bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1 **A multiepitope fusion protein-based p-ELISA method for diagnosing bovine and**

2 **goat brucellosis**

3

4 Dehui Yin[1#], Qiongqiong Bai[1#], Xiling Wu[1], Han Li[2], Jihong Shao[1], Mingjun Sun[3*],

5 Jingpeng Zhang[1*]

6

7 [1] Key Lab of Environment and Health, School of Public Health, Xuzhou Medical

8 University, Xuzhou, 221004, China

9 [2]Department of Infection Control, the First Hospital of Jilin University, Changchun,

10 130021, China

11 [3] Laboratory of Zoonoses, China Animal Health And Epidemiology Center, Qingdao,

12 266032, China

13

14 **Running Head:** P-ELISA for diagnosing animal brucellosis

15

16 # These authors contributed equally to this work

17 *Correspondence:

18 Laboratory of Zoonoses, China Animal Health And Epidemiology Center, No.369

19 Nanjing Road, Qingdao, 266032, China. E-mail: sunmingjun@cahec.cn & School of

20 Public Health, Xuzhou Medical University, No. 129 Tongshan Road, Xuzhou, 221004,

21 China. E-mail: xiaopangpeng@126.com

22

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

23 **Abstract**

24 In recent years, the incidence of brucellosis has increased annually, which has caused
25 tremendous economic losses in agriculture and husbandry in various countries.
26 Therefore, developing rapid, sensitive and specific diagnostic techniques for
27 brucellosis has become critical brucellosis research. Bioinformatics technology was
28 used to predict the B cell epitopes of the main antigen proteins of *Brucella*, and the
29 validity of each epitope was verified by indirect enzyme-linked immunosorbent assay
30 (iELISA). The verified epitopes were connected in series to construct a multiepitope
31 fusion protein, goat, bovine brucellosis sera, and rabbit sera were collected to verify
32 the antigenicity and specificity of this protein. Then, the fusion protein was used as a
33 diagnostic antigen to construct paper-based ELISA (p-ELISA) technology. A total of
34 22 effective epitopes were predicted, and a fusion protein was successfully
35 constructed, which showed good antigenicity and specificity. The constructed
36 p-ELISA method was used for the simultaneous detection of bovine and goat
37 brucellosis. ROC curve analysis showed that the sensitivity and specificity of protein
38 detection in goat serum were 98.85% and 98.51%, respectively. The positive and the
39 negative predictive value was 99.29% and 98.15%, respectively. When assessing
40 bovine serum, the sensitivity and specificity were 97.85% and 96.61%, respectively.
41 The positive and the negative predictive value was 98.28% and 97.33%, respectively.
42 This study combined bioinformatics, fusion protein development and p-ELISA
43 technologies to establish a sensitive and specific rapid diagnosis technology for
44 brucellosis that can be used to assess the serum of bovine, goats and other livestock.

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

45 **Keywords:** brucellosis; bioinformatics technology; fusion protein; p-ELISA;
46 serodiagnosis

47 **IMPORTANCE:** Brucellosis has caused tremendous economic losses in agriculture
48 and husbandry in various countries. Therefore, developing rapid, sensitive and
49 specific diagnostic techniques for brucellosis has become critical brucellosis research.
50 In this study, we used immunoinformatic technology to predict the B cell epitopes in
51 the major outer membrane proteins of Brucella, synthesized polypeptides and coupled
52 them with KLH, screened these polypeptides by iELISA methods, selected effective
53 polypeptides as diagnostic antigens, and established a p-ELISA for brucellosis
54 diagnosis based on a multiepitope fusion protein that can be used to assess the serum
55 of bovine, goats and other livestock.

56

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

57 ## 1 Introduction

58 Currently, a high incidence of brucellosis, a hazardous zoonosis, is reemerging
59 worldwide, especially in developing countries, posing not only a large threat to human
60 health but also tremendous losses in the world economy[1]. *Brucella* infection can be
61 caused by humans directly contacting *Brucella*-infected goats, bovine, pigs and other
62 livestock and their secretions or excreta or eating *Brucella*-contaminated food[2]. Due
63 to the diversity of clinical manifestations of brucellosis and the lack of specific
64 clinical manifestations, the diagnosis of brucellosis is very difficult, and it is easily
65 misdiagnosed as other febrile diseases, such as dengue fever, malaria, or viral
66 bleeding diseases[3,4].

67 There are many methods for diagnosing *Brucella*. Serological diagnostics are the
68 most widely used and mature methods. However, serological diagnosis requires
69 specific and sensitive antigens[5]. Currently, the commonly used antigens include
70 whole-cell antigen and lipopolysaccharide (LPS). However, these antigens easily
71 cross-react with the antibodies of other bacteria, which affects the specificity of the
72 diagnosis. Therefore, it is very important to develop new diagnostic antigens to
73 improve the specificity and sensitivity of serological diagnostic methods[6]. A large
74 number of vaccine studies show that the *Brucella* outer membrane protein has good
75 immunogenicity, which provides a direction for finding new diagnostic antigens[7-9].
76 The rapid development of paper-based enzyme-linked immunosorbent assay
77 (p-ELISA) diagnostic methods based on paper chip technology provides a reference
78 for the development of new brucellosis diagnostic methods[10].

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

79 In this study, five main antigen proteins of *Brucella* were screened, and the
80 possible dominant B cell epitopes of these proteins were predicted by bioinformatics
81 technology. We designed a new *Brucella* multiepitope fusion protein by concatenating
82 the predicted dominant epitopes, making it a candidate antigen for the serological
83 diagnosis of brucellosis. A rapid, sensitive and specific p-ELISA diagnostic technique
84 for brucellosis that can detect protein in serum of bovine, goats and other livestock
85 was successfully constructed.
86

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

87 ## 2. Materials and methods

88 ### 2.1 Serum samples

89 A total of 140 goat serum samples that were *Brucella* positive, 54 goat serum samples
90 that were *Brucella* negative, 116 bovine serum samples that were *Brucella* positive,
91 and 75 serum samples that were *Brucella* negative were provided by the China
92 Animal Health and Epidemiology Center (Qingdao, China). All *Brucella*-positive and
93 *Brucella*-negative sera were verified to be positive by the tube agglutination test and
94 the Rose Bengal plate agglutination test (RBPT). All experiments involving animals
95 or animal samples were fully compliant with ethical approval granted by the Animal
96 Care and Ethics Committee of Xuzhou Medical University.

97 ### 2.2 Prediction and synthesis of peptide epitopes

98 The *Brucella* outer membrane proteins (Omp) Omp16, Omp25, Omp31, Omp2b
99 and BP26 were selected, and their amino acid sequences were obtained through the
100 protein database at NCBI (https://www.ncbi.nlm.nih.gov/protein/). The conservation
101 of the amino acid sequences was assessed by BLASTing. The prediction of B cell
102 epitopes was carried out by using the B cell epitope prediction tool BepiPred linear
103 epitope prediction 2.0 at IEDB (http://tools.iedb.org/bcell/). The predicted B cell
104 epitope peptides were synthesized by Sangon Biotech (Shanghai, China) and coupled
105 with keyhole limpet hemocyanin (KLH) with a purity of more than 90%.

106 ### 2.3 Epitope antigenicity screening

107 Forty-five bovine and goat *Brucella*-positive sera were randomly selected to
108 identify the antigenicity of the peptides through an indirect enzyme-linked

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

109 immunosorbent assay (iELISA). In addition, a KLH carrier negative control and an
110 LPS positive antigen control were established. For the procedure, in a 96-well
111 microtiter plate (NUNC, Denmark), 100 μL of peptide (30 μg/mL in carbonate buffer
112 solution (CBS), pH 9.6) was added to each well and incubated overnight at 4°C. The
113 wells were blocked with 300 μL/well of 5% skimmed milk powder (Sangon,
114 Shanghai) at 37°C for 2 hours, and then, 100 μL/well of *Brucella* serum was added
115 (1:400 dilution with PBS) and incubated at 37°C for 1 hour. HRP-labeled protein G
116 (diluted 1:5000, PBS) (Thermo, USA) was then added and incubated at room
117 temperature for 30 min, after which an EL-TMB kit was utilized (Sangon). Optical
118 density was measured at 450 nm (OD450) using an ELISA plate reader (BioTek,
119 USA). After each step, the plates were washed 3 times with PBST.

120 **2.4 Preparation of the fusion protein**

121 The effective peptides were selected to be connected in series, and adjacent
122 peptides were linked by the 'GGGS' linker. For the concatenated amino acid sequence,
123 the molecular weight (https://web.expasy.org/compute_pi/), spatial conformation
124 (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and other parameters were
125 predicted. According to the amino acid sequence after concatenation, the codon was
126 reversed, and the prokaryotic expression was optimized. The plasmid was constructed
127 by whole gene synthesis and subcloned into expression vector pET30a (Beijing
128 Protein Innovation, Beijing) and then transferred into competent cells (BL21 cells) for
129 IPTG-induced expression. Specifically, competent cells (BL21 cells) (100 μL), stored
130 at -80°C, were slowly thawed on ice, after which the ligation product was added to the

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

131 cells and mixed well; the cells were then placed on ice for 30 min, heat shocked at
132 42°C for 90 s and then incubated in an ice bath for 2 min. Subsequently, 800 μL of
133 nonresistant LB medium was added, incubated at 37°C for 45 min and centrifuged at
134 5000 rpm for 3 min. The majority of the supernatant was discarded, leaving
135 approximately 100-150 μL, which was used to resuspended the cell pellet. The
136 resuspended cells were added to LB plates with the corresponding resistance
137 antibiotic and spread over plates, which were air-dried and cultured upside down and
138 placed in an incubator at 37°C overnight. Then, the transformed BL21 cells were
139 selected and cultured in 1.5 mL of LB liquid medium at 37°C and shaken at 200 rpm.
140 The cells were incubated until the OD600=0.6, at which time they were induced by
141 IPTG (0.5 mM) and cultured for 2 hours at 37°C. One milliliter of induced bacterial
142 solution was centrifuged at 12000 rpm for 1 min, the supernatant was discarded, and
143 the precipitate was resuspended in 50-100 μL of 10 mM Tris-HCl (pH 8.0) solution
144 (the amount of added buffer was dependent on the amount of bacteria). Loading
145 buffer equal to twice the volume of the resuspended precipitate was added, after
146 which the sample was boiled at 100°C for 5 min and then assessed by SDS-PAGE
147 electrophoresis.

148 After validation, 2 μL of activated bacterial solution was transferred to 750 mL of
149 LB liquid medium at 37°C, spun at 200 rpm and incubated until the absorbance
150 reached OD600 = 0.6-0.8. IPTG (0.5 mM) was added for overnight induction at 16°C.
151 After centrifugation at 6000 rpm for 5 min, the supernatant was discarded, and the
152 bacteria were collected. The bacteria were resuspended with 20-30 mL of 10 mM

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

153 Tris-HCl (pH 8.0) solution and fragmented by ultrasonication (500 W, 60 times, 10 s
154 each time, 15 s intervals). One hundred microliters of bacterial suspension was
155 collected after ultrasonic treatment and centrifuged at 12000 rpm for 10 min. Fifty
156 microliters of supernatant was poured into another EP tube. After the supernatant was
157 removed, the precipitate was resuspended with 50 μL of 10 mM Tris-HCl (pH 8.0)
158 solution and assessed by SDS-PAGE electrophoresis.

159 **2.5 Purification of fusion protein**

160 A nickel column (Ni Sepharose 6 Fast Flow, GE Healthcare) was washed with
161 deionized water at pH 7.0. The nickel column was adjusted to pH 2~3. The column
162 was washed with deionized water at pH 7.0. The nickel column was equilibrated with
163 10 mM Tris-HCl (pH 8.0) solution (approximately 100 mL). Then, the nickel column
164 was equilibrated with a 10 mM Tris-HCl (pH 8.0) solution containing 0.5 M sodium
165 chloride, (approximately 50 mL). Diluted sample was loaded. The sample contained
166 sodium chloride at a final concentration of 0.5 M. After loading, the column was
167 washed with 10 mM Tris-HCl (pH 8.0) solution containing 0.5 M sodium chloride.
168 The proteins were eluted with a 10 mM Tris-HCl (pH 8.0) (containing 0.5 M sodium
169 chloride) solution containing 15 mM imidazole, 60 mM imidazole, and 300 mM
170 imidazole, and the protein peaks were collected separately. SDS-PAGE
171 electrophoresis was used to assess the effect of protein purification.

172 **2.6 Antigenicity assessment of the fusion protein**

173 The iELISA method was used to assess the antigenicity of the purified protein.
174 For the procedure, in a 96-well ELISA plate (NUNC, Denmark), 100 μL of fusion

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

175 protein (2.5 μg/mL, CBS) was added to each well, and 100 μL of LPS (1 μg/mL, CBS)
176 was added to the wells with positive antigen control and incubated overnight at 4°C.
177 For blocking, 300 μL of 5% skimmed milk (PBS) was added per well and incubated at
178 37°C for 2 hours. Then, 100 μL of *Brucella*-positive serum (1:400 dilution, PBS) was
179 added and incubated at 37°C for 1 hour; next, 100 μL of HRP-labeled protein G
180 (diluted 1:8000, PBS) was added and incubated at room temperature for 30 min. After
181 color development with the EL-TMB color kit, the absorbance of the wells was
182 measured at OD450. After each step, the plates were washed 3 times with PBST.
183 At the same time, we used rabbit sera containing other pathogenic bacteria that
184 easily cross-react with *Brucella*, including *Yersinia enterocolitica* O9, *Escherichia*
185 *coli* O157:H7, *Salmonella*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Listeria*
186 *monocytogenes*. All rabbit sera samples were purchased from Tianjin Biochip
187 Corporation (Tianjin, China). The specificity of the protein was verified by iELISA. A
188 1:10000 dilution of HRP-labeled goat anti-rabbit secondary antibody (Bioworld, USA)
189 was used in this assay, and the remainder of the steps were the same as outlined
190 above.

191 **2.7 Establishment of the p-ELISA method**

192 A puncher was used to make a round sheet of Whatman No. 1 filter paper with a
193 diameter of 10 mm, and a small hole (a diameter of 6 mm) was punched out of A4
194 plastic packaging paper. The 10 mm filter paper was placed in the center of the 6 mm
195 hole in the plastic packaging paper, and a laminating machine joined the filter sheet
196 and packaging paper, fixing and cutting the combined papers it into small strips with 3

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

197 holes in each strip. The fellow steps have been described in the literature[11] Five
198 microliters of chitosan deionized water solution (0.25 mg/mL) was added to the round
199 holes with Whatman No. 1 filter paper and dried at room temperature; then, 5 μL of
200 2.5% glutaraldehyde solution (PBS) was added, incubated at room temperature for 2
201 hours, and then washed 3 times with 20 μL of deionized water. Then, 5 μL of fusion
202 protein solution (2.5 μg/mL, PBS) was added to each well, incubated at room
203 temperature for 30 min, and washed 3 times with 20 μL of deionized water. Next, 20
204 μL of 5% skimmed milk powder was added and incubated for blocking at room
205 temperature. Subsequently, 5 μL of serum (1:400 dilution) was added; after washing 3
206 times with PBST, 5 μL of HRP-labeled protein G (1:8000 dilution, PBS) was added,
207 incubated at room temperature for 210 s, and washed 3 times with PBST. Finally, 5
208 μL of TMB substrate solution was added, and after 10 min, an HP Laser Jet Pro MFP
209 M227 was used to scan the samples to obtain images. ImageJ software was used to
210 perform gray intensity analysis for quantitation. The collected bovine and goat serum
211 samples were assessed according to the established p-ELISA method, and ROC
212 curves were used to analyze the diagnostic effect of the established method, including
213 sensitivity and specificity.

214 **2.8 Statistical analysis**

215 Dot plot and receiver operating characteristic (ROC) analyses were performed
216 using GraphPad Prism version 6.05 for Windows. The significance of gray intensity
217 differences was determined by Student's t-test (unpaired t-test). Differences were
218 considered statistically significant when $P < 0.05$.

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

219

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

3 Results

3.1 B cell epitope peptide prediction and antigenicity verification

A total of 22 B cell epitopes were predicted, including BP26, Omp16, Omp25, Omp31, and Omp2b, which were predicted to have 6, 2, 5, 5, and 4 epitopes (table S1), respectively. Indirect ELISA results showed that all 22 peptides recognized portions of the serum (Fig. 1)

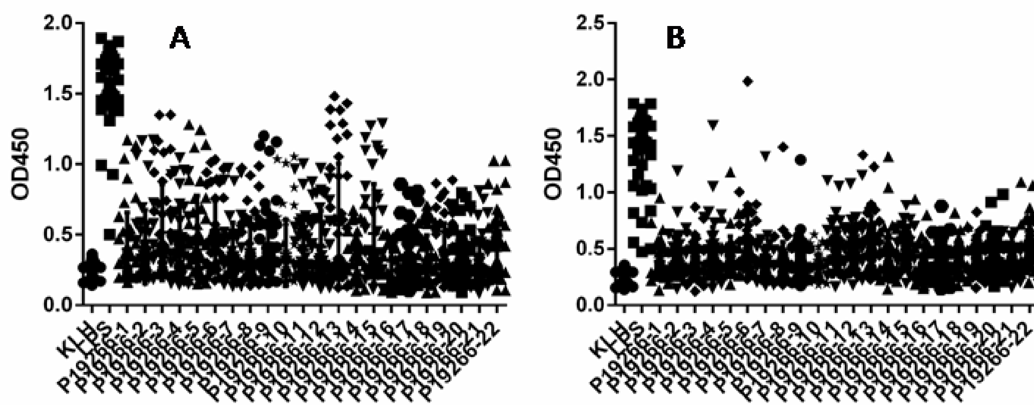


Fig. 1 The results of iELISA of each peptide identification-positive brucellosis serum. (A) Sheep brucellosis serum. (B) Bovine brucellosis serum.

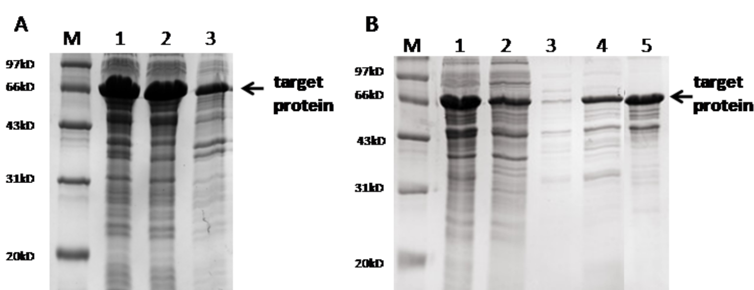
3.2 Preparation of the multiepitope fusion protein

The 22 epitopes were connected in series, and the adjacent peptides were connected with the 'GGGS' linker to form an amino acid sequence of the fusion protein (Figure S1). Prokaryotic expression was induced by IPTG, and the target protein was expressed in the supernatant. SDS-PAGE (15%) electrophoresis showed a protein band with an approximate molecular weight of 66 kD. After mass spectrometry analysis, it was confirmed that this band was the target protein. After purification, most of the miscellaneous bands were removed, and gray intensity

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

240 analysis showed that the purity of the purified protein was approximately 90% (Fig.

241 2).



241

247 **Fig. 2 SDS-PAGE analysis of fusion protein.** (A) Protein expression results. M,
248 marker; lane 1, whole bacteria after ultrasound; lane 2, supernatant after ultrasound;
249 lane 3, precipitation after ultrasound. (B) SDS-PAGE after protein purification. M,
250 marker; lane 1, the original protein before purification; lane 2, flow-through solution;
251 lane 3,15mM imidazole elution fraction; lane 4,60mM imidazole elution fraction; lane
252 5,300mM imidazole elution fraction.

248 **3.3 Antigenicity assessment of the fusion protein**

259 After iELISA analysis, when the fusion protein was used as an antigen to test its

260 diagnostic ability with goat serum, the area under the ROC curve was 0.9799 (95% CI,

261 0.9654 to 0.9944), and the cutoff value calculated by the Youden Index was 0.4675. In

262 this case, the diagnostic sensitivity was 87.14% (95% CI, 0.8044 to 0.9220), and the

263 specificity was 100.0% (95% CI, 0.9340 to 1.000). The positive predictive value was

264 100.0%, and the negative predictive value was 75.00%. When LPS was used as an

265 antigen to test its diagnostic ability with goat serum, the area under the ROC curve

266 was 0.9514 (95% CI, 0.9191 to 0.9836), the cutoff value was 0.8890, the diagnostic

267 sensitivity was 82.00% (95% CI, 0.7305 to 0.8897), and the specificity was 95.83%

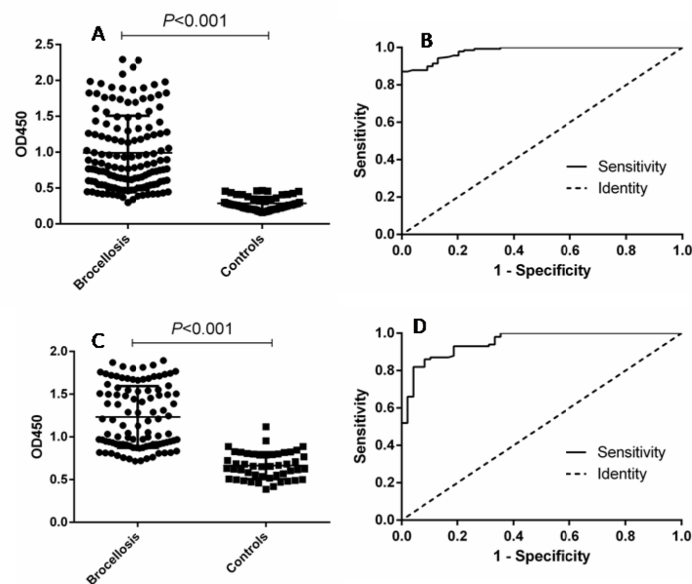
268 (95% CI, 0.8575 to 0.9949). The positive predictive value was 98.39%, and the

269 negative predictive value was 74.29% (Fig. 3 and table 1).

260 To test its diagnostic ability with bovine serum, the area under the ROC curve

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

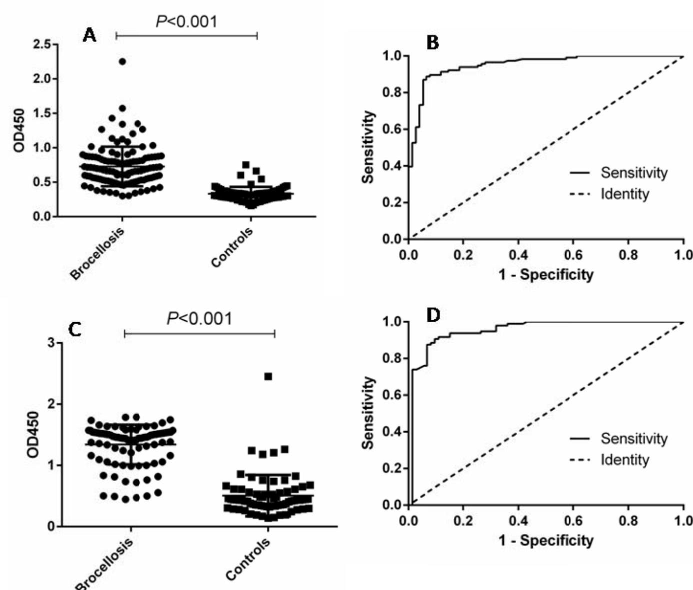
269 was 0.9518 (95% CI, 0.9224 to 0.9812), and the cutoff value calculated by the Youden
270 index was 0.4530. In this case, the diagnostic sensitivity was 88.79% (95% CI, 0.8160
271 to 0.9390), and the specificity was 93.33% (95% CI, 0.8512 to 0.9780). The positive
272 predictive value was 95.37%, and the negative predictive value was 84.34%. When
273 LPS was used as an antigen to diagnose bovine serum, the area under the ROC curve
274 was 0.9528 (95% CI, 0.9187 to 0.9868), and the cutoff value was 0.8105. In this case,
275 the diagnostic sensitivity was 90.63% (95% CI, 0.8295 to 0.9562), and the specificity
276 was 90.28% (95% CI, 0.8099 to 0.9600). The positive predictive value was 93.86%,
277 and the negative predictive value was 88.31% (Fig. 4 and table 1).



270

273 **Fig. 3** ELISA analysis of goat serum samples. (A) Dotplot of the fusion protein ELISA assay. (B)
274 ROC analysis of fusion protein IELISA assay results. (**C**) Dotplot of the LPS antigen ELISA assay.
275 (D) ROC analysis of LPS antigen ELISA assay results.

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

274 

277 **Fig 4** ELISA analysis of cattle serum samples. (A) Dotplot of the fusion protein ELISA assay. (B)
278 ROC analysis of fusion protein IELISA assay results. (**C)** Dotplot of the LPS antigen ELISA assay.
279 (D) ROC analysis of LPS antigen ELISA assay results.

278 Table 1. Positive and negative predictive values of the test calculated for different cut-off values

Cut-off value	Positive		Negative		PPV (%)	NPV (%)
	TP	FN	TN	FP		
≥0.4675(Fusion protein)[a]	122	18	54	0	100.0	75.00
≥0.8890(LPS)a	122	18	52	2	98.39	74.29
≥0.4530(Fusion protein)[b]	103	13	70	5	95.37	84.34
≥0.8105(LPS)[b]	107	9	68	7	93.86	88.31
≥34.12(p-ELISA)[a]	139	1	53	1	99.29	98.15
≥30.21(p-ELISA)[b]	114	2	73	2	98.28	97.33

281 a, gaot sera; b, cattle sera. TP, true positives; TN, true negatives; FP, false positives; FN, false
282 negatives; PPV, positive predictive value (TP/TP+FP)×100; NPV, negative predictive value
283 (TN/TN+FN) ×100.

282

283 **3.4 Determining the cross-reactivity with the fusion protein**

284 To verify whether the fusion protein as a diagnostic antigen shows

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

284 cross-reactivity with other bacteria, we selected 6 zoonotic pathogens for a
285 cross-reactivity test. The results showed that the fusion protein did not cross-react
286 with other bacteria according to an S/N (OD450, sample/negative) > 2.1, which
287 indicated that the fusion protein as a diagnostic antigen has good specificity (table 2).
288 Table 2 Specific cross-reactivity test results of the indirect ELISA diagnostic method
289 for the fusion protein.

Rabbit sample	OD450	S/N
Vibrio parahaemolyticus	0.1230	1.64
Escherichia coli O157:H7	0.0457	0.61
Salmonella	0.1267	1.69
Vibrio cholerae	0.0598	0.80
Yersinia enterocolitica O9	0.0443	0.59
Listeria monocytogenes	0.0758	1.01
Negative	0.0751	--

290

291 **3.5 Evaluation of the diagnostic ability of the p-ELISA**

292 To verify the effectiveness of the established p-ELISA method, the collected
293 serum samples were tested. When diagnosing goat sera, the area under the ROC curve
294 was 0.9986 (95% CI, 0.9957 to 1.002). The cutoff value was 34.12, the diagnostic
295 sensitivity was 98.85% (95% CI, 0.9376 to 0.9997), and the specificity was 98.51%
296 (95% CI, 0.9196 to 0.9996). The positive predictive value was 99.29%, and the
297 negative predictive value was 98.15% (table 1). When testing its activity in bovine

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

298 serum, the area under the ROC curve was 0.9964 (95% CI, 0.9910 to 1.002), and the
299 cutoff value calculated by the Youden index was 30.21. In this case, the diagnostic
300 sensitivity was 97.85% (95% CI, 0.9245 to 1.002), and the specificity was 96.61% (95%
301 CI, 0.8829 to 0.9959). The positive predictive value was 98.28%, and the negative
302 predictive value was 97.33% (table 1).
303

304 **4 Discussion**

305 Brucellosis is a serious zoonotic disease. Bovine and goats are the most
306 commonly infected animals[12]. Currently, culling infected livestock is widely used
307 to eradicate brucellosis worldwide[13]. The accurate diagnosis of livestock is
308 essential for the prevention and control of the disease and for reducing unnecessary
309 economic losses. Particularly in China, where the base of bovine and goats is
310 relatively large, fast and efficient screening methods are of great significance[14].
311 Serological diagnostic techniques, mainly the agglutination test, RBPT, complement
312 fixation test (CFT), ELISA, immunochromatographic diagnostic test (ICDT), and
313 fluorescence polarization assay (FPA), are currently the commonly used screening
314 methods for brucellosis[15,16]. Serological methods have the advantages of high
315 sensitivity and a short operation time. Serological methods are the most commonly
316 used methods for diagnosing brucellosis, but the existing serological diagnostic
317 methods have the shortcomings of cross-reactivity with other bacteria (such as
318 *Escherichia coli* O157:H7 and *Yersinia enterocolitica* O9)[17], which easily results in

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

319 false positives. Reducing cross-reactions with other bacterial species is the key to
320 improving the diagnostic specificity of serological methods.

321 ELISA is currently the most widely studied serological diagnosis method, even as
322 diagnostic confirmation in brucellosis[16]. The main problem with using ELISAs for
323 the diagnosis of brucellosis is the choice of antigen, but to date, ELISA-based
324 diagnoses lack a single standard antigen[18]. Currently, the most commonly used
325 diagnostic antigens used in ELISA are whole bacteria or extracts. These diagnostic
326 antigens are prone to cross-reactivity with other bacteria, have poor specificity and
327 have considerable defects. Therefore, the development of new diagnostic antigens is
328 key to improving the diagnostic effect of ELISAs.

329 B cell epitopes refer to antigen regions that can be recognized by B cell receptors
330 (BCRs) or antibodies produced by the humoral immune system. Epitope recognition
331 is an important aspect of immunology. The rapid development of bioinformatics
332 provides convenient ways and methods for epitope prediction[19]. Bioinformatics
333 tools can be used to select potential cell epitopes without cultivating pathogens, which
334 has the advantages of rapid testing and low cost[20]. Currently, B cell epitope
335 prediction tools include databases, algorithms, and web servers. In this study, five
336 main antigen components of *Brucella* were selected, and 22 effective epitopes were
337 successfully predicted using the IEDB website. After iELISA verification, each
338 peptide was used to identify a certain sample of brucellosis serum. Therefore,
339 combining these peptides to construct new protein antigens can theoretically increase

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

340 the recognition of proteins in serum. The results of the iELISA confirmed our
341 hypothesis. The diagnostic specificity of this protein was higher than that of LPS, and
342 it did not cross-react with other bacteria. The fusion protein we constructed has
343 potential value as a diagnostic antigen.

344 In recent years, new disease diagnosis and pathogen detection technologies have
345 been developed based on ELISA. Among these technologies, the p-ELISA has been
346 rapidly developed and widely used in related fields such as health testing and
347 diagnosis[21]. The p-ELISA method is a new technology developed based on the
348 traditional ELISA method principle, using paper as the solid-phase carrier[22].
349 Compared with the traditional ELISA method, p-ELISA is faster and can be used to
350 detect proteins within 1 hour. Less reagent and only 2-5 μL of the samples are
351 required, no special instruments such as microplate readers are needed (only smart
352 phones or cameras are needed to take photos, combined with Photoshop and other
353 software to complete the data analysis), and the carrier is replaced with harmless
354 easy-to-handle paper, which greatly saves costs and provides a new research direction
355 for the development of new methods for the diagnosis of brucellosis [23]. Currently,
356 the most common paper-processing method of the p-ELISA method involves
357 constructing hydrophilic and hydrophobic areas through wax printing technology.
358 This method often requires expensive printers, which limits the application of this
359 method. We use plastic-encapsulated paper to construct the hydrophobic area,
360 punching small holes into it, filling the small holes with hydrophilic paper sheets to
361 make a sandwich structure. The paper can be processed with an ordinary laminator;

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

362 therefore, the cost is low. Through serum detection, p-ELISA has a better diagnostic
363 outcome. Compared with the traditional ELISA method, it has improved sensitivity
364 and specificity, and the dosage of the reagents and the time of the operation are
365 significantly reduced. This method can simultaneously detect bovine and goat
366 brucellosis and has certain application prospects.

367 In conclusion, we predicted 22 B cell epitopes by using bioinformatics
368 technology and successfully constructed a *Brucella* diagnostic antigen. Combined
369 with this antigen, we constructed a p-ELISA method that can be used to diagnose
370 bovine and goat brucellosis simultaneously. However, currently, it is extremely
371 difficult to identify all affected animals from epidemiological units through
372 serological tests, and there is no serological test able to differentiate between affected
373 and vaccinated animals. The p-ELISA method we have constructed cannot yet solve
374 the two questions mentioned above. In addition, our study is only preliminary, the
375 number of samples is small, and the selected samples, especially *Brucella*-positive
376 samples, represent highly positive samples. The effectiveness of this method needs
377 further study, such as by testing a large number of random samples.

378 **Acknowledgments**

379 **Conflict of Interest**

380 The authors declare no potential conflicts of interest with respect to the research,
381 authorship, and/or publication of this article.

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

382 **Funding**

383 This work was supported by the National Natural Science Foundation of China
384 (Grant number 81802101). The funders had no role in the study design, data
385 collection and analysis, decision to publish, or preparation of the manuscript.

386

387 **References**

388 1. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global
389 map of human brucellosis. Lancet Infect Dis. 2006;6:91-99.
390 2. Olsen SC, Palmer MV. Advancement of knowledge of Brucella over the past 50
391 years. Vet Pathol. 2014;51:1076-1089.
392 3. Deng Y, Liu X, Duan K, Peng Q. Research Progress on Brucellosis. Curr Med
393 Chem. 2019;26:5598-5608.
394 4. Franco MP, Mulder M, Gilman RH, Smits HL. Human brucellosis. Lancet Infect
395 Dis. 2007;7:775-786.
396 5. Araj GF. Update on laboratory diagnosis of human brucellosis. Int J Antimicrob
397 Agents. 2010;36 Suppl 1:12-17.
398 6. Yagupsky P, Morata P, Colmenero JD. Laboratory Diagnosis of Human Brucellosis.
399 Clin Microbiol Rev. 2019;33:e00073-19.
400 7. Gupta S, Singh D, Gupta M, Bhatnagar R. A combined subunit vaccine comprising
401 BP26, Omp25 and L7/L12 against brucellosis. Pathog Dis 2019;77:ftaa002.
402 8. Rezaei M, Rabbani-Khorasgani M, Zarkesh-Esfahani SH, Emamzadeh R, Abtahi H.
403 Prediction of the Omp16 Epitopes for the Development of an Epitope-based Vaccine

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

404 Against Brucellosis. Infect Disord Drug Targets 2019;19:36-45.

405 9. Verdiguel-Fernández L, Oropeza-Navarro R, Ortiz A, Robles-Pesina MG,
406 Ramírez-Lezama J, Castañeda-Ramírez A, Verdugo-Rodríguez A. Brucella melitensis
407 omp31 Mutant Is Attenuated and Confers Protection Against Virulent Brucella
408 melitensis Challenge in BALB/c Mice. J Microbiol Biotechnol 2020;30:497-504.

409 10. Cheng CM, Martinez AW, Gong J, Mace CR, Phillips ST, Carrilho E, Mirica KA,
410 Whitesides GM. Paper-based ELISA. Angew Chem Int Ed Engl 2010;49:4771-4774.

411 11. Wang S, Ge L, Song X, Yu J, Ge S, Huang J, Zeng F. Paper-based
412 chemiluminescence ELISA: lab-on-paper based on chitosan modified paper device
413 and wax-screen-printing. Biosens Bioelectron 2012;31:212-218.

414 12. Zheng R, Xie S, Lu X, Sun L, Zhou Y, Zhang Y, Wang K. A Systematic Review
415 and Meta-Analysis of Epidemiology and Clinical Manifestations of Human
416 Brucellosis in China. Biomed Res Int. 2018; 2018:5712920.

417 13. Zhang N, Huang D, Wu W, Liu J, Liang F, Zhou B, Guan P. Animal brucellosis
418 control or eradication programs worldwide: A systematic review of experiences and
419 lessons learned. Prev Vet Med. 2018;160:105-115.

420 14. Zhong Z, Yu S, Wang X, Dong S, Xu J, Wang Y, Chen Z, Ren Z, Peng G. Human
421 brucellosis in the People's Republic of China during 2005-2010. Int J Infect Dis.
422 2013;17:e289-92.

423 15. Khan MZ, Zahoor M. An Overview of Brucellosis in Cattle and Humans, and its
424 Serological and Molecular Diagnosis in Control Strategies. Trop Med Infect Dis.
425 2018;3:65.

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

426 16. Nielsen K, Yu WL. Serological diagnosis of brucellosis. Prilozi.2010;31:65-89.

427 17. Chart H, Okubadejo OA, Rowe B. The serological relationship between
428 Escherichia coli O157 and Yersinia enterocolitica O9 using sera from patients with
429 brucellosis. Epidemiol Infect. 1992;108:77-85.

430 18. Ducrotoy MJ, Muñoz PM, Conde-Álvarez R, Blasco JM, Moriyón I. A systematic
431 review of current immunological tests for the diagnosis of cattle brucellosis. Prev Vet
432 Med. 2018;151:57-72.

433 19. D'Annessa I, Di Leva FS, La Teana A, Novellino E, Limongelli V, Di Marino D.
434 Bioinformatics and Biosimulations as Toolbox for Peptides and Peptidomimetics
435 Design: Where Are We? Front Mol Biosci 2020;7:66.

436 20. Agyei D, Tsopmo A, Udenigwe CC. Bioinformatics and peptidomics approaches
437 to the discovery and analysis of food-derived bioactive peptides. Anal Bioanal Chem
438 2018;410:3463-3472.

439 21. Pang B, Zhao C, Li L, Song X, Xu K, Wang J, Liu Y, Fu K, Bao H, Song D, Meng
440 X, Qu X, Zhang Z, Li J. Development of a low-cost paper-based ELISA method for
441 rapid Escherichia coli O157:H7 detection. Anal Biochem 2018;542:58-62.

442 22. Wang S, Ge L, Song X, Yu J, Ge S, Huang J, Zeng F. Paper-based
443 chemiluminescence ELISA: lab-on-paper based on chitosan modified paper device
444 and wax-screen-printing. Biosens Bioelectron 2012;31:212-218.

445 23. Hsu MY, Hung YC, Hwang DK, Lin SC, Lin KH, Wang CY, Choi HY, Wang YP,
446 Cheng CM. Detection of aqueous VEGF concentrations before and after intravitreal
447 injection of anti-VEGF antibody using low-volume sampling paper-based ELISA. Sci

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.04.434020; this version posted March 5, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

448 Rep. 2016;6:34631.