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Title page

Antigenicity and Potential Use of A Novel Brucella Multiepitope Recombinant Protein in the Diagnosis of Brucellosis

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Antigenicity and Potential Use of A Novel Brucella Multiepitope Recombinant Protein in the Diagnosis of Brucellosis

Abstract

Currently, brucellosis is a reemerged zoonotic infectious disease with an increased incidence in recent years. A simple, rapid and sensitive method for diagnosing brucellosis has important significance for early diagnosis and early treatment, which can help to reduce medical burden and economic loss. Previously, a multiple epitope recombinant protein was constructed based on linear B-cell epitope prediction tools. In this study, the recombinant protein was used as an antigen to study the immune response produced by immunized mice, and goat serum was used to verify its diagnostic accuracy.The production of antibodies was successfully induced in the vaccinated mice. Through analyzing the serum antibody subtypes, the primary antibody was identified as IgG. Flow cytometric analysis revealed that the percentage of CD4+, CD8+ and the CD4+/CD8+ ratios were increased by T cell subsets in mouse splenocytes, indicating that the recombinant protein induced a strong immune response in mice and that it had strong immunoreactivity. Using indirect ELISA, the recombinant protein correctly diagnosed positive and negative brucellosis samples. Compared with the whole bacterial antigen, the recombinant protein had a weaker sensitivity but a stronger specificity.In this study, animal experiments showed that the recombinant protein had good antigenicity, and indirect ELISA indicates that it can be used as an antigen to diagnose brucellosis. Therefore, the recombinant protein is a

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35 potential candidate antigen for brucellosis vaccine development and serological

36 diagnosis.

37 **Key words**: brucellosis; recombinant protein; serological diagnosis; vaccine

38

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Introduction

Currently, a high incidence of brucellosis, a hazardous zoonosis, is reemerging worldwide, especially in developing countries. There are more than 500,000 new cases every year in the world[1], and the number of cases in China is also increasing annually, with approximately 37,000 new cases reported in 2018 (http://www.nhc.gov.cn/jkj/s3578/201904/050427ff32704a5db64f4ae1f6d57c6c.shtml). Brucellosis causes not only human health problems but also extensive losses in agriculture and livestock husbandry. Therefore, it is of great importance to strengthen the prevention and diagnosis of brucellosis.

In recent years, various diagnostic methods based on detecting specific antibodies in patient sera have been used for the diagnosis of brucellosis. The Rose Bengal plate test (RBPT), plate agglutination test (PAT), standard tube agglutination test (SAT), complement fixation test (CFT), Coombs anti-Brucella test and enzyme-linked immunosorbent assay (ELISA) are the main methods for the serological diagnosis of brucellosis. However, these methods usually use the whole cell or smooth lipopolysaccharides (S-LPS) as the antigen to detect *Brucella* antibodies in the patient's serum, which can lead to false positives and cross-reactivity. The existing brucellosis vaccines have some potential safety hazards. Additionally, most brucellosis diagnostic methods are time-consuming, of low-sensitivity, and cross-reactive[2,3]. The key to improving brucellosis prevention and diagnosis is to find suitable antigens. Therefore, the development of new antigens is crucial for improving the safety of brucellosis vaccines and the specificity and sensitivity of

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diagnostic methods. A large number of studies in immunology have shown that *Brucella* outer membrane proteins (OMPs) are very immunogenic, and they are potentially new subunit vaccine and diagnostic antigens for brucellosis[4,5]. With the rapid development of computer science and the large-scale development of genome and proteome sequencing, a new discipline has emerged called bioinformatics. Bioinformatics is an interdisciplinary subject that integrates computer science, life sciences, and mathematics. It is widely used in genomics and proteomics. In terms of proteomics, bioinformatics can predict the structure and function of proteins and has been used for predicting protein structure and B cell and T cell epitopes[6,7].

In previous work, a new *Brucella* multiepitope fusion protein was successfully designed using the predominant B-cell epitopes of the main OMPs of *Brucella* and a new *Brucella* recombinant multiepitope protein (rMEP) containing B-cell epitopes of *Brucella* OMP16, OMP2b, OMP31 and BP26 was successfully designed using bioinformatics-related technology[8]. Based on previous work, the immunogenicity and antigenicity, the ability to recognize *Brucella* serum antibodies and the potential applications of the recombinant protein were assessed and evaluated in this study.

Material and methods

Animals and ethics statement

A total of 20 female SPF BALB/c mice, 6-8 weeks, weighing 18±2.0 g, were purchased from Beijing Hua Fukang Biological Polytron Technologies Inc. After the study, all mice were euthanized by CO_2. All animal experiments were fully compliant with an ethical approval granted by the Animal Care and Ethics Committee of Jilin

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University (permit number：LSXK2015101) and the Institutional Research Ethics Committee of Medicine, the School of Public Health, Jilin University (permit number: JLU2014-0303).

Serum samples

A total of 93 goat serum samples included 57 brucellosis-positive sera (*Brucella melitensis, B. melitensis*) that were determined serologically positive by plate agglutination tests (PATs) and standard tube agglutination tests (SATs), which are standard methods for diagnosing brucellosis, and 36 brucellosis-negative sera. All samples were provided by the China Animal Health And Epidemiology Center (Qingdao, China).

Identification of immunogenicity of rMEP

Mouse vaccination

Before the start of the experiment, the BALB/c mice were kept in cages with a 12-h light-dark cycle and with water and food *ad libitum* for 1 week. BALB/c mice were randomly divided into two groups consisting of 10 mice per group. Group 1 was immunized by intraperitoneal (i.p.) injection of 25 μg of rMEP, and group 2 was intraperitoneally injected with phosphate-buffered saline (PBS). Antigens and PBS were mixed with equal volumes of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) on day 0 and with equal incomplete Freund's adjuvant (Sigma-Aldrich) on days 15, 30, and 45. Sera were obtained before each immunization and stored at -70℃ until assayed.

Humoral immune response

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As described previously[9], enzyme-linked immunosorbent assay (ELISA) was performed to measure total IgG, IgM, IgG1, IgG2a, IgG2b, and IgG3 levels of antigen-specific serum according to manufacturer instructions (Southern Biotech, Birmingham, AL, USA), and 96-well plates (Corning, NY, USA) were coated with 100 μL/well purified protein [final concentration 10 μg/mL in PBS (pH 7.4)] and incubated overnight at 4°C. Wells were washed three times with PBS containing 0.05 % Tween-20, and plates were blocked with PBS containing 1 % bovine serum albumin for 2 h at room temperature (RT). The plates were later incubated with 100 μL of mouse sera at a 1:100 dilution for 1 h at RT and washed three times as described. Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG, IgM, IgG1, IgG2a, IgG2b, and IgG3 (100 μL/well) were added and incubated for 1 h at RT. After a final wash step, 0.1 mL of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [15 mg ABTS powder dissolved in 1 mL of distilled water and diluted with 9 mL of substrate buffer consisting of 2.1 g of citric acid dissolved in 100 mL of H_2O and 10 μL of 30 % hydrogen peroxide (pH 4.0)] substrate solution was added to each well. The optical density (OD) at 405 nm (OD405) of each well was recorded at 10 min and 20 min after substrate addition.

Flow cytometry analysis of lymphocyte subtypes

Splenocytes (100 μL; 1 × 10^6 cells/mL) were stained with allophycocyanin-cyanine 7 (APC-Cy7)-labeled anti-mouse CD3, fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4, and phycoerythrin (PE)-labeled anti-mouse CD8 (Invitrogen, USA). FITC-, PE-, and APC-Cy7-conjugated primary

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127 antibodies were used as isotype controls. Based on their scattering profiles,
128 lymphocytes were gated, and the percentages of CD3-, CD4-, and CD8-positive cells
129 were quantified. Data analysis was performed using NovoExpress software (Acea
130 Biosciences, San Diego, CA, USA).

131 **Identification of the diagnostic accuracy of rMEP**

132 To verify the sensitivity and specificity of rMEP in the diagnosis of goat
133 brucellosis, an indirect ELISA assay was performed as described previously[8].
134 Meanwhile, the antigen in the SAT (the whole bacteria antigen was supplied by China
135 Animal Health And Epidemiology Center) was used to evaluate the effectiveness of
136 the rMEP protein as a diagnostic antigen for brucellosis by indirect ELISA.
137 The whole bacterial antigen (dilution with 1:160) and the rMEP protein (100 μg/mL)
138 were used as antigens in coating buffer (0.01 M PBS, pH 7.4) and incubated in
139 96-well microtiter plates (Corning) overnight at 4 °C. After washing 4 times with
140 PBST (PBS containing 0.05 % Tween-20), the plates were blocked with 0.1 %
141 ovalbumin (OVA, TCI, Japan) for 1.5 h at 37 °C, washed with PBST 4 times, and then
142 incubated with a 1:400 dilution of goat serum at 37 °C for 60 min. After washing 4
143 times with PBST, the plates were incubated with HRP-conjugated rabbit anti-goat
144 (Abcam, UK) immunoglobulin IgG antibodies at 1:10,000 dilutions for 1 h at 37 °C.
145 The plates were washed and 100 μL TMB (Trimethylbenzene) substrate solution was
146 added to each well. Reactions were left for 15 min at RT in the dark. Finally, 50 μL of
147 stop solution (2 M H_2SO_4) was added to each well. Optical density values were
148 measured at 450 nm using an ELISA plate reader (BioTek). All samples were tested

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149 in replicates.

150 **Statistical Analysis**

151 Dot plot and receiver-operating characteristic (ROC) analyses were performed
152 using GraphPad Prism version 6.05 for Windows. The OD405, OD450 and the
153 percentages of lymphocyte subtypes were determined by Student's t-test (unpaired
154 t-test). Differences were considered statistically significant when $p < 0.05$.

155

156 # Results

157 **Immunogenicity of rMEP**

158 After mice were immunized with rMEP, the serum titer of the mice rapidly
159 increased compared with the control group, indicating that the recombinant protein
160 used in the present study had good immunogenicity as shown in Fig. 1. Further
161 analysis of antibody subtypes in serum showed that the main antibody subtypes were
162 IgG, IgG1, IgG2a, IgG2b, and IgG3, which all had higher OD values, as shown in Fig.
163 2. The analysis of T cell subtypes in mouse spleen cells showed the experimental
164 group had significantly higher CD4 differentiation than the control, and the
165 CD4+/CD8+ ratio was increased, indicating that the recombinant protein significantly
166 increased the Th1 response in mice. The recombinant protein has good
167 immunogenicity, as shown in Fig. 3 and Table 1.

168

169 **Diagnosis accuracy of rMEP**

170 To evaluate the accuracy of the rMEP for diagnosis of brucellosis, 93 serum

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171 samples were tested by indirect ELISA. A dot plot diagram (Fig. 4a) and receiver
172 operating characteristic (ROC) curve analysis (Fig. 4b) were performed to evaluate
173 the sensitivity and specificity. Based on the ROC curve analysis, the area under the
174 curve (AUC) for rMEP was 0.9798 (95 % confidence interval (CI), 0.9569 to 1.003).
175 In addition, a diagnostic sensitivity of 96.49 % (95 % CI, 8789 to 99.57) and a
176 specificity of 94.44 % (95 % CI, 81.34 to 99.32) were obtained from an optimum
177 cutoff value (0.6195). With this cutoff value, 55 of 57 brucellosis cases were
178 diagnosed correctly as positive, and 34 of 36 negative cases were diagnosed correctly
179 as negative. Furthermore, SATs using the whole bacteria antigen were used to
180 diagnose brucellosis in parallel with indirect ELISA with the rMEP, and a dot plot
181 diagram (Fig. 4c) and ROC curve (Fig. 4d) were constructed. The AUC was 0.9484
182 (95 % CI, 0.9040 to 0.9927), and a diagnostic sensitivity of 100.0 % (95 % CI, 93.73
183 to 100.0) and a specificity of 77.78 % (95 % CI, 60.85 to 89.88) were obtained from
184 an optimum cutoff value (0.8875). With the cutoff value, all brucellosis cases were
185 diagnosed correctly; however, only 28 of 36 negative cases were diagnosed correctly.
186 A cross-table with absolute numbers of positive and negative samples with these
187 cutoff values is listed in Table 2. The results of the ELISA indicate that it is possible
188 to clearly and correctly differentiate between positive and negative brucellosis
189 samples with this assay. Compared with the whole bacterial antigen, rMEP has a
190 weaker sensitivity but a stronger specificity.
191

192 ## Discussion

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193 At present, many vaccine studies have shown that many proteins or other
194 components of *Brucella* have strong antigenicity. Animal experiments show that these
195 components have a certain degree of immunoprotection against *Brucella* and are good
196 candidates for vaccine development. Therefore, these components also provide
197 direction for researchers to develop new brucellosis diagnostic antigens, and the
198 development of immunoinformatics technology provides tools for the development of
199 new diagnostic antigens. Serological methods are the most commonly used methods
200 for diagnosing brucellosis, but serological diagnostic methods have some
201 disadvantages, such as low sensitivity and specificity. Thus, improving the diagnostic
202 accuracy of antigens is key to improving the diagnostic sensitivity and specificity of
203 serological methods. Specificity using *Brucella* OMPs in the diagnosis of brucellosis
204 has certain advantages, but the sensitivity of a single OMP as an antigen for the
205 diagnosis of brucellosis is restricted[10]. Combining multiple OMPs can significantly
206 improve the diagnostic accuracy and increase the sensitivity[11]. To ensure its
207 diagnostic accuracy, the rMEP contained B-cell epitopes of *Brucella* OMP16, OMP2b,
208 OMP31 and BP26. Recombinant proteins are different from native proteins and may
209 lose some function after expression. Therefore, after purification of the rMEP protein,
210 relevant experiments were performed to verify its validity. The mouse model is the
211 most commonly used to verify whether fusion proteins are immunogenic or
212 antigenic[12,13,14]. The fusion protein constructed in this study was composed of the
213 predicted linear epitopes of B-cells and was used to immunize mice. The antibodies
214 were produced in the serum of the immunized mice and verified by indirect ELISA.

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Antibody levels against rMEP specifically were measured in sera obtained 45 days after the first immunization. Antibody titer results demonstrated that antibodies in mouse serum were produced by rMEP. Further analysis of the antibody subtypes found that IgG was the main antibody produced in response to *Brucella* infection, accounting for more than 90 % of all antibodies[15]. The analysis of mouse T cell subtypes showed that the ratio of CD4+/CD8+ was significantly higher and the fusion protein was very immunogenic. After immunization, both strong humoral and cellular immune responses were induced in mice. Vaccine candidates should stimulate cell-mediated immunity, such as the induction of Th1 and Th2 immune responses, which are extremely important in controlling intracellular organisms such as *Brucella*. However, one of the major flaws in this study was the deficiency of in-depth research on cell-mediated immunity, such as cytokine secretion measurements (IFN-γ and IL-6), so the specific potential applications of rMEP should be considered cautiously.

In China, the government requires serological methods such as PAT, SAT, and iELISA for primary screening, and a general positive result will initiate eradication of the positive livestock or the whole farm, so SAT or iELISA is still used in China.

Although the current golden test of diagnosis is the pathogen culture, the positive rate of culture is too low, and the government does not mandate the identification of pathogens as long as the serological positive results are available, which is related to China's strict management strategy. According to the epidemiological study of brucellosis, *Brucella* spp., including *B. melitensis*, *B. abortus*, *B. canis*, and *B. suis*, can infect humans and domestic animals[16], and human *B. melitensis* is the main

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Brucella species worldwide, which accounts for 80 % to 90 % of the total number of brucellosis cases in China[17,18]. Therefore, brucellosis sera infected by *B. melitensis* were used in this study to further verify the diagnostic accuracy of the recombinant protein. The results of indirect ELISA showed that the recombinant protein had good ability to recognize the positive- and negative-brucellosis sera. To investigate the sensitivity and specificity of the rMEP, ROC curve analysis was performed, and the results indicated that the rMEP had better specificity than the whole bacterial antigen. The AUC indicated that both the recombinant protein and the whole bacterial antigen could distinguish positive samples from negative samples with a high level of accuracy ($0.9 < AUC < 1$)[19]. The following work should be to expand the range of strains to further verify their specificity.

Conclusion

The recombinant protein was used to immunize mice and successfully induced the production of antibodies in mice, resulting in a strong immune response, demonstrating that the recombinant protein had good immunogenicity. The indirect ELISA results proved that the recombinant protein had good sensitivity and specificity for diagnosing brucellosis in goat serum. It is a potential candidate antigen for brucellosis vaccine development and serological diagnosis.

Declaration of conflicting interests

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

Authors' contributions

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D-HY, Q-QB, KX and JL conceived and designed the study. D-HY, Q-QB and LL performed the animal and ELISA assays. D-HY, Q-QB and LL analyzed the data. D-HY drafted the manuscript. L-CX, JL and KX reviewed and made improvements in the manuscript. All authors read and approved the final version of the paper.

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337 **Figure Legends**

338 **Fig. 1 Changes in serum titer levels after immunization in mice**

339 Test: rMEP; Control: PBS

340

341 **Fig. 2 Antibody subtypes in mouse serum**

342 Test: rMEP; Control: PBS; ***p<0.001

343

344 **Fig. 3 Flow cytometric analysis results**

345 Test: rMEP; Control: PBS

346

347 **Fig. 4 Indirect ELISA analysis of serum samples. The analysis was performed**

348 **using positive brucellosis (57 sera) and negative control (36 sera) sera from goats.**

349 **(**a) Dot plot of the rMEP, (b) ROC analysis of rMEP, (c) Dot plot of the SAT antigen,

350 (d) ROC analysis of SAT antigen

351

Table 1 Percentage of CD3+, CD4+, CD8+ and CD4+/CD8+ by flow cytometric analysis (%)

Group	CD3+	CD4+	CD8+	CD4+/CD8+
Test	44.69±0.81*	27.57±0.99*	10.94±0.14	2.54±0.059*
Control	40.54±0.27	20.17±2.75	10.19±0.39	2.08±0.302

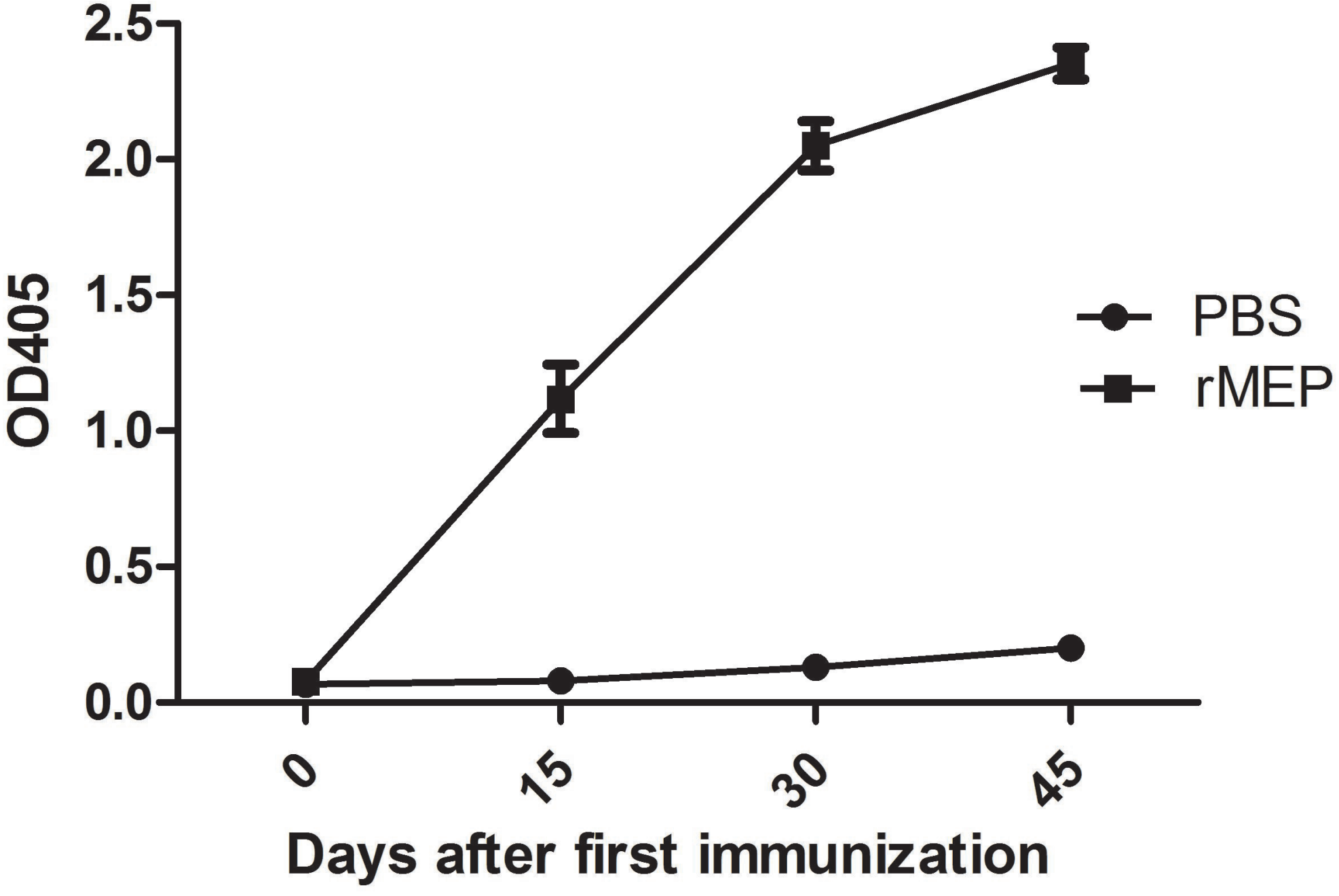
*Compared with the control group, $p<0.05$

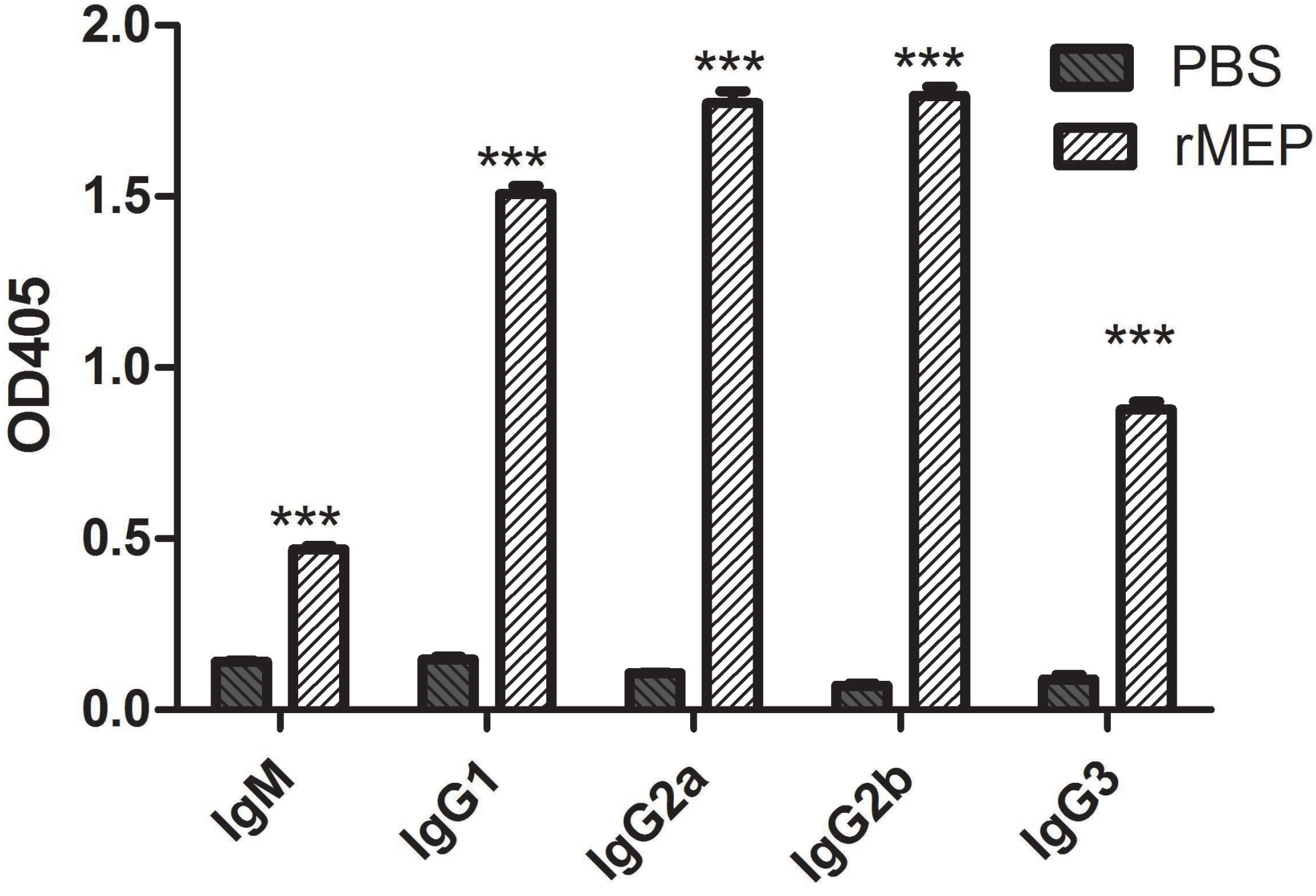
Table 2 Positive and negative predictive values of different cutoff values

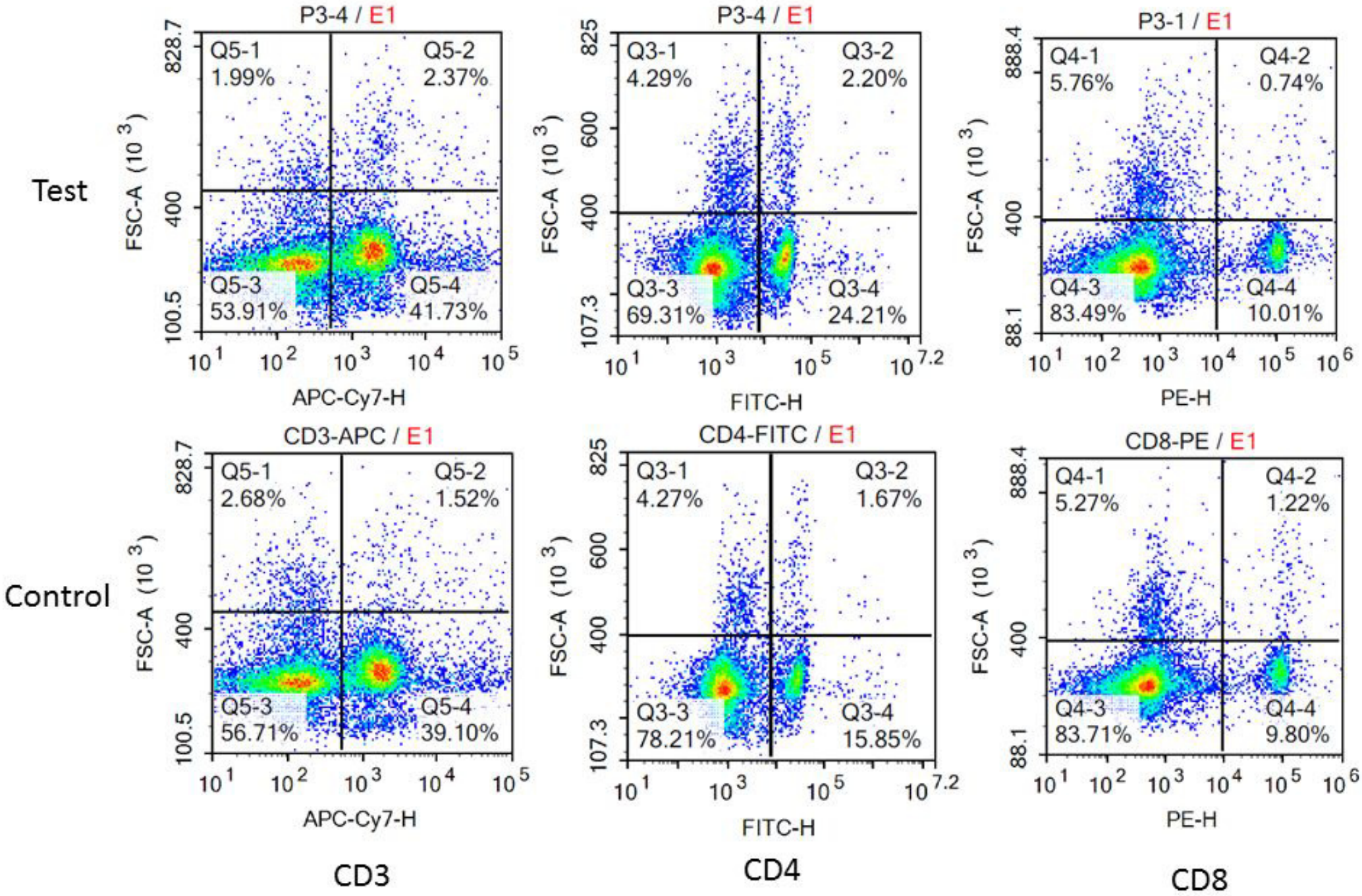
Cutoff value	positive		negative		PPV(%)	NPV(%)
	TP	FN	TN	FP		
0.611[a]	55	2	34	2	96.49	94.44
0.887[b]	57	0	26	8	87.69	100

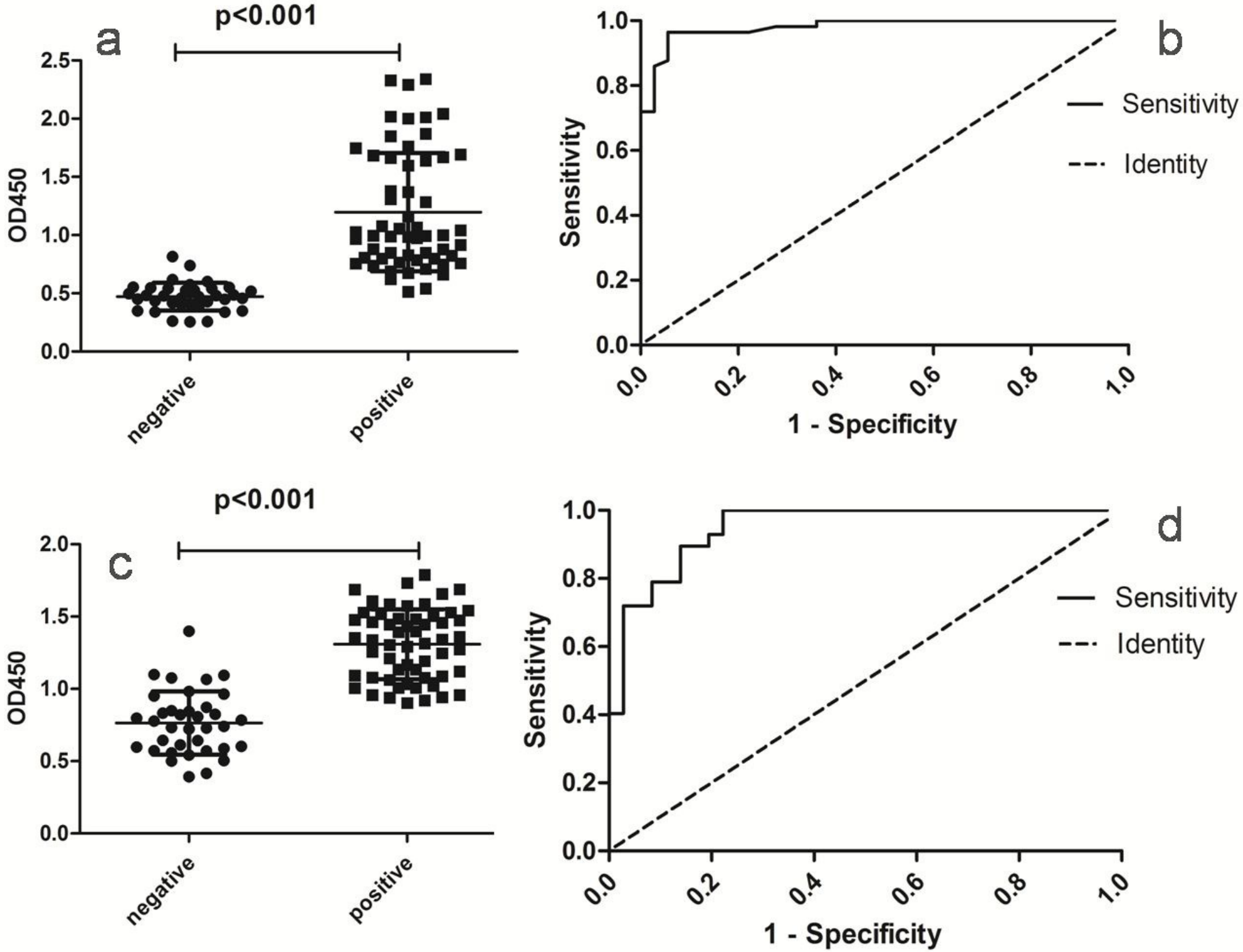
TP, true positives; TN, true negatives; FP, false positives; FN, false negatives; PPV, positive predictive value (TP/(TP + FP)) × 100; NPV, negative predictive value (TN/(TN + FN)) × 100

a, cutoff value is calculated by rMEP; b, cutoff value is calculated by antigen of SAT


2.5
2.0
1.5
1.0
0.5
0.0
OD405
0
15
30
45
Days after first immunization
PBS
rMEP


2.0
1.5
1.0
0.5
0.0
OD405
***
***
***
***
***
PBS
rMEP
IgM
IgG1
IgG2a
IgG2b
IgG3


Test
Control
P3-4 / E1
Q5-1
1.99%
Q5-2
2.37%
Q5-3
53.91%
Q5-4
41.73%
FSC-A (10 3)
828.7
400
100.5
APC-Cy7-H
P3-4 / E1
Q3-1
4.29%
Q3-2
2.20%
Q3-3
69.31%
Q3-4
24.21%
825
600
400
107.3
FITC-H
P3-1 / E1
Q4-1
5.76%
Q4-2
0.74%
Q4-3
83.49%
Q4-4
10.01%
888.4
400
88.1
PE-H
CD3-APC / E1
Q5-1
2.68%
Q5-2
1.52%
Q5-3
56.71%
Q5-4
39.10%
CD4-FITC / E1
Q3-1
4.27%
Q3-2
1.67%
Q3-3
78.21%
Q3-4
15.85%
CD8-PE / E1
Q4-1
5.27%
Q4-2
1.22%
Q4-3
83.71%
Q4-4
9.80%
CD3
CD4
CD8


a
p<0.001
OD450
2.5
2.0
1.5
1.0
0.5
0.0
negative
positive
b
Sensitivity
1 - Specificity
Sensitivity
Identity
c
p<0.001
OD450
negative
positive
d
Sensitivity
1 - Specificity
Sensitivity
Identity