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## Paper-based ELISA diagnosis technology for human brucellosis based on a multiepitope fusion protein

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| Abstract: | Background: Brucellosis, as a serious zoonotic infectious disease, has been recognized as a re-emerging disease in the developing countries worldwide. In china, the incidence of brucellosis is increasing each year, seriously threatening the health of humans as well as animal populations. Despite a quite number of diagnostic methods currently being used for brucellosis, innovative technologies are still needed for its rapid and accurate diagnosis, especially in area where traditional diagnostic is unavailable.<br>Methodology/Principal Findings: In this study, a total of 22 B cell linear epitopes were predicted from five Brucella outer membrane proteins (OMPs) using an immunoinformatic approach. These epitopes were then chemically synthesized, and with the method of indirect ELISA (iELISA), each of them displayed a certain degree of capability in identifying human brucellosis positive sera. Subsequently, a fusion protein consisting of the 22 predicted epitopes was prokaryotically expressed and used as diagnostic antigen in a newly established brucellosis testing method, nano-ZnO modified paper-based ELISA (nano-p-ELISA). According to the verifying test using a collection of sera collected from brucellosis and non-brucellosis patients, the sensitivity and specificity of multiepitope based nano-p-ELISA were 92.38% and 98.35% respectively. The positive predictive value was 98.26% and the negative predictive value was 91.67%. The multiepitope based fusion protein also displayed significantly higher specificity than Brucella lipopolysaccharide (LPS) antigen.<br>Conclusions: B cell epitopes are important candidates for serologically testing brucellosis. Multiepitope fusion protein based nano-p-ELISA displayed significantly sensitivity and specificity compared to Brucella LPS antigen. The strategy applied in this study will be helpful to develop rapid and accurate diagnostic method for brucellosis in human as well as animal populations. |
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1 **Paper-based ELISA diagnosis technology for human brucellosis based on a**

2 **multiepitope fusion protein**

3 **Running head：**P-ELISA for diagnosing human brucellosis

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26

27

28 **Abstract**

29 **Background:** Brucellosis, as a serious zoonotic infectious disease, has been
30 recognized as a re-emerging disease in the developing countries worldwide. In china,
31 the incidence of brucellosis is increasing each year, seriously threatening the health of
32 humans as well as animal populations. Despite a quite number of diagnostic methods
33 currently being used for brucellosis, innovative technologies are still needed for its
34 rapid and accurate diagnosis, especially in area where traditional diagnostic is
35 unavailable.

36 **Methodology/Principal Findings:** In this study, a total of 22 B cell linear epitopes
37 were predicted from five *Brucella* outer membrane proteins (OMPs) using an
38 immunoinformatic approach. These epitopes were then chemically synthesized, and
39 with the method of indirect ELISA (iELISA), each of them displayed a certain degree
40 of capability in identifying human brucellosis positive sera. Subsequently, a fusion
41 protein consisting of the 22 predicted epitopes was prokaryotically expressed and
42 used as diagnostic antigen in a newly established brucellosis testing method,
43 nano-ZnO modified paper-based ELISA (nano-p-ELISA). According to the verifying
44 test using a collection of sera collected from brucellosis and non-brucellosis patients,
45 the sensitivity and specificity of multiepitope based nano-p-ELISA were 92.38% and
46 98.35% respectively. The positive predictive value was 98.26% and the negative
47 predictive value was 91.67%. The multiepitope based fusion protein also displayed
48 significantly higher specificity than *Brucella* lipopolysaccharide (LPS) antigen.

49 **Conclusions:** B cell epitopes are important candidates for serologically testing

brucellosis. Multiepitope fusion protein based nano-p-ELISA displayed significantly sensitivity and specificity compared to *Brucella* LPS antigen. The strategy applied in this study will be helpful to develop rapid and accurate diagnostic method for brucellosis in human as well as animal populations.

**Keywords:** brucellosis; B cell epitope; nano-zinc oxide; p-ELISA; serological diagnosis

## Author Summary

Brucellosis is one of the most important zoonosis in the world and has caused tremendous economic losses in agriculture and animal husbandry in many countries. Developing rapid, sensitive and specific diagnostic methods is very important for early detection and treatment of brucellosis patients. In this study, a novel diagnostic technique, nano-ZnO modified paper ELISA, was established. The antigen used in this technique was a fusion protein containing multiple B cell epitopes, which were predicted from *Brucella* major outer membrane proteins such as Bp26, Omp31, Omp16, Omp2b and Omp25. Comparing to traditional LPS antigen, this multiepitope based antigen displayed considerably higher sensitivity and higher specificity in laboratory. With the strategy described in this paper, more efficient epitopes and protein antigen can be identified in the future. Currently, LPS antigen is only prepared from live *Brucella*, while protein antigen can be produced in large quantities in prokaryotic expression system. In addition to nano-p-ELISA, this protein antigen can also be used for development other methods such as fluorescent polarization assay (FPA) and immunochromatographic assay (ICA) to meet the varied demand for

72 brucellosis testing.

73

74 **Introduction**

75 Brucellosis is a reemerging zoonotic infectious disease. It not only seriously
76 threatens the health of the people but also causes huge economic losses to animal
77 husbandry industry. In human, brucellosis often manifests multiple symptoms and a
78 long course of disease. So it is often misdiagnosed and causes increased cost of
79 treatment and waste of medical resources [1]. Therefore, a rapid and accurate testing
80 technology is very important for brucellosis diagnosis and subsequent treatment.

81 Currently, diagnostic methods for brucellosis include bacterial isolation, specific
82 antibody detection and amplification of specific DNA fragments(PCR or qPCR) [2,3].
83 Bacterial isolation needs biosafety level 3 laboratory, and usually takes several
84 weeks[4]. PCR or qPCR are fast and having higher sensitivity and specificity, but
85 nucleic acid contamination often causes false positive result so that expensive
86 facilities must be needed to guarantee the accuracy of testing [5]. On the other hand,
87 antibody detection is the most popularly used method for testing brucellosis as they
88 are easy to handle and suitable for most laboratories. There are several serological
89 methods popularly used for antibody detection, including the agglutination test,
90 complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA),
91 immunochromatographic assay (ICA) and fluorescence polarization assay (FPA)[6].
92 All these methods are based on detecting antibody targeting for lipopolysaccharide
93 (LPS). Although LPS is a major immunogen arousing high level of antibody titer, it
94 contains the common epitope with other gram negative bacillus like *Yersinia* O:9 and
95 *Escherichia* O:157, which greatly reduces its specificity in testing brucellosis.
96 Moreover, as LPS is obtained only through culturing *Brucella* in high-level biosafety

97 facilities, it is not available to most of diagnostic kit manufacturers. Thus, seeking for
98 new antigen to replace LPS is critical for developing easily available brucellosis
99 testing kits.

100 In our previous study, the out membrane proteins such as Bp26 and Omp31
101 demonstrated considerable efficacy in detecting human brucellosis sera[7]. In this
102 study, the B cell epitopes from five out membrane proteins were predicted and
103 synthesized for the purpose of designing a more effective antigen. Paper-based
104 enzyme-linked immunosorbent assay (p-ELISA) is an emerging technology. Due to
105 the small reagent required and special equipment independence, it has attracted
106 increasing attention from diagnostic reagent developers[8-10]. Here, using a
107 prokaryotically expressed protein consisting of multiple B cell epitopes, a nano-ZnO
108 modified p-ELISA (nano-p-ELISA) was established. The efficiency of this new
109 method in detecting human brucellosis was evaluated against a collection of human
110 sera. Hopefully, the epitope based protein can be applied in development of other fast
111 and low cost diagnostic methods for brucellosis.

## 112 Methods

### 113 Human serum samples

114 121 human brucellosis sera were gifted by the School of Public Health of Jilin
115 University. 90 negative control sera were collected by Infection Department of the
*sera from healthy individuals
116 First Clinical Hospital of Jilin University, including 50 *healthy sera and 40 patient
117 sera confirmed by blood culture to be infected with other pathogens(S1 Table). All
118 experiments were approved by the Animal Care and Ethics Committee of Xuzhou

119 Medical University.

120 **B cell epitope prediction and synthesis**

121 The amino acid sequences of *Brucella* out membrane protein Bp26, Omp2b,
122 Omp16, Omp25 and Omp31 were download from NCBI website
123 (https://www.ncbi.nlm.nih.gov/protein/). *Brucella* species and protein accession
124 numbers were listed in supplementary S2 Table. The conserved amino acid sequences
125 of these proteins were used to predict B cell epitopes using BepiPred tool in IEDB
126 (http://tools.iedb.org/bcell/). Prediction threshold is 0.350(default value), above this
127 threshold is possible epitope. Peptides longer than 6 amino acids were assumed as
128 effective epitope and selected. Each of selected B cell epitope was chemically
129 synthesized and coupled to keyhole limpet hemocyanin (KLH) in Sangon Biotech
130 Company (Shanghai, China). The purity of each polypeptide-KLH was more than
131 90%.

132 **Evaluation of B cell epitopes**

133 121 human brucellosis positive sera were used to verify the diagnostic effect of
134 predicted epitopes by indirect ELISA (iELISA). Each peptide-KLH was diluted with
135 carbonate buffer (pH = 9.6) to final concentrations of 30 μg/mL. 100 μL of
136 peptide-KLH was added to 96-well plate (Corning, USA) and incubated overnight at
137 4°C. 300 μL blocking solution (5% skimmed milk in PBS) was then added to plate
138 and incubated at 37°C for 1 h. After washing 3 times with PBST, 100 μL of 1:400
139 diluted serum was added and incubated at 37℃ for 1 h. After washing 3 times with
140 PBST, 1:5000 diluted HRP-labeled protein G (Thermo, USA) was added to plate and

141 incubated at room temperature for 30 min. In the coloring step, 100 μL of TMB
142 substrate solution was added to each well and incubated for 15 min at room
143 temperature. Coloring was terminated by adding 50 μL of stopping solution (2 M
144 $H_2SO_4$). The optical density was measured at 450 nm ($OD_{450}$) using ELISA plate
145 reader (BioTek, USA). At the same time, KLH (30 μg/mL, sigma) and
146 lipopolysaccharide (LPS, 1 μg/mL provided by China Animal Health and
147 Epidemiology Center) were used as controls in this experiment.

148 **Fusion protein preparation and verification**

149 Selected peptides were concatenated together and adjacent peptides were
150 connected by a 'GGGS' linker(Fig S1). The DNA fragment corresponding to full
151 length of concatenated peptides was synthesized and cloned into the expression vector
152 pET-21a(+). Fusion protein containing concatenated peptides was expressed and
153 purified from *E. coli* BL21(DE3) cells according to the optimized procedures (Sangon
154 Biotech). The specific steps are described below.

155 After transferring the recombinant plasmid into BL21(DE3), 800 μL of
156 nonresistant LB medium was added, followed by incubation at 37°C for 45 min and
157 centrifugation at 5000 rpm for 3 min. Most of the supernatant was discarded (leave
158 approximately 100-150 μL), the bacteria were resuspended, the LB plate with
159 corresponding resistance was selected, and it was coated. After air-drying, it was
160 inverted and cultured overnight in a 37°C incubator. The monoclonal colonies on the
161 plate were chosen, placed into 10 mL of LB liquid medium and incubated at 37°C and
162 200 rpm. The cultured bacterial solution was transferred to 750 mL of LB liquid

163 medium at 37°C and 200 rpm, cultured to $OD_{600}$=0.6-0.8 with IPTG (0.5 mM) at
164 16°C and induced overnight. Then, the cells were centrifuged at 6000 rpm for 5 min,
165 the supernatant was discarded, and the bacteria were collected. Bacteria were blown
166 away with 20-30 mL 10 mM Tris-HCl (pH = 8.0) solution and ultrasonically broken
167 (500 W, 60 times, 10 s each time, 15 s interval). After sonication, 100 μL of the
168 bacterial suspension was centrifuged at 12000 rpm for 10 min, and 50 μL of
169 supernatant was transferred to another EP tube. After the supernatant was removed,
170 the precipitate was blown away with 50 μL of 10 mM Tris-HCl (pH = 8.0) solution.
171 SDS-PAGE and Western blotting were used to detect protein expression. A nickel
172 column (Ni Sepharose 6 Fast Flow, GE Healthcare) for affinity chromatography was
*Please rephrase the sentence
173 used for protein purification. *Taking 5 mL of Ni-NTA, the equilibrium column was
174 washed with 5 times the column bed volume of binding buffer at a flow rate of 5
175 mL/min. The crude protein was incubated with the equilibrated column packing for 1
176 h; the incubated product was loaded onto the column and the effluent liquid was
177 collected; the equilibrium column was washed with binding buffer; the column was
178 washed with washing buffer, and the effluent liquid was collected; with the column
179 was eluted with elution buffer, and the effluent liquid was collected; and the crude
180 protein was treated, washed with effluent and eluted with effluent separately, followed
181 by sample preparation and, SDS-PAGE and WB detection. The concentrated protein
182 was divided into 1 mL/tube and stored at -80°C.

183 **Evaluation of the diagnostic effect of fusion protein**

184 Diagnostic effect of the fusion protein was evaluated according to the iELISA

185 method described in **Evaluation of B cell epitopes section**. In this experiment, 1 μg
186 fusion protein was coated to each well in 96-well plate, while other conditions were
187 not changed.

188 **Establishment of nano-p-ELISA**

189 ZnO nanorods were synthesized on Whatman No. 1 filter paper by a
190 hydrothermal method[11]. Whatman filter paper was soaked in 100 mM zinc acetate
191 solution for 60 s and then annealed at 100 ℃ for 1 h to form a seed layer. Then, the
192 filter paper was transferred to a hydrothermal reaction vessel containing 100 mM
193 hexamethylenetetramine and $Zn(NO_3)_2.6H_2O$. Whatman filter paper was left at 90 ℃
194 for 5 h for formation of ZnO nanorods. Filter paper was then immersed in an hydrous
195 toluene solution with 1% APTES for 5 min, dried at 100 ℃ for 15 min and then
196 silanized. Scanning electron microscopy (SEM, JSM-7500F), X-ray diffraction (XRD,
197 Bruker D8) and X-ray photoelectron spectroscopy (XPS, Escalable250Xi) were used
198 to evaluate the nanorods structure on the surface of the paper. Nano-ZnO modified
199 Whatman filter paper was punched into circular pieces with a diameter of 10 mm and
200 A4 plastic packaging paper was punched into small holes with a diameter of 6 mm.
201 The 10 mm circle paper was placed in the center of the 6 mm holes of the plastic
202 packaging paper, fixed by a plastic packaging machine, and cut into small strips for
203 further use.

204 **Evaluation of the diagnostic effect of nano-p-ELISA**

205 Five microliters of fusion protein solution (30 μg/mL in PBS) was placed in each
206 well, followed by incubation at room temperature for 30 min, washing with 20 μL of

deionized water 3 times, and blocking with 20 μL of 5% skimmed milk powder at room temperature for 15 min. After washing 3 times with PBST, 5 μL of serum was added (diluted with 1:400) to the paper and incubated for 30 min. After washing 3 times with PBST, 5 μL of HRP labeled protein G was added (diluted with 1:8000), followed by incubation at room temperature for 210 s. After washing another 3 times, 5 μL of TMB substrate solution was added and incubated for 10 min. HP Laser Jet Pro MFP M227 was used to scan the paper. Image J software was used to carry out intensity analysis on developed color. To compare with the nano-p-ELISA method, the traditional p-ELISA (tra-p-ELISA) method was also performed according to literature[12]. Five microliters of chitosan dissolved in deionized water (0.25 mg/mL) was placed onto Whatman No.1 filter paper and air dried at room temperature. Then, 5 μL of 2.5% glutaraldehyde solution was added to the paper and incubated at room temperature for 2 h. The remaining steps are same as described in nano-p-ELISA.

**Statistical analysis**

Dot plot and receiver operating characteristic (ROC) curve analyses were performed using GraphPad Prism version 6.05. The gray intensity were determined by Student's t-test (unpaired t-test). $P$-values $< 0.05$ were considered to be significantly different.

## Results

**B cell epitope prediction and evaluation**

From five *Brucella* antigen proteins, BP26, Omp16, Omp25, Omp31 and Omp2b predicted 14, 8, 14, 12, 19 epitopes respectively, and finally a total of 22 epitopes

229 were selected(Table 1). The length of these peptides ranged from 9 to 28 amino acid.

230 The 22 polypeptide epitopes were synthesized and coupled to KLH. The results of

231 iELISA showed that these epitopes have different ability in identifying human

232 brucellosis positive sera(Fig 1). Average $OD_{450}$ value of KLH plus 3 times Standard

233 Deviation (SD) was used as threshold to distinguish positive and negative samples,

234 here the threshold value being calculated as 0.5178, the epitope with the highest

235 capability was P19266-6, as from 121 human brucellosis serum samples, 79 samples

236 were detected as above the threshold. The other six epitopes (P19266-5, 12, 13, 15, 16

237 and 18) displayed medium capability with 37, 43, 44, 33, 24 and 33 samples were

238 identified respectively. The remaining epitopes showed only limited capability, with

Please add space between 'detected' and '(Table 1)'

239 no more than 20 positive samples being detected(table 1).

240 Table 1 Information about 22 predicted B cell epitopes

| Protein | Epitope (amino acid sequence) | Start-end position | Length | Peptide ID | Number of positive sera recognized (n=121) |
|---|---|---|---|---|---|
| BP26 | AFAQENQMTTQPARIAV | 26-42 | 17 | P19266-1 | 18 |
| | KAGIEDRDLQTGGIN | 88-102 | 15 | P19266-2 | 17 |
| | QPIYVYPDDKNNLKEPTITGY | 104-124 | 21 | P19266-3 | 17 |
| | GVNQGGDLNLVNDNPSAVIN | 151-170 | 20 | P19266-4 | 19 |
| | LSRPPMPMP | 204-212 | 9 | P19266-5 | 37 |
| | AAAPDNSVPIAAGENSYNVSV NVVFE | 223-248 | 26 | P19266-6 | 79 |
| Omp2b | SGAQAADAIVAPEPEAVEY | 31-49 | 19 | P19266-7 | 10 |
| | DVKGGDDVYSGTDRNGWDK | 79-97 | 19 | P19266-8 | 12 |
| | NNSGVDGKYGNETSSGTV | 129-146 | 18 | P19266-9 | 10 |
| | TVTPEVSYTKFGGEWKNTVAE DNAWGGI | 341-368 | 28 | P19266-10 | 11 |
| Omp16 | AAAPGSSQDFTV | 44-55 | 12 | P19266-11 | 1 |
| | SRGVPTNRMRTISYGNERPVA VCD | 125-148 | 24 | P19266-12 | 42 |
| Omp25 | GRAKLENRTNGGTS | 56-69 | 14 | P19266-13 | 44 |

| | GNPVQTTGETQ | 115-125 | 11 | P19266-14 | 1 |
|---|---|---|---|---|---|
| | GGIKNSLRIGGEESSKSKTQT | 154-174 | 21 | P19266-15 | 33 |
| | GWTVGAGIEYAA | 175-186 | 12 | P19266-16 | 24 |
| | TDYGKKNFGLNDLDTRGSFKTNDIR | 199-223 | 25 | P19266-17 | 6 |
| | VSEPSAPTAAPVDTFSWTGGYIGINA | 24-49 | 26 | P19266-18 | 33 |
| | GKFKHPFSSFDKEDNEQVSGSL | 53-75 | 23 | P19266-19 | 8 |
| Omp31 | TGSISAGASGLEGKAE | 112-127 | 16 | P19266-20 | 7 |
| | GDDASALHTWSDKTKAGWTLGAGAEYA | 168-194 | 27 | P19266-21 | 4 |
| | DLGKRNLVD | 209-217 | 9 | P19266-22 | 8 |

241

242 **Fig 1.** Dotplot result of 22 epitopes in identifying 121 human brucellosis positive

243 serum by iELISA

244 **Fusion protein preparation and its diagnostic effect**

245 As each of 22 predicted epitopes demonstrated some extent discerning capability

246 for brucellosis sera, all these epitopes were included for constructing a fusion protein.

247 SDS-PAGE showed that the molecular weight of prokaryotically expressed fusion

248 protein was approximately 66 kDa (Figs 2A and 2B). Western blotting using anti-his

249 tag antibody showed the same result (Fig 2C). Further mass spectrometry verified that

250 the fusion protein was correctly expressed.

251 The diagnostic effect of the purified fusion protein was verified using121 human

252 brucellosis sera and 90 control sera. For the fusion protein, the area under the ROC

253 curve was 0.9877 (95% CI: 0.9758 to 0.9996), while the area under the ROC curve

254 for LPS was 0.9174 (95% CI: 0.8796 to 0.9552) (Fig 3), indicating that fusion protein

255 has higher diagnostic effectiveness than LPS. The optimal cutoff value was also

256 calculated by the Youden index, under which the positive predictive value (PPV) and

257 negative predictive value (NPV) of fusion protein were higher than those of
258 LPS(Table 2). Under the cutoff value, 3 negative samples were misdiagnosed as
259 positive with fusion protein. However, 20 negative samples was misdiagnosed as
260 positive with LPS. This data indicated that the fusion protein had better specificity
261 than LPS.

262 Table 2. Positive and negative predictive values of the test calculated for different
263 cutoff values

| Cutoff value | Positive | | Negative | | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|
| | TP | FN | TN | FP | | |
| ≥0.470 (fusion protein) | 117 | 4 | 87 | 3 | 95.90 | 95.51 |
| ≥0.4095 (LPS) | 115 | 6 | 70 | 20 | 85.19 | 92.10 |
| ≥50.98 (nano-p-ELISA) | 113 | 8 | 88 | 2 | 98.26 | 91.67 |
| ≥45.66 (tra-p-ELISA) | 113 | 8 | 87 | 3 | 97.41 | 91.58 |

264 TP, true positives; TN, true negatives; FP, false positives; FN, false negatives; PPV,
265 positive predictive value (TP/TP+FP)×100; NPV, negative predictive value
266 (TN/TN+FN) ×100.

267

268 **Fig 2.** SDS-PAGE and Western blot analysis of fusion protein. (A)SDS-PAGE result
269 of fusion protein in the process of purification (M, marker; lane1, loading solution;
270 lane2, flow-through solution; lane3-4, 20 mM imidazole elution fraction; lane5, 50
271 mM imidazole elution fraction; lane6, 500 mM imidazole elution fraction). (B)
272 SDS-PAGE result of purified fusion protein (M, marker; lane1, purified protein). (C)

273 Western blot result of purified fusion protein (M, marker; lane1, purified protein).

274

275 **Fig 3.** Comparison of fusion protein and LPS in detecting human brucellosis positive
276 and negative sera. (A) Dotplot result of iELISA assay with fusion protein. (B) ROC
277 analysis of iELISA assay with fusion protein.(C) Dotplot result of iELISA assay with
278 LPS antigen. (D) ROC analysis of iELISA assay with LPS antigen.

279 **Characterization of nano-ZnO**

280 XRD results showed that the main composition of nano-ZnO was successfully
281 formed(Fig 4A). Scanning electron microscopy showed the shape of nano-ZnO on the
282 surface of Whatman filter paper (Fig 4B). XPS showed that the concentration of Zn
283 atoms was 40.79% and the concentration of oxygen atoms was 59.21%, which further
284 indicated that the nano-crystal was composed of ZnO.

285

286 **Fig 4.** Characterization of prepared nano-ZnO. (A) XRD analysis of nano-ZnO. (B)
287 The shape of nano-ZnO acquired by scanning electron microscope.

288 **Evaluation of the diagnostic effect of nano-p-ELISA**

289 Using 211 human brucellosis positive and negative sera, the ROC curve was
290 obtained for nano-p-ELISA. Under the optimal cutoff value, 113 out of 121 positive
291 samples were accurately diagnosed, and 88 out of the 90 negative samples were
292 correctly identified. The positive predictive value of the nano-p-ELISA was 98.26%,
293 and the negative predictive value was 91.67% (Table 2). There was a significant
294 difference between the positive and negative samples($P<0.001$) (Fig 5A). The area

295 under the curve was 0.9900 (95% CI, 0.9816 to 0.9984), indicating that this method
296 performed well in diagnosing human brucellosis. The optimal cutoff value was 50.98,
297 under which the sensitivity of this method was 92.38% (95% CI, 0.8554 to 0.9665)
298 and the specificity was 98.35% (95% CI, 0.9416 to 0.9980)(Fig 5B).

299 Correspondingly, the gray intensities and ROC curve of tra-p-ELISA
300 demonstrated a similar performance as nano-p-ELISA(Figs 5C and 5D). Sensitivity
301 and specificity analysis also supported this conclusion(Table 2).

302

303 **Fig 5.** P-ELISA analysis of human serum samples. (A)Dotplotresult of the
304 nano-p-ELISA assay. (B) ROC analysis of nano-p-ELISA assay results. (**C**) Dotplot
305 result of tra-p-ELISA assay. (D) ROC analysis of tra-p-ELISA assay results.

306 **Discussion**

307 At present, the most of commercially available brucellosis detecting kits are
308 based on the LPS which is polysaccharides compound on the surface of *Brucella*.
309 Although single antigenic epitope of LPS can be chemically synthesized[13], under
310 most circumstances, acquisition of LPS is only achieved by culturing live *Brucella* in
311 high-level biosafety laboratories, which is an insurmountable obstacle for most
312 diagnostics manufacturers. Therefore, seeking for new candidate antigen to replace
313 LPS is of great significance for developing brucellosis detecting kits which can be
314 easily produced and available to all users.

315 According to literatures, many *Brucella* OMPs manifested strong capacity in
316 arousing humoral immune response [14-16], and some of these proteins have also

been used for development of subunit vaccine against brucellosis[17,18]. In our previous study, some *Brucella* OMPs showed quite satisfactory result in diagnosing brucellosis, basically comparable to LPS antigen [7]. The data in this paper reflected that not only entire OMPs, the linear short peptides in these OMPs also maintain good antigenicity. Moreover, multiple epitopes were more effective than single epitopes in identifying human brucellosis positive sera. It seems that higher sensitivity of epitope-based protein antigen could be achieved by increasing the number of epitopes. But, more epitopes could imply lower specificity as the chance of including common epitopes with other pathogens can be simultaneously increased. Therefore, there is still a lot of trimming work to be done on this fusion protein, so that the most suitable epitopes are selected for future commercial use.

The immunoinformatic analysis is an emerging science that integrates life sciences, computer science and mathematics to accelerate the process for vaccine design, disease diagnosis and treatment, as well as diagnostic agent screening[19,20]. The online immunoinformatic tool (Bepipred Linear Epitope Prediction) used in this study were proved to be feasible in discerning viable B cell epitopes, as a quite number of predicted epitopes were subsequently confirmed to be effective in laboratory. On the other hand, there were still many predicted epitopes just showing limited diagnostic effects. Hopefully in the future, a combination of immunoinformatic tools can be set up and used to improve the efficiency of epitope prediction.

338 Fast and easy-to-perform are the most concerned features in developing novel
339 brucellosis detection technologies, especially in the point-of-care testing of medical
340 diagnosis, environmental surveillance and food safety analysis. P-ELISA has attracted
341 the attention from many researchers due to its higher specificity, simplicity, rapidity,
342 portability and low cost [22,23]. Currently, nanomaterial modified p-ELISA are more
343 widely used as modification can increase the surface area of the paper[10, 24-26].
344 Although tra-p-ELISA and nano-p-ELISA demonstrated similar antigenic capability,
345 the latter method is more suitable for rapid on-site detection as it uses fewer reagent
346 and can be stored at room temperature for a long time.

347 In summary, using bioinformatic technology combined with nanomaterials, this
348 performance has established a new type of brucellosis diagnostic technology, which
349 has good potential application value. However, the brucellosis sera selected in this
350 study were all clinically screened positive sera, and the number was limited. The
351 diagnostic validity of this method requires a large number of clinical random samples
352 for verification. Beside nano-p-ELISA, there are some other methods suitable for
353 rapid on-site testing, such as FPA and ICA. Combining the multiepitope based antigen
354 and well established fast testing methods, more brucellosis testing kits would be
355 produced in the future to meet the varied demand for brucellosis testing.

356

357 **Acknowledgments**

358 We thank the School of Public Health of Jilin University for their gift of the

359 brucellosis serum samples.

360 **Competing interests**

361 The authors declare that they have no competing interests

362 **Funding**

363 This work was supported by Young Scientists Fund of the National Natural Science

364 Foundation of China (Grant number 81802101).

365 **Author Contributions**

366 **Conceptualization:** Dehui Yin, Mingjun Sun, Hai Jiang, Jingpeng Zhang.

367 **Data Curation:** Dehui Yin, Qiongqiong Bai, Xiling Wu, Han Li.

368 **Formal Analysis:** Dehui Yin, Qiongqiong Bai, Jihong Shao.

369 **Funding Acquisition:** Dehui Yin.

370 **Investigation:** Dehui Yin, Mingjun Sun.

371 **Methodology:** Dehui Yin, Qiongqiong Bai, Xiling Wu, Han Li, Jihong Shao, Mingjun

372 Sun.

373 **Project Administration:** Dehui Yin, Mingjun Sun, Jingpeng Zhang.

374 **Resources:** Dehui Yin, Mingjun Sun, Han Li, Jingpeng Zhang.

375 **Supervision:** Dehui Yin, Mingjun Sun, Jingpeng Zhang.

376 **Validation:** Dehui Yin, Mingjun Sun.

377 **Writing – Original Draft Preparation:** Dehui Yin, Qiongqiong Bai.

378 **Writing – Original Draft Preparation:** Dehui Yin, Mingjun Sun, Hai Jiang,

379 Jingpeng Zhang.

380

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454

455 **Supporting information**

456 **S1 Fig. Fig S1.** Comprehensive sequence of amino acids of the fusion protein; the
457 linkers are in red font.

458 **S2 Fig. Fig S2.** Results of p-ELISA. (A) Positive of nano-p-ELISA. (B) Negative of
459 nano-p-ELISA. (**C**) Positive of tra-p-ELISA. (D) Negative of tra-p-ELISA.

460 **S1 Table. Table S1.** Information of the patient

461 **S2 Table. Table S2**. The OMPs' Accession Numbers of *Brucella* in NCBI Protein
462 database

463 **S1 Data. supporting data for figure 1.**

464 **S2 Data. supporting data for figure 3.**

465 **S3 Data. supporting data for figure 5.**

466 **S4 Data. supporting data for XPS**

467 **S5 Data. supporting data for figure 4A.**

468 **S1 File. Medical Ethics Review Report.**

Figure 1

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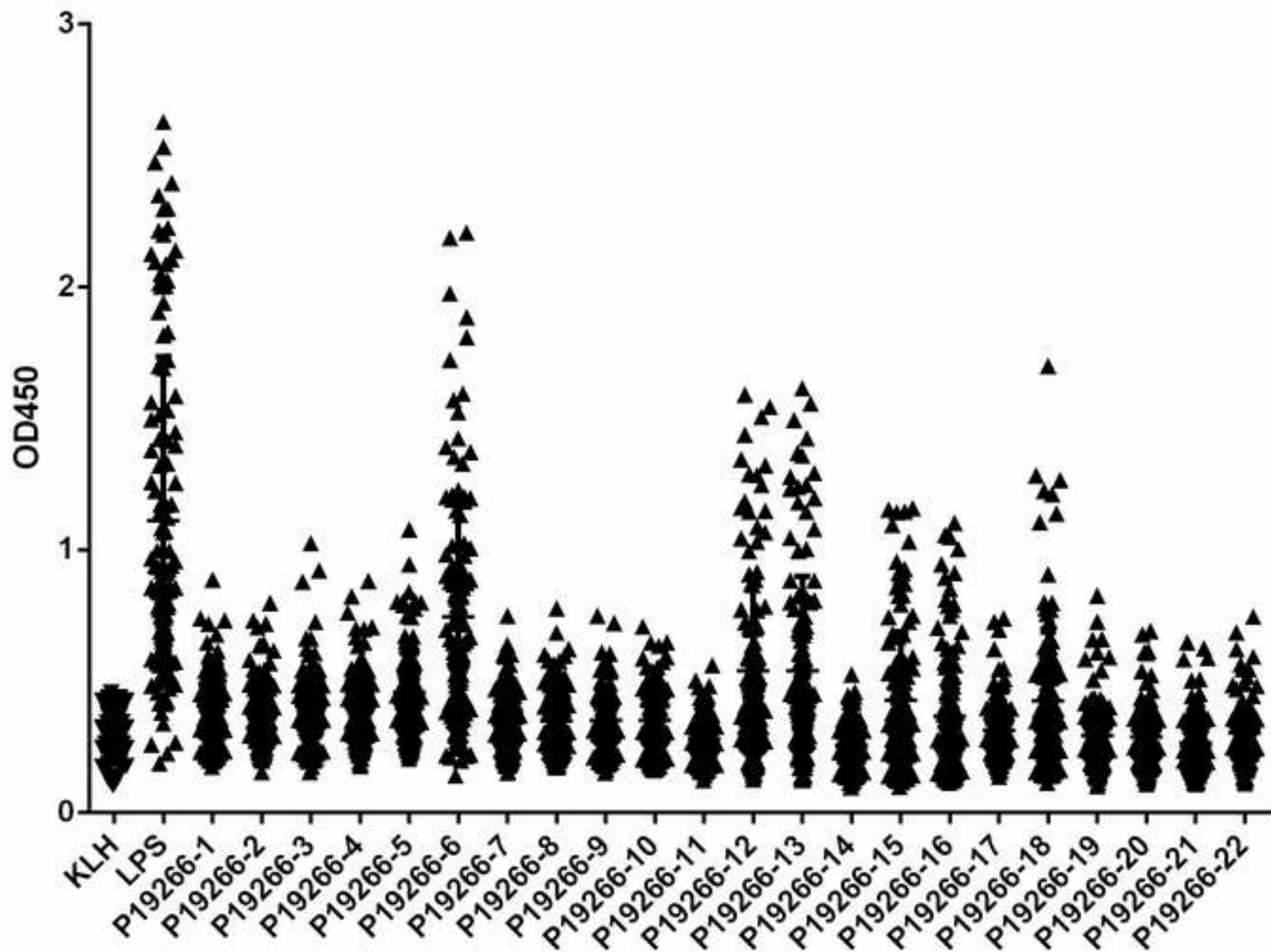

Figure 2

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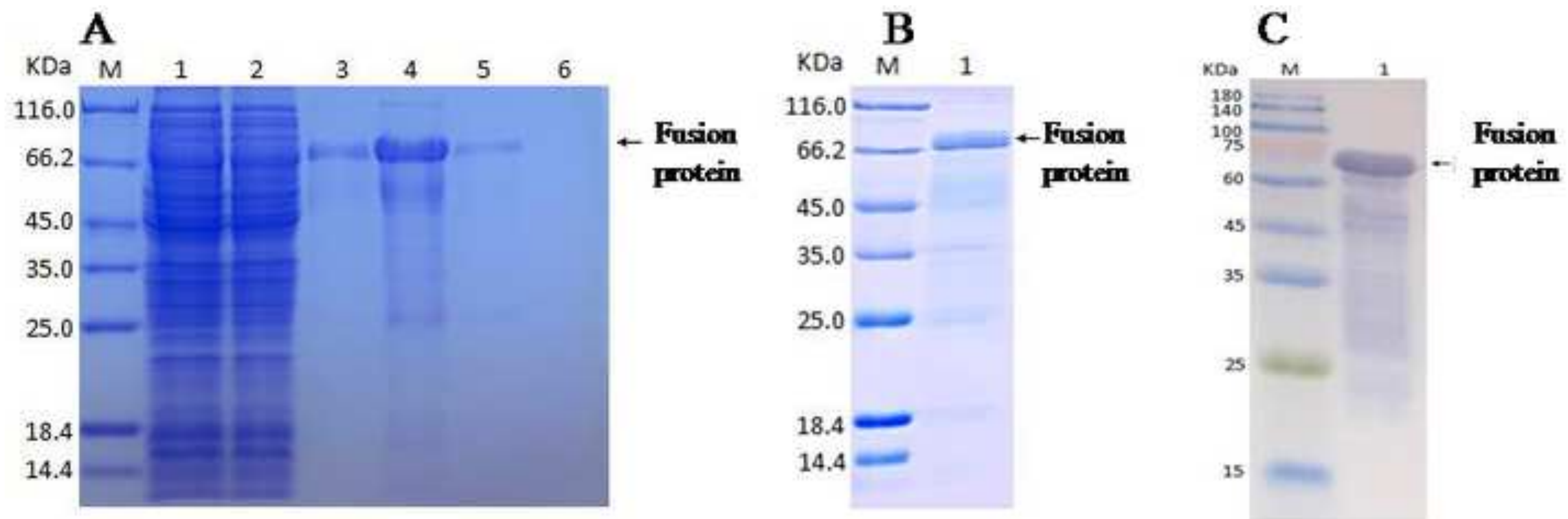

Figure 3

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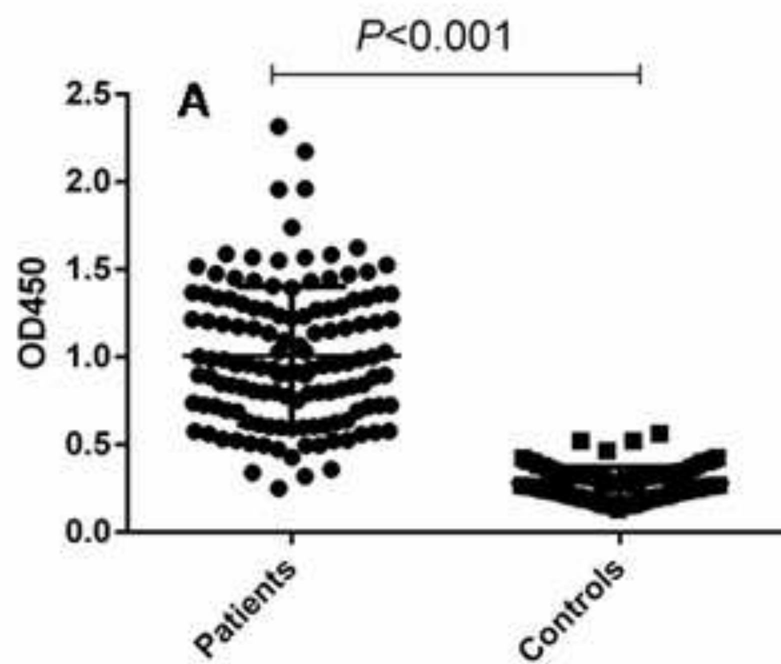


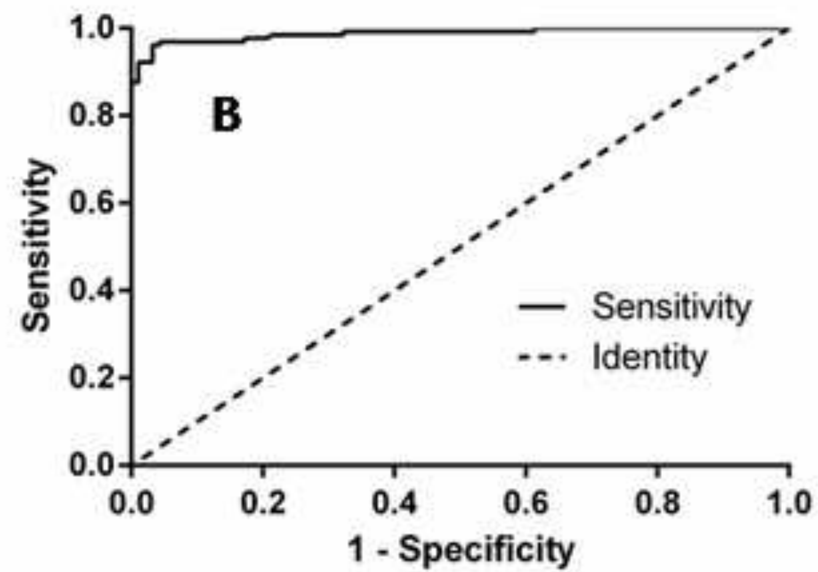


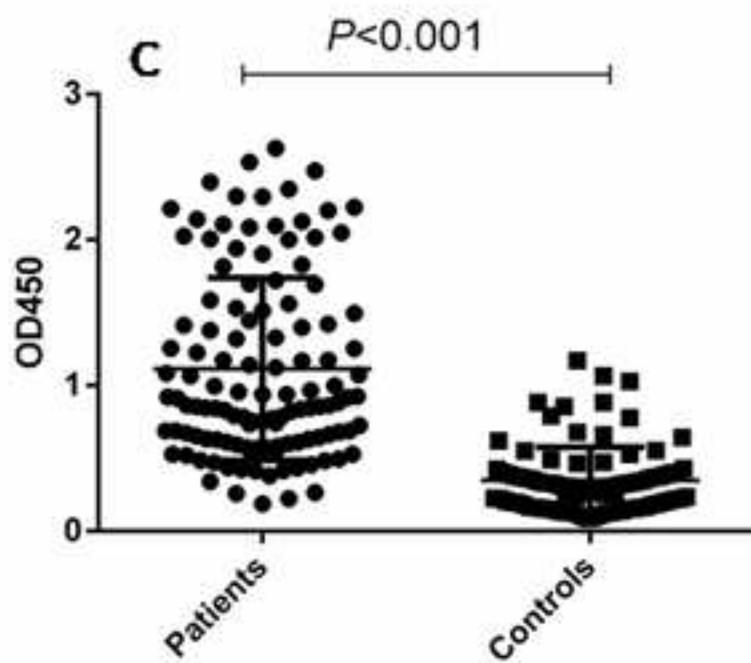


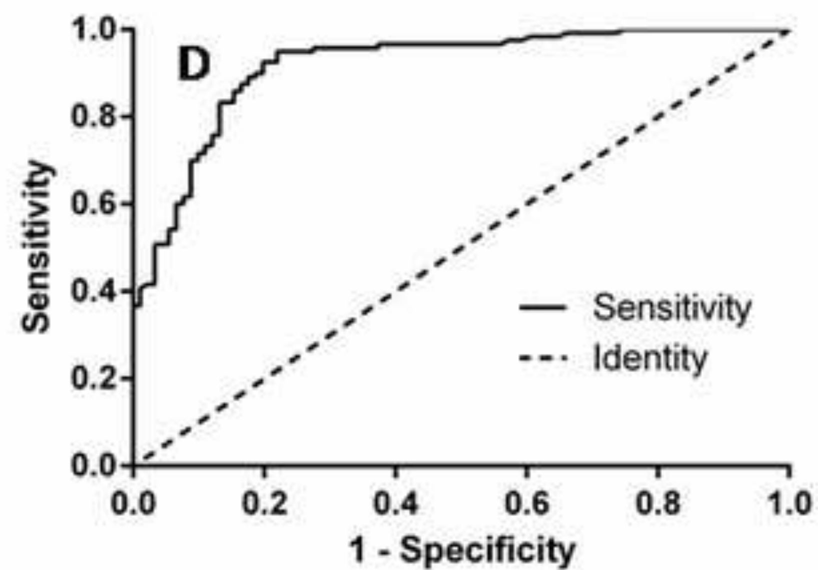

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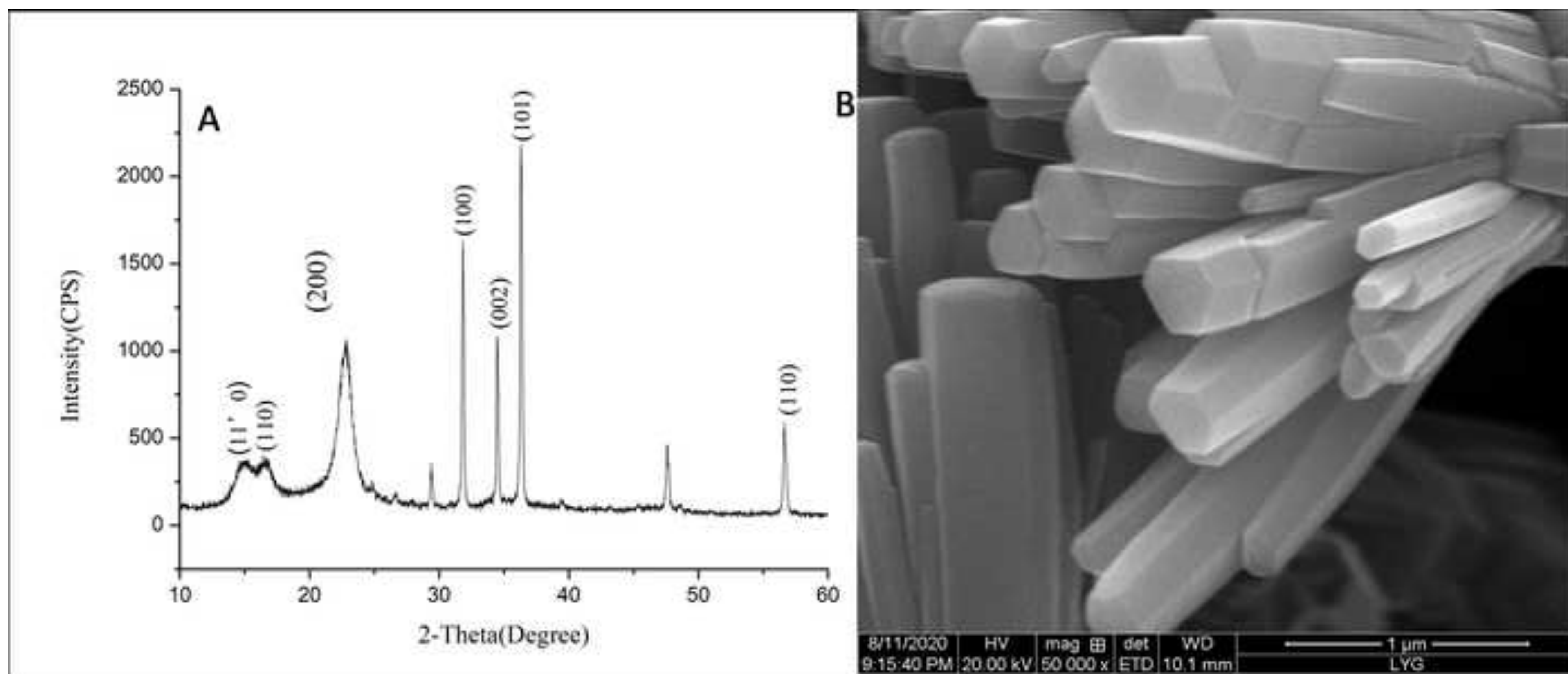

Figure 5

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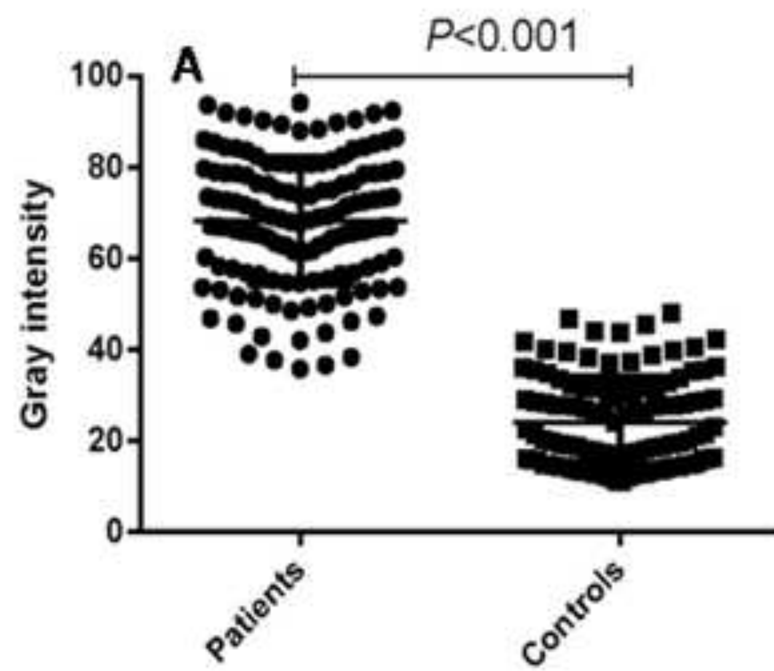


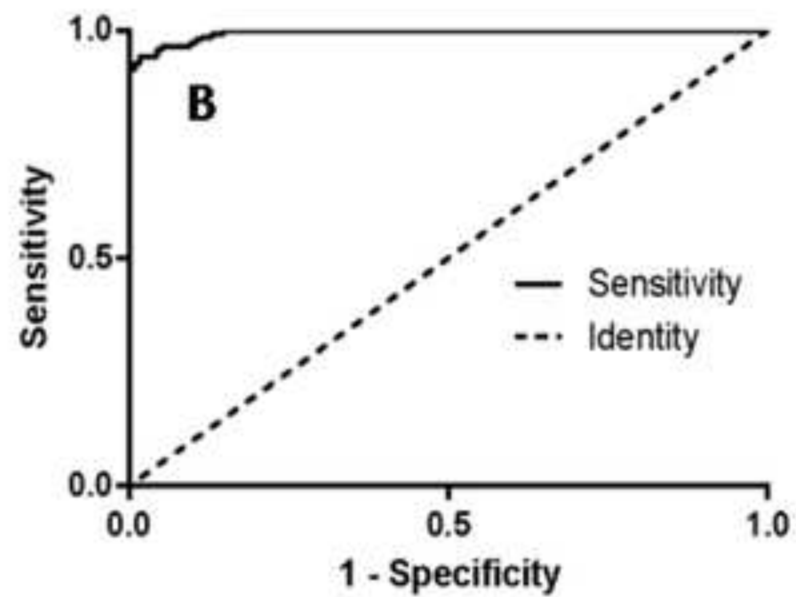


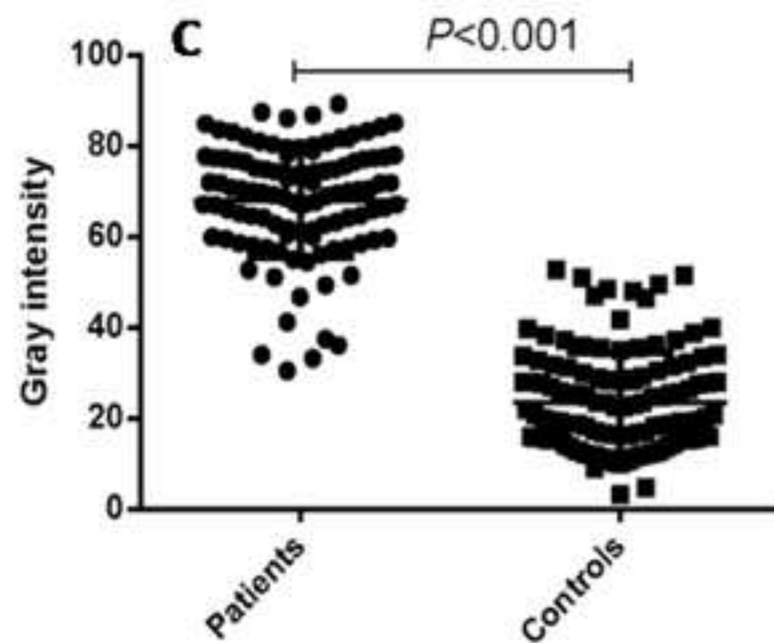


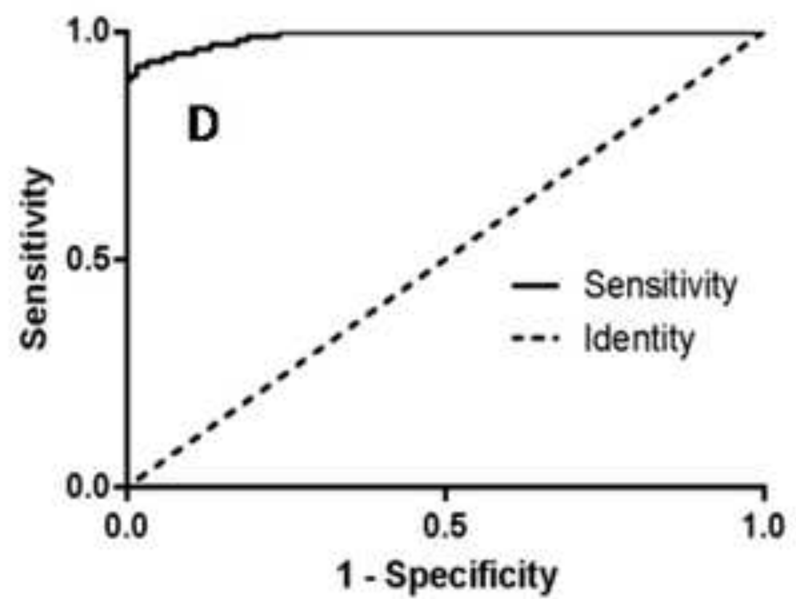

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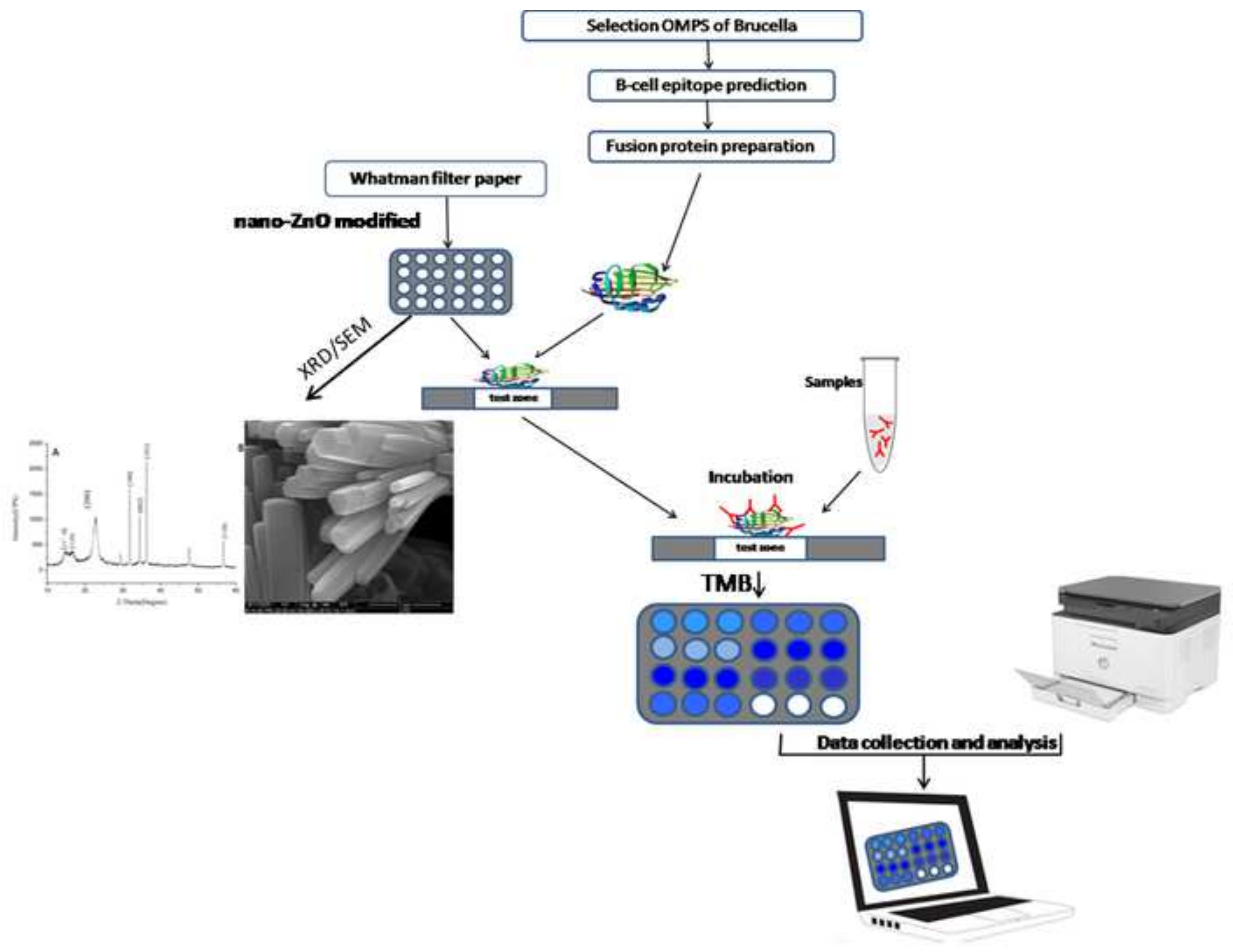

**Dear Editors and Reviewers:**

We really appreciate for your time and comments on our manuscript entitled "Paper-based ELISA diagnosis technology for human brucellosis based on a multiepitope fusion protein" (ID: PNTD-D-21-00330). Considering the reviewers' comments and the fact that English is not our native language, to make it more understandable, we consulted to native English speaker and made extensive revision on the main text, but the results and supporting materials such as tables and pictures were not changed. We hope that the massive text changes would not result in adverse effect that the revised manuscript could be considered as a new manuscript. The previous and revised manuscripts were provided for your consideration.

More attentions were paid to the sections reviewers most concerned, and modification has been made according to the reviewer's suggestion. To the reviewer's comments, our responses were listed as followings:

**Methods**

**Reviewer #1:**

**1. Objectives and hypothesis are not clear in the manuscript. Methods need details and not matching with the results provided.**

**Response:** The objective of this study is to provide a novel diagnostic antigen for brucellosis. To make the objective clearer, we reorganized the Introduction section, and added description about antigen used in current brucellosis testing methods. We hypothesized that the B cell epitopes predicted from *Brucella* out membrane proteins can be used to replace LPS for brucellosis serodiagnosis. So, in this study, a fusion protein containing multiepitopes were produced ant its efficacy in diagnosing human

brucellosis was evaluated with ELISA method. At the same time, the Method sand Results sections were carefully revised and more details were added. We believed that the results were much clearer in revised manuscript.

**2. Section starting from L106 and 111 could be pooled together to make it comprehensive and easy to follow. The algorithm used to determine length and selection of sequence of peptides predicted and selected for the study are not mentioned. Also, the number of peptide epitopes predicted are missing. Were only 22 peptides predicted and used in screening by ELISA and for fusion protein preparation? Or 22 peptides were selected? The final length and details of sequence for fusion protein produced are missing in methods, as provided in table 1.**

**Response:** Line 106 to line 111 has been revised in the new manuscript. The method used to determine length and selection of sequence of peptides were addedto the **Methods** Section. The prediction was mainly depended on the online software BepiPred (http://tools.iedb.org/bcell/) using default parameters. The output epitopes had different number of amino acid and some even contains just single amino acid. So an empirical value was used to choose the epitopes, here epitopes containing more than 6 amino acids were believed to have effective diagnostic capability. From five Omps, a total of 22 predicted peptides were found to be longer than 6 amino acids, and all of them were included in following verifying assay by ELISA. The method section has been modified so that the epitope prediction and selection step can be clearly described(Line 125). The final length and details of sequence for fusion

protein were also added, please see the **Supporting information (Fig S1)**.

**3. L236-Describe the positive sera used for screening of peptides? Is it the reference sera or from 121 known positive sera? Were all sera reacted to all 22 peptides?**

Response: We are very sorry that we did not describe the source of human sera clearly. We added the details in line 132. Reference sera for human brucellosis are not available presently. So, the 121 positive sera used for screening of peptides. According to the result, not all sera reacted strongly to these peptides, but each peptide can recognize a proper portion of these positive sera. We revised the data in Results section so that it can be clearly demonstrated(Table 1).

**4. L213-nanomodified p-ELISA (nano-p-ELISA) method comparison using traditional p-ELISA is described, however the details for nano-p-ELISA is not mentioned in the manuscript.**

**Response:** The method for nano-p-ELISA has been reorganized and described in Method Section (Line 167 to 213). The verifying data was displayed in Table 2. The nano-p-ELISA showed very similar efficacy in detecting human brucellosis as tra-p-ELISA, so we just described briefly the comparison result in Discussion section.

**5. Sample size is sufficient and population is also clearly described. The statistical analysis support the findings. No concerns about ethical and regulatory requirements.**

**Response**: Thank you very much for your comments. Actually, the study has been reviewed and approved by the Ethics Committee of Xuzhou Medical University, and related document was provided at the end of this cover letter.

**Reviewer #2:**

**I would suggest for the authors to add the Figure on the schematic diagram of developed paper-based ELISA for human brucellosis using multiepitope fusion protein.**

**Response:** Thank you very much for your suggestion, the schematic diagram of nano-ZnO modified paper-ELISA has been created, please see the Graphical Abstract Image.

**Results**

**Reviewer #1:**

**1. Results of LPS antigen are shown in figure 3 C and D, but details of LPS antigen are missing in method section and not mentioned elsewhere in the manuscript.**

**Response:** LPS antigen was provided by China Animal Health and Epidemiology Center. We had added the related information to Methods section, and please refer to the line 145 to 146.

**2. L236-Describe the positive sera used for screening of peptides? Is it the reference sera or from 121 known positive sera? Were all sera reacted to all 22 peptides?**

**Response:** We are very sorry that we did not describe the source of human sera clearly. We added the details in line 132. Reference sera for human brucellosis are not available presently. So, the 121 positive sera used for screening of peptides. According to the result, not all sera reacted strongly to these peptides, but each

peptide can recognize a proper portion of these positive sera. We revised the data in Results section so that it can be clearly demonstrated(Table 1)..

3. **Nanomodified p-ELISA (nano-p-ELISA) method comparison using traditional p-ELISA is unclear. In discussion L365, authors also mention comparison with traditional ELISA, please provide details.**

   **Response:** Using 121 brucellosis positive sera and 90 negative sera, the sensitivity and specificity of nano-p-ELISA and tra-p-ELISA were evaluate and displayed in Table 2. As the two mehods showed very similar efficacy in detecting human brucellosis, we just described briefly the comparison result in Discussion section.

**Reviewer #2:**

**Overall the results are clearly presented accept that Figure 1. I would suggest the authors to replace with better quality of the graph to show clearly results of iELISA of each peptide identification-positive brucellosis serum.**

**Response:** Thank you for your suggestion, we have replaced the Figure 1 and with better quality of the graph.

**Conclusions**

**Reviewer #1:**

1. **This section needs to be revised with relevance to findings of the manuscript.L340-341-should it be part of results?**

**Response:** This section has been massively revised with relevance to the main findings. Some sentences apparently more suitable for Introduction or Result sections were removed from this section.

2. **L345-346- need justification for the statement for cross-reactivity.**

**Response:** After careful consideration, more accurate term to describe antigen's reactivity to negative sera is specificity. So, we deleted the word of *cross-reactivity* from the manuscript.

**3.L357-359 should be part of introduction and not discussion.**

**Response:** We made great change to the Discussion section, and these sentences has been deleted from the manuscript.

**Reviewer #2:**

**1. The conclusions are supported the data presented and the authors have explained the public health relevance for human brucellosis by using the developed method (paper-based ELISA on multiepitope fusion protein)**

**Response**: Thank you very much for your comments.

**Editorial and Data Presentation Modifications?**

Use this section for editorial suggestions as well as relatively minor modifications of existing data that would enhance clarity. If the only modifications needed are minor and/or editorial, you may wish to recommend "Minor Revision" or "Accept".

**Reviewer #1:**

**Authors need to carefully review terminology used in the manuscript. The term "antigenicity" is misleading, when throughout the manuscript the fusion peptide was used as a target for detection of antibodies in human sera.**

**Response**: Thank you very much for your comment. In this paper, we discussed mainly the capability of fusion peptides in serodiagnosis of human brucellosis. To avoid misunderstanding, we deleted the word of *antigenicity* from manuscript, and replace it with other words such as diagnostic effect.

**Reviewer #2: Minor revision**

**Summary and General Comments**
**Reviewer #1: (No Response)**

**Reviewer #2:**

**1. This study is important to be published as the developed method will help the human brucellosis diagnosis.**

**Response:** Thank you very much for your comment.

**2. The study is novel and has shown the better performance in terms of sensitivity and specificity of the test.**

**Response:** Thank you very much for your comment.

**3. Please include the details of the ethical approval documents.**

**Response**: Before we applied for this project from National Natural Science Foundation of China in 2018, the application has been reviewed and approved by the Ethics Committee of Xuzhou Medical University. We attached the original ethical documents in picture format as following. At the same time, we also provided the translated version for your consideration.

## 医学伦理审查报告

我校公共卫生学院拟开展“基于多表位融合蛋白的人布鲁氏菌病快速诊断技术研究”科研工作，该项目涉及布鲁氏菌病人血清标本的采集和检测，我校医学伦理委员会对该项目相关医学伦理学问题进行了审查。

**项目信息：**

项目名称：基于多表位融合蛋白的人布鲁氏菌病快速诊断技术研究

承担单位：徐州医科大学，项目负责人：殷德辉，职称：讲师

研究起止日期：2018 年 7 月至 2021 年 6 月

**涉及人体研究的主要内容：**

本项目拟以布鲁氏菌病病人血清为研究对象，利用生物信息学、融合蛋白、蛋白L和p-ELSIA技术，建立一种快速、灵敏的人布鲁氏菌病血清学诊断新技术，为针对性地开展人兽共患传染病-布鲁氏菌病的防治提供有价值的科学依据和新的策略，具有重要的现实意义。

**审查评议意见：**

经我校医学伦理委员会审议，该研究的实验设计和实施方案充分考虑了安全性和公平性原则，研究内容不构成对研究人员等人群的危害，研究内容和结果不存在利益冲突。

**结论：**

该研究中，实验内容符合医学伦理学要求，不存在潜在风险，同意该项研究的工作按计划进行。

徐州医科大学伦理委员会

2018 年 2 月 27 日

**Translated version:**

## Medical Ethics Review Report

School of Public Health of our school intends to carry out the scientific research work of *Study on the rapid diagnosis technology of human brucellosis based on multiepitope fusion protein*. This work involves the collection and detection of serum samples from human. The Ethics Committee of Xuzhou Medical University carefully reviewed the related medical ethics issues about this project.

**Project information:**

**Project name:** Study on rapid diagnosis technology for human brucellosis based on

multiepitope fusion protein

**Institute undertaking this project:** Xuzhou Medical University

**Project leader:** Yin Dehui, Lecturer

**Start and end dates:** From January 2019 to December 2021

**Main content involved in human research:**

This project intends to use the serum of brucellosis patients together with bioinformatics, fusion protein, protein L and p-ELSIA technology to establish a rapid and sensitive new technology for the serological diagnosis of human brucellosis. It is of great significance for the prevention and treatment of the zoonotic infectious disease brucellosis in human, and also providing a valuable strategy for developing novel diagnostic technology for this disease.

**Review comments:**

After careful consideration by the Ethics Committee of Xuzhou Medical University, the research design and implementation fully comply with the principles of safety and fairness. The research content does not constitute any hazard factor to researchers and other populations, and there is no conflict of interest in the research content and results.

**Conclusion:**

In the study, the experimental content completely meets the requirements of medical ethics and there is no potential risk. Agreement is reached that this work can be carried out according to the plan.

Ethics Committee of Xuzhou Medical University

February 27, 2018

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Once again, thank you very much for your comments and suggestions.

Best wishes,

Dehui Yin

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