тронный ресурс]. – Режим доступа: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8123435/. – Дата доступа: 31.01.2024.

4. Henriette, M.C. Betalains: properties, sources, applications, and stability / M.C. Henriette // Intern. J. Food Sci. Technol. – 2009. – Vol. 44. – P. 2365–2376.

5. Соколова, Д.В. Эколого-географическое изучение накопления бетанина у перспективных образцов столовой свеклы коллекции ВИР / Д.В. Соколова // Труды по прикладной ботанике, генетике и селекции. – 2019. – Т. 180 (4). – С. 66–74. DOI: 10.30901/2227-8834-2019-4-66-74.

6. Characterization of betalains, saponins and antioxidant power in differently colored quinoa (*Chenopodium quinoa*) varieties / J. Escribano [et al.] // Food Chem. – 2017, 234. – P. 285–294.

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DEVELOPMENT OF AN INDIRECT ELISA METHOD FOR DETECTION OF BRUCELLA TOTAL ANTIBODY

Brucellosis is an infectious disease caused by Brucella species. Brucellosis is an endemic zoonotic disease in many developing countries, causing great harm to public health and devastating losses to livestock [1, 2]. Brucella is a small aerobic intracellular coccobacilli, its infection often involves multiple organ systems, which can be transmitted to other organisms through meat, milk, urine and other fluids of the infected animals [3]. Brucellosis has nonspecific clinical manifestations, and so the early suspicion and proper diagnosis is necessary for patient recovery and control widespread transmission [4]. Culture is considered to be the "gold standard" for Brucella diagnosis, but serological tests are the primary methods of diagnosis in endemic regions due to their low cost, user-friendliness, and strong ability to provide a negative prediction [5].

In serological tests, ELISA is the most commonly used method [6]. To date, brucella outer membrane proteins (Omp) are commonly used antigens in the establishment of serological detection methods due to their good immunogenicity and immunoreactivity [7, 8]. In this study, we prepared the recombinant fusion antigen of Omp19, Omp22 and Omp28 in Escherichia coli system,

and then established an indirect ELISA method using the recombinant fusion antigen of Omp19, Omp22 and Omp28 as the coating antigen to detect the brucella total antibody. Based on this indirect ELISA method, we attempt to provide a more accurate and sensitive method for early detection and daily monitoring of brucellosis in humans and animals.

Materials and methods. Antigen, reagents and samples. Recombinant fusion antigen of Omp19, Omp22 and Omp28 of Brucella (Ompf), carbonate solution (pH 9.6), 0.01 mol/L phosphate buffer solution (PBS), washing buffer (PBS+0.05% tween 20, PBST) and TMB coloring solution were prepared by our laboratory. 96-wells plate purchased from Costar. Tiger red agglutination antigen, standard negative and positive serum of brucella were purchased from the China Veterinary Drug Administration. The positive serum samples of mycobacterium bovis, bovine viral diarrhea virus (BVDV), foot-and-mouth disease virus (FMDV), bovine leukemia virus (BLV), sheep pox virus (SPV), caprine arthritis-encephalitis virus (CAEV) were from South China Agricultural University.

Establishment of indirect ELISA. Determination of optimal reaction conditions. The ELISA test was conducted on a 96-wells plate. Diluted the purified recombinant fusion proteins with 0.05 mol/L carbonate solution to 0.5 μg/mL, 1.0 μg/mL, 1.5 μg/mL and 2 μg/mL, to determinate the optimal coating concentration. Overnight coating at 4ºC, washed the plate with PBST, and stored below 4ºC for later use. The serum sample dilutions were 1:50, 1:100, 1:200, 1:400, and 1:800, respectively. The primary antibody (serum sample) reaction time was set to 0.5 h, 1 h, 1.5 h and 2 h. The dilution of enzyme-linked secondary antibody were 1:2500, 1:5000, 1:10000, and 1:20000, respectively. The reaction time of enzyme-linked secondary antibody was set to 20 min, 30 min, 45 min and 60 min. The color rendering time was set to 5, 10, 15, and 20 min. Through analyzing and comparing OD450 under different conditions, determined the optimal reaction conditions for indirect ELISA method. The coating, serum sample and secondary antibody were all optimized according to a volume of 100 μL.

Determination of critical value. Selected 35 negative serum samples and tested under the optimal reaction conditions, and calculated the mean and standard deviation (SD) of OD450 values. Repeated three times for each sample. The formula for calculating the critical value is: mean+3SD. According to statistical analysis, if OD450 < mean + 3SD, the serum sample can be determined as negative, and if OD450 ≥ mean + 3SD, the serum sample can be determined as positive.

Specificity assay. Detected the positive serum samples of mycobacterium bovis, BVDV, FMDV, BLV, SPV and CAEV using established ELISA

methods to evaluate the specificity of the established ELISA method. Repeated three times for each sample.

Sensitivity and repeatability assay. Diluted 5 known brucella positive serum at 1:50, 1:100, 1:200, 1:400, and 1:800, and tested according to the optimal reaction conditions in 1.4.1 to determine the sensitivity of the established ELISA method. Using recombinant proteins purified from the same batch and different batches to coat 96-wells plates, and then preformed the detection of 5 known brucella positive serum samples. Each serum sample was repeated three times, and the OD450 value was statistically analyzed. The repeatability of the ELISA method was determined by calculating the coefficient of variation (CV) of the intra-batch and inter-batch repeat tests.

Comparison assay. Detected 62 serum samples using the indirect ELISA method and tiger red plate agglutination test (RBPT), compared the results to assess the coincidence rate.

Statistical analysis. Data were statistically analyzed using SPSS 19.0. All results are presented as the mean or mean ± SD.

Results. Determination of indirect ELISA method. By optimizing the reaction conditions of each step through the square matrix method, the optimal reaction conditions for the ELISA method were ultimately determined to be: coating concentration 1 μg/mL; dilution and reaction time of the serum sample 1:400, 1 h; Dilution and reaction time of the secondary antibody 1:5000, 45 min; Color reaction time 8 min.

In summary, the final test steps were: added 100 μL serum sample at a ratio of 1:400 into the coated well, and then incubated for 1 h. After that, washed 3 times with PBST, added 100 μL secondary antibody at a ratio of 1:5000 into the well and incubated for 45 min. After washing 4 times with PBST, added 100 μL TMB to each well and incubated for 8 min. Finally, 50 μL H2SO4 solution (2 mol/L) to terminate the reaction, and then detected the OD450 value in a microplate reader. The entire ELISA was conducted at 37℃.

Determination of critical values. By conducting ELISA detection on 35 serum samples with negative antibodies against Brucella, we obtained the mean value was 0.11 and the corresponding SD was 0.027. Therefore, when OD450 < 0.19, the serum sample can be determined as brucella antibody negative, and when OD450 ≥ 0.19, the serum sample can be determined as brucella antibody positive. Specificity assay. The established ELISA method was used to detect the positive serum samples of mycobacterium bovis, BVDV, FMDV, bovine BLV, SPV and CAEV, the results showed that only known Brucella positive serum samples were positive, while the remaining serum antibodies were all negative (Table 1), indicating that the established ELISA method has good specificity.

Table 1 – Specificity results

Samples	Mean ± 3*SD*	Results
Mycobacterium bovis	0.13 ± 0.01	Negative
BVDV	0.12 ± 0.025	Negative
0.12	0.14 ± 0.015	Negative
BLV	0.13 ± 0.01	Negative
SPV	0.12 ± 0.015	Negative
CAEV	0.11 ± 0.021	Negative
Positive	2.25 ± 0.095	Positive
Negative	0.11 ± 0.015	Negative

Sensitivity and repeatability assay. Five known brucella positive serum samples at the dilution of 1:50, 1:100, 1:200, 1:400 and 1:800 were tested by the indirect ELISA, ELISA results showed that the positive serum still was positive at 1:400 dilution (OD_{450} > 0.19, Table 2), indicating the indirect ELISA has high sensitivity.

Table 2 – Sensitivity results

No.	Serum dilution				
	1:50	1:100	1:200	1:400	1:800
1	1.56	1.12	0.75	0.26	0.14
2	1.63	1.23	0.86	0.43	0.22
3	1.36	1.02	0.71	0.40	0.18
4	1.47	0.92	0.65	0.34	0.15
5	2.02	1.58	1.16	0.39	0.21

Five known brucella positive serum samples were subjected to intra- and inter-batch repeatability tests, and analyzed using SPSS software. The results showed that the intra-batch CV were all less than 6.55%, and the inter-batches CV were all less than 8.55%. The data indicated the indirect ELISA method has high intra- and inter-batch repeatability. The results were shown in Table 3.

Table 3 – Repeatability results

No.	Intra-batch			Inter-batches		
	mean	SD	CV	mean	SD	CV
1	0.23	0.015	6.55	0.24	0.021	8.55
2	0.43	0.015	3.53	0.45	0.032	7.20
3	0.41	0.015	3.70	0.43	0.02	4.65
4	0.32	0.012	3.65	0.32	0.026	8.27
5	0.35	0.01	2.86	0.34	0.026	7.78

Comparison assay. Performed consistency analysis on the detection results of indirect ELISA and RBPT, and calculated the Kappa value using SPSS 19.0 statistical software. The results showed that the sensitivity of the

ELISA method was 96.55%, and the specificity was 100%, and Kappa = 0.968 (Table 4), indicating the indirect ELISA and RBPT has high consistency.

Table 4 – Comparison of coincidence rate between indirect ELISA and RBPT

		ELISA		
		P	N	Total
RBPT	P	28	1	28
	N	0	33	34
Total		28	34	62
Sensitivity	96.55%			
Specificity	100%			
Kappa value	0.968			

Discussion. Brucellosis remains an important zoonosis in various parts of the world. An evidence-based conservative estimate of the annual global incidence is 2.1 million, and Africa and Asia sustain most of the global risk and cases [9]. Additionally, international migration of humans, animals and trade of animal products induced the emergence of brucellosis in new areas as well as transmission of brucellosis from wild and domestic animals, it has created a challenge for brucellosis spread and diagnosis in non-endemic areas [10]. Incidence of brucellosis was growing up in Iran during the past years, the five-years mean incidence of brucellosis was 46.5 in one hundred thousand [11]. In Greece, brucellosis is endemic in sheep and goats [12]. In the Middle East and Black Sea Basin region, brucellosis continues to be an important burden due to the insufficient infrastructure for brucellosis prevention and control [13]. In Belarus, although the active and risk-based serosurveillance to brucellosis was conducted every three years, it still has a significant risk of brucellosis outbreak due to various factors [14]. Therefore, rapid and accurate brucellosis diagnosis in these areas is needed for early control and prevention.

At present, specific anti-Brucella antibodies testing is the most commonly used indicator for screening brucellosis, and so finding a good antigen is crucial. Several studies have investigated Brucella recombinant proteins, cell wall proteins (outer membrane proteins, Omp) are recognized as the best antigens for diagnosing brucellosis in animals and humans [15,16]. Researchers have established multiple methods for detecting Brucella antibodies using different Omp antigens, such as recombinant Omp19, Omp28 etc.[6, 17]. However, the available results on the specificity and sensitivity of serological tests based on Omp are ambiguous and sometimes contradictory [18]. Perhaps, a single antigen can easily lead to missed detection, while the combination of multiple antigens may bring more ideal detection results [19, 20]. In this study, we established an indirect ELISA method using coating of fusion anti-

gens, results showed this ELISA method has high sensitivity (1:400), high specificity and high consistency with RBPT, the intra-batch and inter-batches CV lower than 10%. These data showed the testing performance of the indirect ELISA has met the conventional detection requirements.

In a word, the indirect ELISA method were developed using recombinant fusion antigens, with the high specificity, sensitivity, repeatability, and consistency. This indirect ELISA method has low sample size, low requirements for experimental conditions, simple and fast operation, and is suitable for rapid screening of brucellosis on site. Therefore, this method has broad application prospects in the prevention and control of brucellosis in humans and animals.

REFERENCE

1. Hayoun MA, Muco E, Shorman M. Brucellosis. 2023 Apr 29. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. PMID: 28722861.

2. Lambert S, Thébault A, Anselme-Martin S, et al. Brucellosis in Alpine ibex: 10 years of research and expert assessments. Med Sci (Paris). 2023;39(10):722−731. French.

3. Jin M, Fan Z, Gao R, Li X, Gao Z, Wang Z. Research progress on complications of Brucellosis. Front Cell Infect Microbiol. 2023;13:1136674.

4. Shi L, Wang S, Li X, Li X, Li Y, Wang Y. Acute Brucella infection associated with splenic infarction: a case report and review of the literature. Front Cell Infect Microbiol. 2023;13:1234447.

5. Elbehiry A, Aldubaib M, Marzouk E, Abalkhail A, Almuzaini AM, Rawway M, Alghamdi A, Alqarni A, Aldawsari M, Draz A. The Development of Diagnostic and Vaccine Strategies for Early Detection and Control of Human Brucellosis, Particularly in Endemic Areas. Vaccines (Basel). 2023;11(3):654.

6. Hans R, Thavaselvam D. Immunoassay-based evaluation of rOmp28 protein as a candidate for the identification of Brucella species. J Med Microbiol. 2023;72(6).

7. Koyuncu I, Kocyigit A, Ozer A, Selek S, Kirmit A, Karsen H. Diagnostic potential of Brucella melitensis Rev1 native Omp28 precursor in human brucellosis. Cent Eur J Immunol. 2018;43(1):81-89.

8. Behroozikhah AM, Dadar M, Asli E, Hosseini SD. Molecular Survey of Brucella melitensis Field Isolates using Sequence-Based PCR of Outer Membrane Protein 31. Arch Razi Inst. 2022;77(3):1261-1267.

9. Laine CG, Johnson VE, Scott HM, Arenas-Gamboa AM. Global Estimate of Human Brucellosis Incidence. Emerg Infect Dis. 2023;29(9):1789-

1797.

10. Khurana SK, Sehrawat A, Tiwari R, Prasad M, Gulati B, Shabbir MZ, Chhabra R, Karthik K, Patel SK, Pathak M, Iqbal Yatoo M, Gupta VK, Dhama K, Sah R, Chaicumpa W. Bovine brucellosis - a comprehensive review. Vet Q. 2021;41(1):61-88.

11. Rajabi J, Hamidi-Farahani R, Mansouri F, Soleiman-Meigooni S. Incidence and Risk Factors of Brucellosis in Kermanshah Province, Iran During 2010-2014. Infect Disord Drug Targets. 2020;20(2):203-207.

12. Kefaloudi C, Mellou K, Dougas G, Vorou R, Mitrou K, Kontopidou F. Human Brucellosis in Greece, 2005-2020: A Persistent Public Health Problem. Vector Borne Zoonotic Dis. 2022;22(3):163-169.

13. Bagheri Nejad R, Krecek RC, Khalaf OH, Hailat N, Arenas-Gamboa AM. Brucellosis in the Middle East: Current situation and a pathway forward. PLoS Negl Trop Dis. 2020;14(5):e0008071.

14. Arede M, Beltrán-Alcrudo D, Aliyev J, Chaligava T, Keskin I, Markosyan T, Morozov D, Oste S, Pavlenko A, Ponea M, Starciuc N, Zdravkova A, Raizman E, Casal J, Allepuz A. Examination of critical factors influencing ruminant disease dynamics in the Black Sea Basin. Front Vet Sci. 2023;10:1174560.

15. Bai Q, Li H, Wu X, Shao J, Sun M, Yin D. Comparative analysis of the main outer membrane proteins of Brucella in the diagnosis of brucellosis. Biochem Biophys Res Commun. 2021;560:126-131.

16. Bulashev A, Akibekov O, Syzdykova A, Suranshiyev Z, Ingirbay B. Use of recombinant Brucella outer membrane proteins 19, 25, and 31 for serodiagnosis of bovine brucellosis. Vet World. 2020;13(7):1439-1447.

17. Golchin M, Mollayi S, Mohammadi E, Eskandarzade N. Serodiagnosis of human brucellosis by an indirect ELISA test using recombinant outer membrane protein 19 kDa (rOMP19) as an antigen. BMC Biotechnol. 2023;23(1):46.

18. Bulashev A, Eskendirova S. Brucellosis detection and the role of Brucella spp. cell wall proteins. Vet World. 2023;16(7):1390-1399.

19. Kong Y, Zheng J, Wang H, et al. Exploration of a rapid test strip for brucellosis. Chinese Journal of Zoonoses. 2019, 35 (5):460-464.

20. Simborio HL, Lee JJ, Bernardo Reyes AW, Hop HT, Arayan LT, Min W, Lee HJ, Yoo HS, Kim S. Evaluation of the combined use of the recombinant Brucella abortus Omp10, Omp19 and Omp28 proteins for the clinical diagnosis of bovine brucellosis. Microb Pathog. 2015;83-84:41-6.