NOTE *Immunology*

Development of a blocking ELISA for detection of *Mycoplasma hyopneumoniae* infection based on a monoclonal antibody against protein P65

Maojun LIU[1,2], Gaimei DU[3], Yue ZHANG[1], Yuzi WU[1], Haiyan WANG[1], Bin LI[1], Yun BAI[1], Zhixin FENG[1], Qiyan XIONG[1], Fangfang BAI[1], Glenn F BROWNING[4] and Guoqing SHAO[1,5]*

[1]*Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences. Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture. National Center for Engineering Research of Veterinary Bio-Products, Nanjing 210014, China*
[2]*Key Laboratory of Food Quality and Safety of Jiangsu Province-State Key Laboratory Breeding Base, Nanjing 210014, China*
[3]*Department of Animal Science and Technology, Jinling Technology Institution, Nanjing 210038, China*
[4]*Asia-Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Victoria 3010, Australia*
[5]*Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, 225009, China*

(Received 30 July 2015/Accepted 17 March 2016/Published online in J-STAGE 14 April 2016)

ABSTRACT. *Mycoplasma hyopneumoniae causes* porcine enzootic pneumonia, an economically important disease of swine. A more sensitive and reliable method for detection of serum antibodies is needed for epidemiological investigations and to evaluate the effect of immunization. We expressed the *M. hyopneumoniae* protein P65 in *Escherichia coli* and produced a monoclonal antibody (mAb) that bound specifically to recombinant P65. Using this mAb, a blocking enzyme linked immunosorbent assay (ELISA) was developed. The blocking ELISA had similar specificity to and sensitivity with the commercial ELISA produced by IDEXX. Thus, this blocking ELISA is a useful test for serological confirmation of *M. hyopneumoniae* infection.
KEY WORDS: blocking ELISA, *Mycoplasma hyopneumoniae*, P65

doi: 10.1292/jvms.15-0438; *J. Vet. Med. Sci.* 78(8): 1319–1322, 2016

Mycoplasma hyopneumoniae causes porcine enzootic pneumonia (PEP), a chronic respiratory disease of pigs prevalent in most swine producing areas [3]. Commercial vaccines, consisting of inactivated whole-cell preparations, are widely used to control disease caused by *M. hyopneumoniae*, but cannot eliminate *M. hyopneumoniae* from infected herds [9], so early and accurate diagnosis of *M. hyopneumoniae* infection is critical to improved control of the disease it causes. Although several commercial kits have been developed for detection of infection with *M. hyopneumoniae* [4, 12], the ELISA kits currently available for *M. hyopneumoniae* serology are expensive and limit the use in the clinic. Therefore, the development of the next generation of serological tests will depend on better characterization of *M. hyopneumoniae* antigens and improved methods for detection of antibody-antigen reactions [12].

Currently available serological methods include complement fixation tests, hemagglutination inhibition tests, growth inhibition assays and ELISAs [2, 5–7, 10], but diagnosis is complicated by cross-reactions between *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* [1], in part because of the complexity of the whole cell antigens generally used in these assays. The use of monoclonal antibodies (mAbs) against *M. hyopneumoniae* antigens can substantially solve this problem. P65, a 65 kDa lipoprotein of *M. hyopneumoniae,* is an immunodominant surface antigen of *M. hyopneumoniae* that is specifically recognized during infection. P65 has been shown previously to be a useful antigen for serological tests [11]. Therefore, we investigated P65 as a target for a mAb blocking ELISA and compared the sensitivity and specificity of a commercial ELISA with this blocking ELISA.

Recombinant *M. hyopneumoniae* P65 was produced in *Escherichia coli* and purified by affinity chromatography using Ni-charged agarose resin (GenScript). A hybridoma line (3G12) that secreted a mAb recognizing P65 was generated and used to produce ascitic fluid as described previously [13]. The mAb was purified from ascitic fluid by protein G affinity chromatography, and its purity was confirmed by SDS-PAGE. The isotype of the mAb was IgG1, and it had κ light chains. The mAb reacted specifically in Western blots with the 85.5 kDa recombinant P65 fusion protein and with native 65 kDa protein in a *M. hyopneumoniae* whole cell protein preparation, but not with any protein in a *M. hyorhinis* whole cell protein preparation nor in extracts of *E. coli* containing the pET-32a (+) vector after induction of expression with IPTG (Fig. 1).

A mAb blocking ELISA was developed using mAb 3G12. All reagents were added in volumes of 100 μl, and incubation was carried out at 37°C unless otherwise stated. The

*CORRESPONDENCE TO: SHAO, G., Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences. Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture. National Center for Engineering Research of Veterinary Bio-Products, Nanjing 210014, China.
e-mail: gqshaojaas@gmail.com

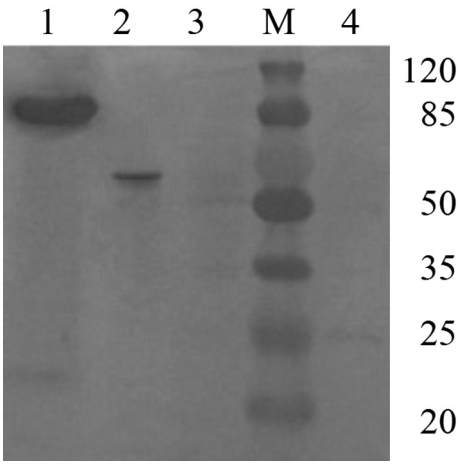

Fig. 1. Western blot of recombinant P65, *M. hyopneumoniae* whole cell proteins, *M. hyorhinis* whole cell proteins and *E. coli* containing the empty expression vector probed with the anti-P65 mAb. Lane M, molecular mass standards (Pierce Pre-stained Protein Molecular Weight Markers); lane 1, recombinant P65; lane 2, *M. hyopneumoniae* whole cell proteins; lane 3, *M. hyorhinis* whole cell proteins; and lane 4, *E. coli* containing the pET-32a (+) vector.

concentrations of coating antigen, samples and mAbs were optimized for the blocking ELISA. The effect of different blocking buffers and reaction times were also compared. Use of a blocking buffer and a dilution buffer composed of 2% casein and a final concentration of 5% PEG 6000 in PBST were found to yield the best signal-to-noise ratio. In the optimized assay recombinant P65 at a concentration of 10 μg/m*l* in 0.05 M sodium carbonate buffer was added to individual wells of 96-well plates, and the plates were incubated at 4°C overnight. After washing four times with phosphate buffered saline −0.05% Tween 20 (PBST), non-specific binding sites were blocked with 200 μl of the optimized blocking buffer for 2 hr. After the wells were washed, serum samples were added at a dilution of 1:5 to the wells and incubated for 120 min. The wells were then washed and incubated with the mAb conjugated to HRP at a dilution of 1:20,000 for 30 min. After washing, substrate was added to the wells, and the plate was incubated at room temperature for 10 min. Color development was stopped by adding 50 μl of 2 M H_2SO_4. The amount of HRP-conjugated mAb bound to P65 was quantified by measuring the absorbance at 450 nm, and the percentage inhibition (PI) was determined using the formula: PI=((OD_{450} for negative control serum −OD_{450} for test serum)/ OD_{450} for negative control serum) × 100.

The blocking ELISA was standardized using sera from field cases that had been confirmed to be serologically positive using the IDEXX M. Hyo. Ab ELISA test kit (IDEXX Laboratories Inc., Westbrook, ME, U.S.A.). The cut-off for discrimination between positive and negative samples was determined by plotting a receiver-operating characteristic (ROC) curve to identify the OD_{450} value that optimized the sensitivity and specificity [8]. The area under the ROC curve (AUC) was calculated to determine the accuracy of the test. This analysis yielded an optimal cut-off at an OD_{450} of 0.55, corresponding to a PI of 36.5%, and this was used for preliminary validation of the test (Fig. 2B). This cut-off resulted in good discriminatory capacity (AUC=0.978) for the blocking ELISA (Fig. 2A), indicating highly accurate discrimination between the negative and positive reference sera.

The cross-reactivity of the blocking ELISA was assessed with 6 antisera samples from each infectious group including *M. hyopneumoniae*, *M. hyorhinis*, *Haemophilus parasuis* (HPS), pseudorabies virus (PRV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV) and foot-and-mouth disease virus (FMDV), respectively. The PI values for antisera against -*M. hyopneumoniae* were not less than 73.87%, while the PI values for antisera against

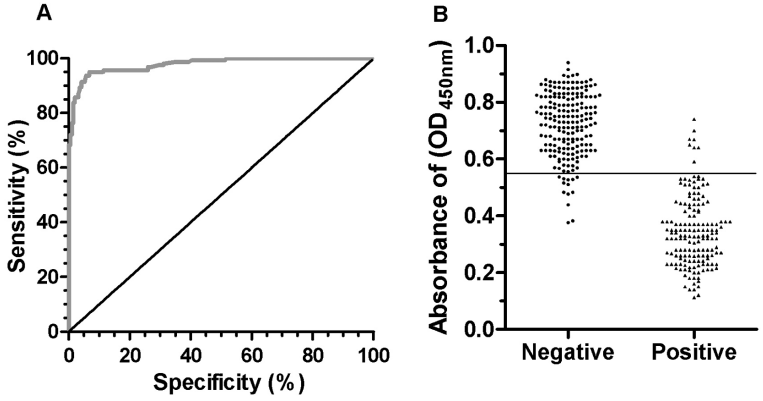

Fig. 2. Receiver-operating characteristic (ROC) curve for anti-P65 blocking ELISA. A. Receiver-operating characteristic (ROC) curve for anti-P65 blocking ELISA. B. The OD_{450} value of positive and negative samples detected by anti-P65 blocking ELISA.

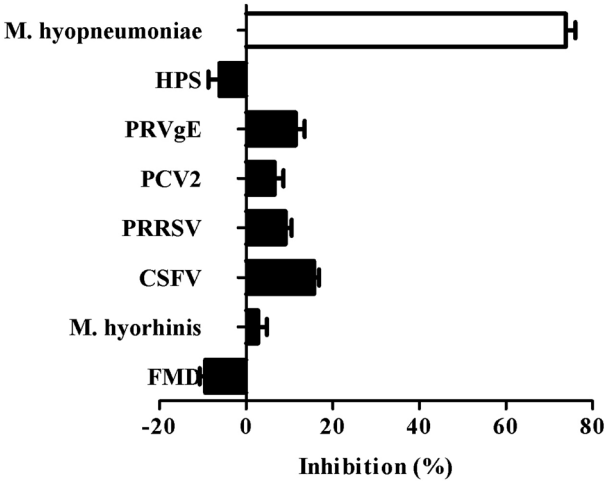

Fig. 3. Specificity analysis of the blocking-ELISA. PI value of antisera against *M. hyopneumoniae, M. hyorhinis, Haemophilus parasuis* (*HPS*), pseudorabies virus (PRV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV) and foot-and-mouth disease virus (FMD) (n=6).

the other pathogens ranged from −9.40% to 15.72% (Fig. 3), indicating that the blocking ELISA was specific for antibodies against *M. hyopneumoniae*. All data were expressed as mean ± SEM. All statistical analyses were performed with SPSS 19.0 for Windows.

To evaluate the sensitivity, specificity, positive predictive and negative predictive values, and the likelihood ratio of the blocking ELISA, serum samples from herds that had pigs with clinical signs of PEP (field samples, n=337) were collected on several pig farms in Jiangsu province and tested using the IDEXX kit and the blocking ELISA. The sensitivities, specificities, negative and positive predictive values, and likelihood ratio of the blocking ELISA, assuming the IDEXX ELISA to be the gold standard, were calculated using GraphPad Prism v6.01 (GraphPad Software, Inc.).

The results obtained for each serum sample in the blocking ELISA and the commercial IDEXX ELISA (Table 1) were compared. A total of 147/155 (94.8%) of the sera found positive in the IDEXX ELISA were also positive in the blocking ELISA. To further investigate the degree of concordance between the blocking ELISA and the IDEXX assay, we also assessed field samples that tested negative in the IDEXX assay (n=182). The blocking ELISA classified 169 of these as negative (92.9%) and 13 as positive (7.1%). Overall, there was a high level of agreement between the blocking ELISA and the IDEXX assay for the same serum samples, with a concordance of 93.8%. The two tests were not found to differ significantly in their performance (P=0.4, binomial test). The blocking ELISA had a high sensitivity (94.8%) and specificity (95.7%) (Table 2).

In conclusion, this study has developed an assay that can meet the requirements for a national test that is accurate and easily performed. The hybridoma produces good yields of the anti-P65 mAb, enabling production of diagnostic kits at a relatively low cost, thus increasing their accessibility.

Table 1. Comparison of the blocking ELISA and the IDEXX ELISA antibody detection kit

Blocking ELISA	IDEXX ELISA		Total
	Positive	Negative	
Positive	147	13	160
Negative	8	169	177
Total	155	182	337

No significant difference detected between the performance of the two tests (P=0.4, binomial test).

Table 2. Characteristics of the blocking ELISA in comparison to the IDEXX ELISA

	Value	95% Confidence Interval
Sensitivity	94.8%	90.1–97.8%
Specificity	92.9%	88.1–96.1%
Positive Predictive Value	91.9%	86.5–95.6%
Negative Predictive Value	95.5%	91.3–98.0%
Likelihood Ratio	13.3	-

CONFLICT OF INTEREST. The authors declare no potential conflict of interest with respect to the research, authorship and/or publication of this article.

ACKNOWLEDGMENTS. This work was supported by the National Nature Science Foundation of China (31100136, 31370208 and 31300155) and the Jiangsu Agricultural Science and Technology Innovation Fund (CX (13) 3066).

REFERENCES

1. Armstrong, C. H., Freeman, M. J. and Sands-Freeman, L. 1987. Cross-reactions between *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*—practical implications for the serodiagnosis of mycoplasmal pneumonia of swine. *Isr. J. Med. Sci.* **23**: 654–656. [Medline]
2. Bereiter, M., Young, T. F., Joo, H. S. and Ross, R. F. 1990. Evaluation of the ELISA and comparison to the complement fixation test and radial immunodiffusion enzyme assay for detection of antibodies against *Mycoplasma hyopneumoniae* in swine serum. *Vet. Microbiol.* **25**: 177–192. [Medline] [CrossRef]
3. Blanchard, B., Vena, M. M., Cavalier, A., Le Lannic, J., Gouranton, J. and Kobisch, M. 1992. Electron microscopic observation of the respiratory tract of SPF piglets inoculated with *Mycoplasma hyopneumoniae*. *Vet. Microbiol.* **30**: 329–341. [Medline] [CrossRef]
4. Erlandson, K. R., Evans, R. B., Thacker, B. J., Wegner, M. W. and Thacker, E. L. 2005. Evaluation of three serum antibody enzyme-linked immunosorbent assays for *Mycoplasma hyopneumoniae*. *Swine Health Prod.* **13**: 198–203.
5. Fano, E., Pijoan, C., Dee, S. and Deen, J. 2012. Longitudinal assessment of two *Mycoplasma hyopneumoniae* enzyme-linked immunosorbent assays in challenged and contact-exposed pigs. *J. Vet. Diagn. Invest.* **24**: 383–387. [Medline]

6. Feng, Z. X., Shao, G. Q., Liu, M. J., Wang, H. Y., Gan, Y. and Wu, X. S. 2010. Development and validation of a SIgA-ELISA for the detection of *Mycoplasma hyopneumoniae* infection. *Vet. Microbiol.* **143**: 410–416. [Medline] [CrossRef]
7. Freeman, M. J., Lopez-Osuna, M., Armstrong, C. H. and Sands-Freeman, L. 1984. Evaluation of the indirect hemagglutination assay as a practical serodiagnostic test for mycoplasmal pneumonia of swine. *Vet. Microbiol.* **9**: 259–270. [Medline] [CrossRef]
8. Hanley, J. A. M. B. J. and McNeil, B. J. 1982. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* **143**: 29–36. [Medline] [CrossRef]
9. Maes, D., Segales, J., Meyns, T., Sibila, M., Pieters, M. and Haesebrouck, F. 2008. Control of Mycoplasma hyopneumoniae infections in pigs. *Vet. Microbiol.* **126**: 297–309. [Medline] [CrossRef]
10. Okada, M., Asai, T., Futo, S., Mori, Y., Mukai, T., Yazawa, S., Uto, T., Shibata, I. and Sato, S. 2005. Serological diagnosis of enzootic pneumonia of swine by a double-sandwich enzyme-linked immunosorbent assay using a monoclonal antibody and recombinant antigen (P46) of *Mycoplasma hyopneumoniae*. *Vet. Microbiol.* **105**: 251–259. [Medline] [CrossRef]
11. Schmidt, J. A., Browning, G. F. and Markham, P. F. 2004. *Mycoplasma hyopneumoniae* p65 surface lipoprotein is a lipolytic enzyme with a preference for shorter-chain fatty acids. *J. Bacteriol.* **186**: 5790–5798. [Medline] [CrossRef]
12. Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F. and Segalés, J. 2009. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. *Vet. J.* **181**: 221–231. [Medline] [CrossRef]
13. Yokoyama, W. M. 2008. Production of Monoclonal Antibody Supernatant and Ascites Fluid. *Current Protocols in Molecular Biology* **83**:II:11.10:11.10.1–11.10.10.