological changes were consistent with the classical pattern of immunogenesis in the spleen, characterized by the coordinated interaction of T and B lymphocytes leading to the differentiation of antibody-producing plasma cells.

3.6. Histological Changes in the Liver

Histological examination of the liver revealed that the overall lobular architecture was largely preserved. The central veins and intralobular sinusoidal capillaries were moderately dilated, indicating mild hemodynamic alterations.

The hepatocytes exhibited predominantly parenchymal granular (protein) degeneration, characterized by cytoplasmic clouding (cloudy swelling) and the

accumulation of fine granular material within the cytoplasm. Nuclear alterations were minimal; however, an increased number of hyperchromatic and binucleated hepatocytes was observed, indicating compensatory regenerative activity. These compensatory and regenerative changes suggest an adaptive response of the hepatic parenchyma to prolonged antigenic stimulation.

Moderate proliferation of Kupffer cells (sinusoidal macrophages) was observed, and hemosiderin pigment accumulation was detected in some cells. Inflammatory infiltrates composed of lymphocytes, plasma cells, and neutrophils were present within the periportal areas. A notable histopathological finding was the presence of microgranulomas composed of epithelioid macrophages located in the periportal regions and along the hepatic sinusoids; focal areas of minimal necrosis were also identified. These microgranulomas represent a characteristic tissue response to brucellosis or exposure to Brucella antigens (Figure 3).

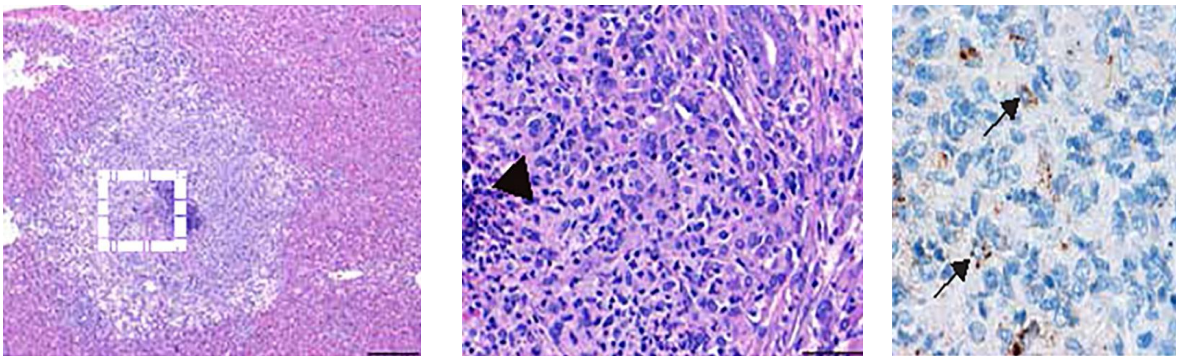

Figure 3. Histological section of the rabbit liver 28 days after vaccination with the live Brucella abortus BA19 strain, showing periportal inflammatory infiltrates, microgranulomas, and focal areas of minimal necrosis. Hematoxylin and eosin (H&E) staining. Original magnifications: ×4, ×20, and ×40.

3.7. Histological Changes in the Kidneys

Histological examination of the kidneys confirmed preservation of the overall histoarchitecture, including the normal cortical and medullary organization. Nevertheless, several morphological alterations associated with systemic antigenic stimulation were identified.

Within the renal corpuscles (glomeruli), moderate proliferation of the glomerular capillary endothelium and widening of the Bowman's capsule urinary space were observed. These changes suggest an adaptive response of the glomerular filtration apparatus to increased functional demand induced by antigenic stimulation.

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The epithelial cells of the proximal and distal renal tubules exhibited features of parenchymal granular degeneration, including cytoplasmic clouding, accumulation of fine protein granules, and indistinct cell boundaries. Focal tubular dilatation and limited areas of epithelial necrosis were observed in some regions.

Moderate lymphoid infiltration was present within the renal interstitium. Overall, these histological changes were predominantly compensatory and reversible in nature. Preservation of the general renal architecture indicates that the observed alterations were not associated with severe structural damage (Figure 4)

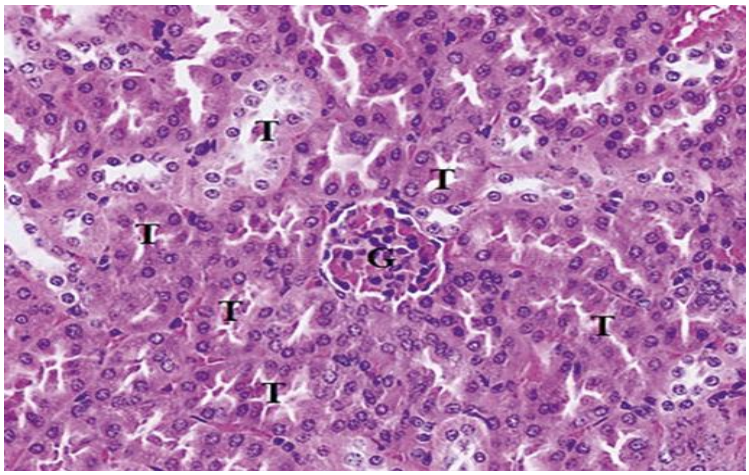

Figure 4. Histological section of the rabbit kidney 28 days after vaccination with the live Brucella abortus BA19 strain, showing focal tubular epithelial necrosis (indicated by asterisks) and dilatation of the renal tubular lumina. Hematoxylin and eosin (H&E) staining. Original magnification: ×40.

4. DISCUSSION

The results obtained in this study demonstrated that the three-stage hyperimmunization scheme using an incomplete Freund's adjuvant combined with the Brucella abortus 19 (BA19) strain is highly effective for producing high-titer specific antibodies suitable for diagnostic purposes. This finding aligns with data reported in the literature: Kiel et al. [15] and Moriyón et al. [16] also emphasized that hyperimmunization with the BA19 strain induces high titers of agglutinating antibodies in rabbits. However, unlike many authors who recommend prolonged immunization schedules lasting 3–6 months with repeated cycles, we have shown that a shortened 28-day scheme can achieve titers as high as 1:6400.

The dynamics of antibody titers corresponded to classical immunological principles: IgM reached its maximum during the first two immunizations in the primary immune response phase, while IgG peaked after the third immunization. This fully matches the classic "primary – secondary immune response" model [17]. It is noteworthy that the fourth immunization did not further increase antibody titers. This phenomenon can be explained by immune tolerance or "overloading" of B cells due to the formation of antigen-antibody complexes [18]. Consequently, our results indicate that three immunization cycles are optimal for the production of diagnostic hyperimmune serum, allowing significant savings in time and resources.

The progressive increase in globulins and total protein during the immunization process is directly related to the heightened activity of antibody-producing plasma cells. These biochemical changes occurred in complete parallel with the morphological alterations observed in the spleen: lymphoid follicle hyperplasia, activation of germinal centers, and an increase in the number of plasma cells—all of which represent the tissue manifestation of intense antibody production. These findings confirm the classical principles of immunobiology regarding the central role of the spleen in systemic antibody production, as highlighted by Tizard [19].

The detection of microgranulomas in the liver deserves special attention. Granulomas—focal reactions composed of macrophages, epithelioid cells, and lymphocytes—are a characteristic tissue response to Brucella spp. infection or its antigens. It has been established that Brucella lipopolysaccharide and other pathogen-associated molecular patterns (PAMPs) are recognized by liver macrophages (Kupffer cells), leading to granuloma formation [20]. These morphological changes are clinically relevant, as they are also observed in natural brucellosis infections. However, the alterations identified in our study resulted from a single exposure to the antigen and were characterized by minimal necrotic changes, distinguishing them from those seen in clinical disease.

The morphological changes in the kidneys—glomerular proliferation and tubular dystrophy—may represent early manifestations of immune complex nephropathy. The deposition of antigen-antibody complexes in the glomeruli and its association with glomerulopathy is well described in the literature [21]. Nevertheless, in our study, these changes were reversible, as evidenced by the preservation of overall histoarchitectonics. This suggests that short-term hyperimmunization does not cause long-term destructive effects on renal function.

Limitations of the study: First, the small sample size (n=3) limits statistical power and requires confirmation in future studies with a larger number of animals. Second, only hematoxylin-eosin staining was used; immunohistochemical investigations (e.g., with anti-CD20 and anti-CD3 antibodies) would have allowed more precise characterization of cell populations. Third, the in vivo diagnostic efficacy of the obtained sera was not tested under practical field conditions.

5. CONCLUSION

The following main conclusions can be drawn from this study:

1.The three-stage hyperimmunization scheme using the live Brucella abortus 19 (BA19) vaccine strain combined with incomplete Freund's adjuvant, administered over 28 days with 7-day intervals, ensures the production of high-titer diagnostic agglutinating hyperimmune sera in experimental rabbits, reaching titers up to 1:6400 in the Wright reaction.

2.The dynamics of antibody titers followed classical immunological patterns: the maximum IgM level was observed after the first and second immunizations (primary immune response), while the peak IgG level occurred after the third immunization (secondary immune response).

3.The fourth immunization did not significantly increase antibody titers and had no additional effect on biochemical parameters. This allows the three-dose hyperimmunization protocol to be evaluated as the optimal scheme for obtaining diagnostic sera.

4.Active immunogenesis developed in the spleen, manifested by lymphoid follicle hyperplasia, formation of germinal centers, and accumulation of plasma cells. All these changes constitute the morphological basis of intense antibody production.

5.In the liver, periportal inflammation, proliferation of Kupffer cells, and the formation of microgranulomas were detected. In the kidneys, predominantly compensatory and reversible morphological changes were recorded. The preservation of histoarchitectonics indicates that these alterations are non-destructive.

6.The data obtained provide a scientifically grounded optimal protocol for the production of high-quality diagnostic agglutinating sera used in the serological diagnosis of brucellosis. They hold significant practical value as a guide in the fields of applied immunology and diagnostics.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in the conduct of this study or the preparation of this article for publication.

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AUTHOR CONTRIBUTIONS

Yusupov A.P. — study design and coordination, development of the hyperimmunization protocol, writing and editing of the manuscript; as well as conducting laboratory tests (serological and immunological analyses) and data analysis. Qoziev O.J. — preparation of histological specimens, microscopic analysis, and interpretation of morphological data. All authors have read and approved the final version of the manuscript.

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