Veterinary Microbiology 143 (2010) 410–416

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Development and validation of a SIgA-ELISA for the detection of *Mycoplasma hyopneumoniae* infection

Zhi-Xin Feng [a,b,c,1], Guo-Qing Shao [a,b,c,*], Mao-Jun Liu [a,b,c], Hai-Yan Wang [a,b,c], Yuan Gan [a,b,c], Xu-Su Wu [a,b,c]

[a] Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China
[b] Key Laboratory of Animal Diseases Diagnostic and Immunology, Ministry of Agriculture, Nanjing 210014, China
[c] National Center for Engineering Research of Veterinary Bio-products, Nanjing 210014, China

ARTICLE INFO

Article history:
Received 19 August 2009
Received in revised form 24 November 2009
Accepted 27 November 2009

Keywords:
Mycoplasma hyopneumoniae
Adhesin P97R1
ELISA
SIgA

ABSTRACT

An alternative indirect enzyme-linked immunosorbent assay (ELISA) for the detection of *Mycoplasma hyopneumoniae* secretory IgA (SIgA) antibody (SIgA-ELISA) was developed using an adhesin (P97R1) of *M. hyopneumoniae* produced in *Escherichia coli*. The SIgA-ELISA assay was validated by the comparison with a nested-PCR assay and a commercial *M. hyopneumoniae* antibody detection kit (IgG-ELISA). Two hundred and sixty nasal swab samples, bronchoalveolar lavage fluids or serum samples were prepared for SIgA-ELISA validation from a *M. hyopneumoniae*-free farm, a *M. hyopneumoniae* vaccinated farm and two *M. hyopneumoniae* contaminated farms. The results showed that the SIgA-ELISA assay could distinguish the *M. hyopneumoniae* infection from *M. hyopneumoniae* vaccinated pigs, which was impossible for the current commercial *M. hyopneumoniae* antibody detection kits. The diagnostic sensitivity (DSN), specificity (DSP) and accuracy of the SIgA-ELISA were 97.0%, 94.4% and 95.8%, respectively and were compared with nested-PCR on 260 field nasal swab samples. The results of repeatability tests revealed that the coefficients of variation of swab samples within and between runs were less than 10%. This SIgA-ELISA is a needle-free detection methodology for large-scale surveys of *M. hyopneumoniae* infection.

1. Introduction

Mycoplasma hyopneumoniae is the primary pathogen of mycoplasmal pneumonia of swine (MPS), a chronic respiratory disease in pigs. For the diagnosis of *M. hyopneumoniae*, the isolation from affected lungs by bacteriological culture is considered the 'gold standard' diagnostic technique (Thacker, 2006), but *M. hyopneumoniae* culture is laborious, time-consuming (isolation from field samples requires 4–8 weeks) and frequently culture media can overgrow with *Mycoplasma hyorhinis* or *Mycoplasma flocculare* (Maes et al., 1996). A blocking ELISA (IDEI, Mycoplasma hyopneumoniae EIA kit, Oxoid) and two indirect ELISA kits (HerdCheck, IDEXX and Tween 20-ELISA) are the most frequently used serological tests to detect IgG antibodies to *M. hyopneumoniae*. However, inactive vaccine inoculation could also induce an anti-*M. hyopneumoniae* IgG antibody reaction. Consequently it is difficult to distinguish between vaccination and infection by *M. hyopneumoniae* using IgG detection kits.

M. hyopneumoniae could be introduced into the herd by airborne transmission (Goodwin, 1985). Adherence of *M. hyopneumoniae* to the ciliated epithelium is a prerequisite for the initiation of infection. Mucosal immune system

* Corresponding author. Tel.: +86 25 84391973; fax: +86 25 84390330.
E-mail addresses: zhixinf2000@yahoo.com.cn (Z.-X. Feng), gq_shao@yahoo.com (G.-Q. Shao).
[1] Tel.: +86 25 84390334; fax: +86 25 84390330.

0378-1135/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2009.11.038

activation, producing an antigen-specific IgA response, is the first signal of *M. hyopneumoniae* infection, but not vaccination (Herremans et al., 1999). Secretory IgA (SIgA) detection had been used to monitor diseases diagnosis (Parida et al., 2006; Cox et al., 2004; Pramod et al., 1999; Yasuda et al., 1998; Lee et al., 1995).

P97 protein was identified as an important adhesin responsible for the adherence of *M. hyopneumoniae* to the respiratory ciliated epithelial cells in swine (Zhang et al., 1995). Furthermore, a repeat region of P97 called R1 (P97R1) has been identified as containing both cilium- and antibody-binding sites (Hsu and Minion, 1998; Hsu et al., 1997).

In this study, we developed a P97R1 adhesin-based ELISA assay (SIgA-ELISA) for *M. hyopneumoniae* secretory immunoglobulin type A (SIgA) detection to monitor the infection by *M. hyopneumoniae*.

2. Materials and methods

2.1. Animal source

The samples used in the study were collected from four farms. The pigs in Farm A were *M. hyopneumoniae*-free and unvaccinated with *M. hyopneumoniae* vaccines. Good management practices, housing conditions and antimicrobial medication strategies were applied. No MPS-like clinical syndromes or lung lesions were observed. On Farm A, *M. hyopneumoniae* was not detected by culture, nested-PCR or commercial ELISA assays for 2 years. The pigs from Farm B were inoculated with a commercial *M. hyopneumoniae* inactive vaccine. MPS sporadically occurred here. The pigs in Farm C and D were *M. hyopneumoniae* contaminated according to clinical observation, lung examination and pathogen isolation. They were not inoculated by any *M. hyopneumoniae* vaccine, and about 70% pigs here showed MPS-like clinical syndromes.

2.2. Sample collection

The nasal swabs were collected from pigs of different ages by inserting cotton swabs deeply into two nasal cavities. Each swab was placed in a tube containing 1 ml of sterile phosphate buffered saline (PBS) and stored at 4 °C overnight. Swab suspensions were centrifuged at 10,000 × *g* for 5 min and the supernatant was collected for anti-*M. hyopneumoniae* SIgA detection. The nasal swabs from SPF piglets were collected and used as negative controls in the SIgA-ELISA, and those from *M. hyopneumoniae* artificially challenged piglets as positive control.

Bronchoalveolar lavage fluids (BALFs) were taken by fiberoptic bronchoscopy as described by previous reports (Hensel et al., 1994; Ganter and Hensel., 1997). The collected BALFs were stored at −20 °C for *M. hyopneumoniae* detection by the nested-PCR. The samples were concentrated by centrifugation at 12,000 × *g* for 30 min, the pellets resuspended in lysis buffer and treated as described by Blanchard et al. (1996).

The pig sera were collected for *M. hyopneumoniae* IgG antibody detection (IgG-ELISA) with a commercial ELISA kit (IDEXX Inc., USA).

2.3. Nested-PCR for M. hyopneumoniae detection

A 427-bp fragment specific for the *M. hyopneumoniae* P36 genome was amplified with the nested-PCR primers (outer primers: F1: 5′-TTAGTGTCTCCCGTTATG-3′, R1: 5′-GAAATCCGTATTCTCCTC-3′; inner primers: F2: 5′-TTACAGCGGGAAGACC-3′, R2: 5′-CGGCGAGAAACTGGATA-3′) (Liu et al., 2008; Lu et al., in press). Briefly, 5 μl of treated tracheobronchiolar washings was used as a template for the first PCR. One microlitre of undiluted PCR product was transferred to a new tube for nested-PCR. The second amplified products were analyzed by electrophoresis in a 1% agarose gel containing ethidium bromide (0.5 mg/ml).

2.4. Antigen preparation

The P97R1 protein with the weight of 29 kDa including His-Tag was produced as described previously (Liu et al., 2005). Briefly, the recombinant pET-32a (+) vector containing adhesin gene R1 region of *M. hyopneumoniae* strain 168 (FJ914570) was transferred into *Escherichia coli* strain BL21 (Amersham). The P97R1 protein was expressed with the induction of 0.1 mM isopropylthio-β-D-thiogalactoside (IPTG) for 4 h at 37 °C and purified by Ni affinity chromatography (Amersham) according to the manufacturer's protocol. The purified P97R1 protein was identified by SDS-PAGE and Western blot assays as described previously (Liu et al., 2005). The concentration of the P97R1 protein was determined by the Bradford assay with bovine serum albumin (BSA, Amresco, Inc., St. Louis, USA) as a standard, and stored at −70 °C.

2.5. Optimization of SIgA-ELISA procedure and working conditions

High binding 96-well microtiter plates (Costar, USA, Catalog No. 2592) were coated with 100 μl P97R1 protein (from 2.5 to 40 μg/ml) in 0.05 M bicarbonate/carbonate buffer (pH 9.6) overnight at 4 °C after incubation for 1 h at 37 °C. Following three washes in PBS containing 0.05% Tween 20 (PBST), the plates were blocked with 5% skimmed milk, 1% BSA, 1% gelatin or 1% ovalbumin at 37 °C for 2 h, then incubated again with 100 μl swab suspensions at 37 °C for different times (from 0.5 to 2 h). Following five washes with PBST, 100 μl goat anti-pig IgA antibody (Bethyl Inc., USA, diluted from 1:1000 to 1:7000) was added per well and incubated at 37 °C for different times (from 15 to 90 min). After five washes with PBST, the plates were conjugated with 100 μl HRP-labeled rabbit anti-goat immunoglobulin (Boster Inc.) diluted in PBST (from 1:2000 to 1:10,000) at 37 °C for different times (from 15 to 90 min). The plates were washed as described above, and a colorimetric reaction was induced by the addition of 100 μl chromogenic substrate (0.1 mg/ml tetramethylbenzidine (TMB, Sigma), 100 mM acetate buffer, pH 5.6, and 1 mM urea hydrogen peroxide) for different times (from 5 to 25 min) at 37 °C. Color development was stopped with 50 μl H_2SO_4 (2 M), and the optical density at 450 nm (OD450) was recorded using a universal Microplate Reader ELx800 (Bio-Tek Instruments, Inc., Winooski, VT, USA). Each working condition in the bracket above was

optimized and determined with the highest OD_{450} ratio between positive and negative samples (P/N value).

2.6. Reproducibility experiments

Evaluation of assay reproducibility within and between runs was performed as proposed by Jacobson (1998). Sixteen field nasal swab samples (10 nested-PCR positive samples, 6 nested-PCR negative samples) were selected for the reproducibility experiments. For intra-assay (within-plate) reproducibility, three replicates of each swab sample were assigned in the same plate. For inter-assay (between-run) reproducibility, three replicates of each sample were run in different plates. Mean S/P ratio, standard deviation (SD) and coefficient of variation (CV) were calculated. The S/P value was calculated by the following formula: S/P = (OD_{450} of sample − OD_{450} of negative control)/(OD_{450} of standard positive control − OD_{450} of negative control).

2.7. Validation of SIgA-ELISA

To set a negative–positive cut-off value for this assay, 260 field nasal swab samples were also tested by the SIgA-ELISA in duplicate. The S/P ratios of 260 field swabs obtained from the SIgA-ELISA were compared with the nested-PCR results. A receiver-operating characteristic analysis (ROC) was performed with the Medcalc statistical software version 10.3, and a cut-off point was determined so that the diagnostic sensitivity (DSN) and specificity (DSP) were maximized while the sum of false-negative and false-positive results was minimized.

The nested-PCR was used as the reference method to detect the presence of *M. hyopneumoniae* (Liu et al., 2008; Lu et al., in press). The DSN, DSP and accuracy of the ELISA were calculated using the following formulae: DSN = TP/(TP + FN) × 100, DSP = TN/(TN + FP) × 100 and accuracy = (TP + TN)/total number of swab samples tested × 100, 100, where TP, FN, TN and FP indicated true-positive, false-negative, true-negative and false-positive, respectively.

2.8. Comparison of SIgA-ELISA and IgG-ELISA

The nasal swabs and serum samples from 260 pigs were collected for *M. hyopneumoniae* SIgA and IgG detection with the SIgA-ELISA and a commercial *M. hyopneumoniae* ELISA kit (IgG-ELISA), respectively. The results of SIgA-ELISA and IgG-ELISA were assessed according to the result of nested-PCR and immunity background of pigs.

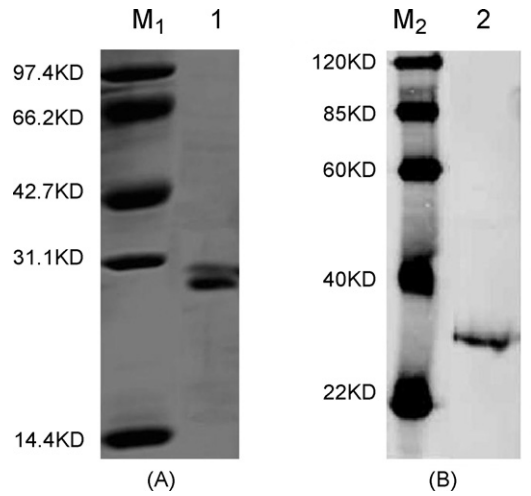

Fig. 1. Identification of purified P97R1 protein of *Mycoplasma hyopneumoniae* by SDS-PAGE (A) and Western blot (B). (A) M_1. Mid-ranged molecular weight protein marker; 1. The purified P97R1 protein. (B) M2. Easy Western protein standard; 2. Purified P97R1 protein reacted strongly with rabbit anti-Mhp polyclonal antibodies.

3. Results

3.1. Results of M. hyopneumoniae detection in BALFs samples by nested-PCR

The results of *M. hyopneumoniae* detection in BALFs samples tested by the nested-PCR are summarized in Table 1. Out of 260 pigs, 135 pigs were positive for *M. hyopneumoniae* infection and 125 pigs negative. In Farm A (*M. hyopneumoniae* free), no *M. hyopneumoniae* genomes were detected (0/85). In Farm B (vaccinated), 90% (36/40) pigs were negative for *M. hyopneumoniae*. However, more severe infection of *M. hyopneumoniae* in Farm C (58/60) and Farm D (73/75) was detected.

3.2. Purification of P97R1 antigen

The recombinant P97R1 protein was expressed as a soluble form and purified by affinity chromatography. As shown in Fig. 1, a 29 kDa protein was eluted from Ni affinity column and could react strongly with rabbit anti-*M. hyopneumoniae* serum in Western blot analysis. In addition, the reactivity of P97R1 protein with rabbit anti-*M. hyorhinis* serum or rabbit anti-*M. flocculare* serum was not observed by Western blot assay (data not shown).

Table 1
Detection of IgG antibody, IgA antibody and genome of *M. hyopneumoniae* by ELISA and nest-PCR in swine serum, nasal swab and BALFs samples obtained from four farms.

Farm	Number of pigs	PCR results of BALFs		IgA-ELISA results of nasal swab samples		IgG-ELISA results of serum samples	
		+	−	+	−	+	−
A	85	0	85	2	83	1	84
B	40	4	36	5	35	36	4
C	60	58	2	57	3	57	3
D	75	73	2	74	1	68	7
Total	260	135	125	138	122	162	98

3.3. Optimization of SIgA-ELISA procedure

In the checkboard ELISAs, the optimal antigen concentration was set at 5 μg/ml (Fig. 2A), based on the standard that the OD_{450} value of positive sample was above 0.5, the OD_{450} ratio between positive and negative sample (P/N value) was highest and the background is lower. Using the same standard, the optimal dilution of the

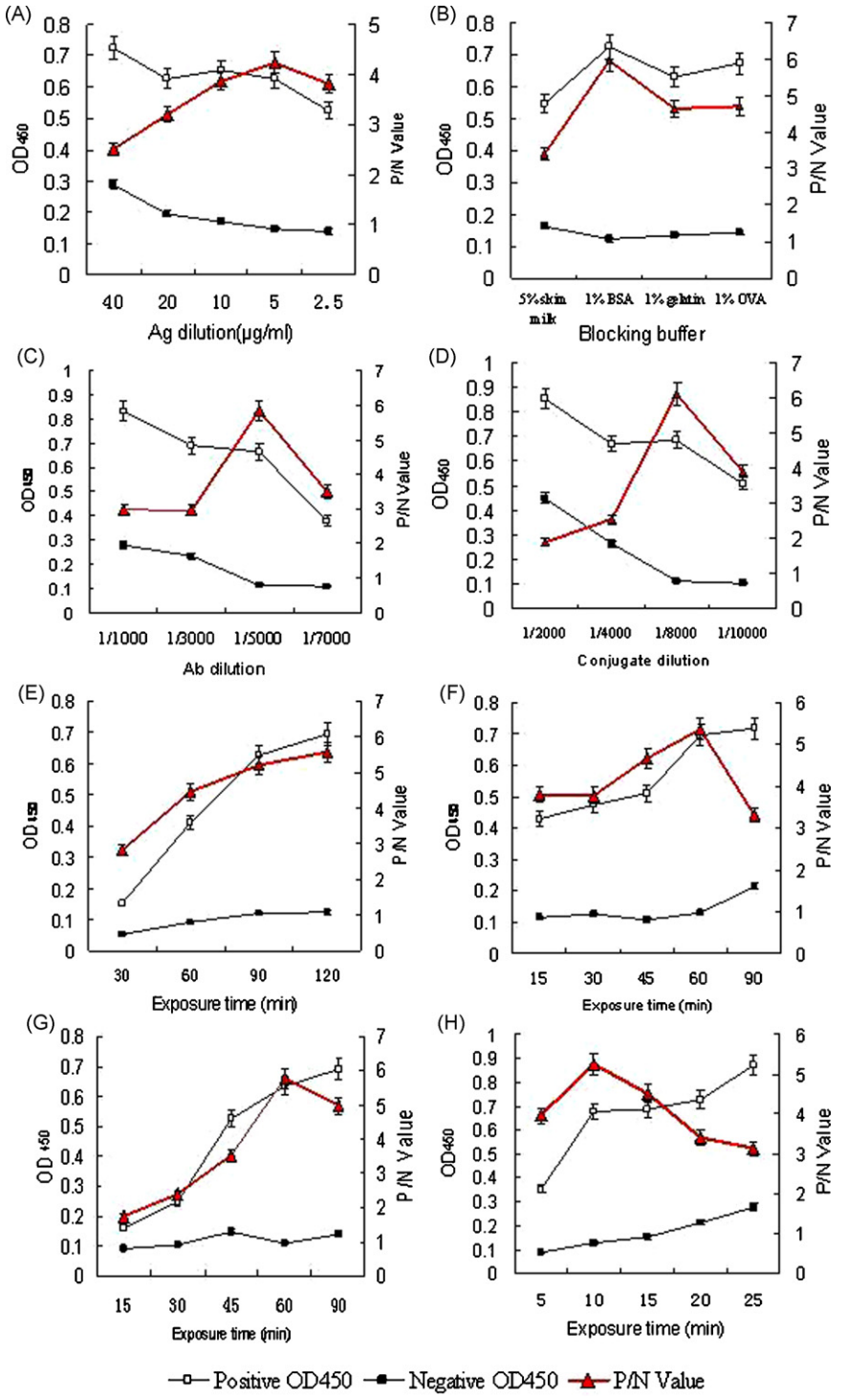

Fig. 2. Optimization of ELISA working conditions. (A) Optimization of P97R1 protein dilutions. (B) Optimization of coating buffers: 5% skimmed milk, 1% BSA, 1% gelatin and 1% ovalbumin. (C) and (D) Optimization of goat anti-pig IgA antibody and HRP-labeled rabbit anti-goat IgG dilution. (E)–(G) Optimization of exposure time of samples, goat anti-pig IgA antibody and HRP-labeled rabbit anti-goat IgG. (H) Optimization of colorimetric reaction time.

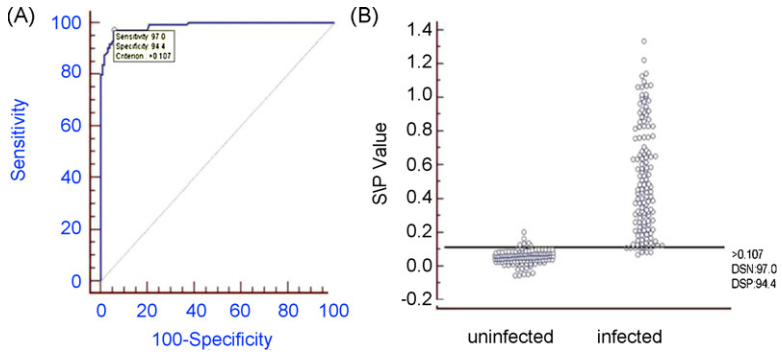

Fig. 3. Receiver-operating characteristic (ROC). Analysis of P97R1 adhesin-based SIgA-ELISA by the Medcalc statistical software version 10.3. (A) ROC curve analysis. (B) Interactive dot diagram.

goat anti-pig IgA antibody and HRP-labeled rabbit anti-goat IgG was defined as 1:5000 (Fig. 2C) and 1:8000 (Fig. 2D), respectively.

After the above-mentioned conditions were checked, the blocking buffer was optimized. The result found that 1% BSA was the best blocking buffer, giving the highest P/N value, compared with other blocking buffers (Fig. 2B). The optimal exposure time of swab samples and two antibodies (the goat anti-pig IgA antibody and HRP-labeled rabbit anti-goat IgG) was also determined with the same method as 120 min (Fig. 2E) and 60 min (Fig. 2F and G), respectively. Fig. 2H shows that 10 min was the optimal colorimetric reaction time that produced the highest P/N ratio.

3.4. Reproducibility of SIgA-ELISA

The reproducibility of the test was determined by comparing S/P ratios of each nasal swab sample. The intra-assay CV of 10 positive and 6 negative samples ranged from 1.45% to 6.61%, whereas the inter-assay CV of these samples ranged between 2.35% and 9.18%. These data showed that this assay was repeatable and yielded a low and acceptable variation.

3.5. Validation of SIgA-ELISA

Based on ROC analysis of the SIgA-ELISA, the S/P ratios of nasal swab samples varied from a minimum of −0.056 to a maximum of 0.205 for negative sample and from a minimum of 0.069 to a maximum of 1.334 for positive swab. A cut-off of 0.107 was found to give an optimal result with a DSP of 94.4% and a DSN of 97.0% (Fig. 3).

Table 2
Comparison of the SIgA-ELISA with nested-PCR for nasal swab samples and BALFs samples from 260 pigs.

SIgA-ELISA[a]	Nest-PCR		
	Positive	Negative	Total
Positive	131	7	138
Negative	4	118	122
Total	135	125	260

[a] The negative–positive cut-off was 0.107. The DSP, DSN and accuracy of the SIgA-ELISA was 94.4%, 97.0% and 95.8%, respectively.

Thus, samples with S/P ratios less than or equal to 0.107 were considered negative, those with ratios of more than 0.107 were considered positive. Based on this cut-off, out of 260 nasal swab samples, 138 samples were classified as positive and 122 samples as negative, respectively (Table 2). The accuracy of the SIgA-ELISA was 95.8% compared with the result of nested-PCR.

3.6. Comparison of SIgA-ELISA and IgG-ELISA

When the samples were detected by SIgA-ELISA and IgG-ELISA, 138 of 260 swab samples were detected as *M. hyopneumoniae* SIgA antibody positive and 122 swab samples as antibody negative by the SIgA-ELISA at a cut-off of 0.107, while 162 of 260 serum samples were judged as *M. hyopneumoniae* IgG antibody positive and 98 serum samples as antibody negative by the commercial *M. hyopneumoniae* ELISA kit (IgG-ELISA). The major difference was present in the samples from Farm B (*M. hyopneumoniae* vaccinated). Only 5 of 40 samples were positive for *M. hyopneumoniae* SIgA antibody, however, 36 of 40 serum samples were positive for *M. hyopneumoniae* IgG antibody in Farm B (Table 1).

4. Discussion

M. hyopneumoniae is the primary pathogen of MPS. Detecting *M. hyopneumoniae* with specific diagnostic tools is necessary to elucidate the course of infection. Currently there are two commercial ELISAs routinely used for serodiagnosis of *M. hyopneumoniae*, the indirect ELISA (Ross et al., 1999) and the blocking ELISA (Feld et al., 1992), both of them judging the infection of *M. hyopneumoniae* by IgG antibody detection. However, the occurrence of anti-*M. hyopneumoniae* IgG could be the result of infection or vaccine inoculation. To date, the inactive *M. hyopneumoniae* whole-cell vaccines are widely applied worldwide. In many countries, vaccination for controlling *M. hyopneumoniae* infections is applied in more than 70% of the pig herds. The presence of anti-*M. hyopneumoniae* IgG induced by vaccination would cause the misjudgment of *M. hyopneumoniae* infection when IgG-ELISA was used, which was confirmed in this study. 90% (36/40) of the serum samples from Farm B (vaccine inoculation) were IgG antibody positive by commercial IgG-ELISA kit, however, only 12.5% (5/40) of

swab samples from the same pigs were positive for *M. hyopneumoniae* infection according to the SIgA-ELISA. Nested-PCR data also suggested low infection rates (4/40) on Farm B, which supports the results of SIgA-ELISA.

M. hyopneumoniae infection occurs through direct contact with respiratory secretions from carrier animals and airborne transmission (Stark et al., 1998; Straw et al., 1999; Hege et al., 2002). *M. hyopneumoniae* initiates disease following interaction with the mucosal surface lining the respiratory tract. The primary defense of this tissue is the mucosal immune system. Mucosal immune responses are characterized by the production of SIgA, which prevents the interaction of the pathogens with receptors on the mucosal cell surface. However, this immunoglobulin may not occur after inactive vaccine inoculation alone (Herremans et al., 1999). In this study, SIgA detection instead of IgG was developed to judge the infection of *M. hyopneumoniae*.

P97 protein, the adhesin of *M. hyopneumoniae*, is involved in the cilium-binding specificity. The cilium-binding motif of P97, which resides in the carboxyl-terminal R1 repeat region, contains various copies of the repeated five amino acid motif, AAKPV(E) (Minion et al., 2000). Different strains of *M. hyopneumoniae* display different numbers of R1 repeats (Wilton et al., 1998; Stakenborg et al., 2006). The research also reported that eight copies of motif are needed for binding cilia and three copies of the R1 repeat are required to bind antibody (Minion et al., 2000). The P97R1 protein used in this work contains 11 copies of motif, which ensure the good reaction between the protein and specific antibody. The deduction was confirmed by the result of western blot. In addition, the result of western blot assay (data not shown) between *M. hyopneumoniae* P97R1 protein and all the porcine *Mycoplasma* spp antibodies showed that P97R1 protein was potentially useful for immunodetection of *M. hyopneumoniae*, which has also been confirmed by other reports (Young and Ross, 1987; Scarman et al., 1997; Kim et al., 2006). In this study, the P97R1 protein expressed in *E. coli* was used as an envelope antigen to detect the anti-*M. hyopneumoniae* SIgA for early diagnosis. High correlation (95.8%) was shown between the results of nested-PCR and SIgA-ELISA.

Nasal swab collection from two nasal cavities is needle-free and noninvasive, making it easier to apply in the field and enhancing the welfare of the animals compared to serum collection.

Acknowledgements

This work was supported by the grants from China Postdoctoral Science Fund (20070421022), Natural Science Foundation of Jiangsu Province (BK2007711) and from Three Projects Fund of Jiangsu Province (SX2007082).

References

Blanchard, B., Kobisch, M., BoveÂ, J., Saillard, C., 1996. Polymerase chain reaction for *Mycoplasma hyopneumoniae* detection in tracheobronchiolar washings from pigs. Mol. Cell. Probes 10, 15–22.

Cox, A.J., Gleeson, M., Pyne, D.B., Saunders, P.U., Clancy, R.L., Fricker, P.A., 2004. Valtrex therapy for Epstein–Barr virus reactivation and upper respiratory symptoms in elite runners. Med. Sci. Sports. Exerc. 36 (7), 1104–1110.

Feld, N., Quist, P., Ahrens, P., 1992. A monoclonal blocking ELISA detecting serum antibodies to *Mycoplasma hyopneumoniae*. Vet. Microbiol. 30, 35–46.

Ganter, M., Hensel, A., 1997. Cellular variables in bronchoalveolar lavage fluids (BALF) in selected healthy pigs. Res. Vet. Sci. 63, 215–217.

Goodwin, R.F., 1985. Apparent reinfection of enzootic-pneumonia-free pig herds: search for possible causes. Vet. Record. 116, 690–694.

Hege, R., Zimmermann, W., Scheidegger, R., Stark, K.D., 2002. Incidence of reinfections with *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* in pig farms located in respiratory-disease-free regions of Switzerland-identification and quantification of risk factors. Acta Vet. Scand. 43, 145–156.

Hensel, A., Ganter, M., Kipper, S., Krehon, S., Wittenbrink, M.M., Petzoldt, K., 1994. Prevalence of aerobic bacteria in bronchoalveolar lavage fluid from healthy pigs. Am. J. Vet. Res. 55, 1697–1702.

Herremans, T.M., Reimerink, J.H., Buisman, A.M., Kimman, T.G., Koopmans, M.P., 1999. Induction of mucosal immunity by inactivated poliovirus vaccine is dependent on previous mucosal contact with live virus. J. Immunol. 162 (8), 5011–5018.

Hsu, T., Minion, F.C., 1998. Identification of the cilium binding epitope of the *Mycoplasma hyopneumoniae* P97 adhesin. Infect. Immun. 66, 4762–4766.

Hsu, T., Artiushin, S., Minion, F.C., 1997. Cloning and functional analysis of the P97 swine cilium adhesin gene of *Mycoplasma hyopneumoniae*. J. Bacteriol. 179, 1317–1323.

Jacobson, R.H., 1998. Validation of serological assays for diagnosis of infectious diseases. Rev. Sci. Techn. 17, 469–526.

Kim, T.J., Cho, H.S., Park, N.Y., Lee, J.I., 2006. Serodiagnostic comparison between two methods. ELISA and surface plasmon resonance for the detection of antibody titres of *Mycoplasma hyopneumoniae*. J. Vet. Med. B. Infect. Dis. Vet. Public. Health 53 (2), 87–90.

Lee, B.W., Bey, R.F., Baarsch, M.J., Larson, M.E., 1995. Class specific antibody response to influenza A H1N1 infection in swine. Vet. Microbiol. 43 (2–3), 241–250.

Liu, M.J., Shao, G.Q., Zhang, Y., Nie, X.T., 2005. Cloning and expressing of R1 region of P97 gene in *Mycoplasma hyopneumoniae*. (in Chinese, with English abstract). Jiangsu J. Agric. Sci. 21 (3), 207–211.

Liu, M.J., Li, D.M., Shao, G.Q., Feng, Z.X., Wu, X.S., Gan, Y., Liu, D.X., 2008. Establishment of PCR Method for the Identification of *Mycoplasma hyopneumoniae* (in Chinese, with English abstract). Jiangsu J. Agric. Sci. 24 (3), 99–101.

Lu, X.M., Feng, Z.X., Liu, M.J., Wu, X.S., Gan, Y., Zhang, Y., Shao, G.Q., in press. Establishment of a nested PCR assay for detection of *Mycoplasma hyopneumoniae* (in Chinese, with English abstract). Jiangsu J. Agric. Sci.

Maes, D., Verdonck, M., Deluyker, H., de Kruif, A., 1996. Enzootic pneumonia in pigs. Vet. Q. 18, 104–109.

Minion, F.C., Adams, C., Hsu, T., 2000. R1 region of P97 mediates adherence of *Mycoplasma hyopneumoniae* to swine Cilia. Infect. Immun. 68, 3056–3060.

Parida, S., Anderson, J., Cox, S.J., Barnett, P.V., Paton, D.J., 2006. Secretory IgA as an indicator of oro-pharyngeal foot-and-mouth disease virus replication and as a tool for post vaccination surveillance. Vaccine 24 (8), 1107–1116.

Pramod, N.P., Dhevahi, E., Sudhamathi, K., Kannan, K., Thyagarajan, S.P., 1999. Tear secretory IgA: evaluation of usefulness as a diagnostic marker in herpetic keratitis. Ocul. Immunol. Inflamm. 7 (2), 61–67.

Ross, R.F., 1999. Mycoplasmal diseases. In: Straw, et al. (Eds.), Disease of Swine. 8th ed. Iowa State University Press, Ames, Iowa, USA, pp. 495–501.

Scarman, A.L., Chin, J.C., Eamens, G.J., Delaney, S.F., Djordjevic, S.P., 1997. Identification of novel species-specific antigens of *Mycoplasma hyopneumoniae* by preparative SDS-PAGE ELISA profiling. Microbiology 143, 663–673.

Stark, K.D.C., Nicolet, J., Frey, J., 1998. Detection of *Mycoplasma hyopneumoniae* by air sampling with a nested PCR assay. Appl. Environ. Microbiol. 64, 543–548.

Stakenborg, T., Vicca, J., Maes, D., Peeters, J., de Kruif, A., Haesebrouck, F., Butaye, P., 2006. Comparison of molecular techniques for the typing of *Mycoplasma hyopneumoniae* isolates. J. Microbiol. Methods 66, 263–275.

Straw, B.E., D'Allaire, S., Mengeling, W.L., Taylor, D.J. (Eds.), 1999. Diseases of Swine. 8th ed. Iowa State University Press, Ames, Iowa, USA, pp. 505–520.

Thacker, E., 2006. Mycoplasmal diseases. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine. 9th ed. Blacwell Publishing Ltd, Oxford, UK, pp. 701–717.

Wilton, J.L., Scarman, A.L., Walker, M.J., Djordjevic, S.P., 1998. Reiterated repeat region variability in the ciliary adhesin gene of *Mycoplasma hyopneumoniae*. Microbiology 144, 1931–1943.

Yasuda, S., Iwasaki, M., Oka, S., Naganawa, S., Nakasone, T., Honda, M., Sata, T., Kojima, A., Matsuda, S., Takemori, T., Tsunetsugu-Yokota, Y., 1998. Detection of HIV-Gag p24-specific antibodies in sera and saliva of HIV-1-infected adults and in sera of infants born to HIV-1-infected mothers. Microbiol. Immunol. 42 (4), 305–311.

Young, T.F., Ross, R.F., 1987. Assessment of antibody response of swine infected with *Mycoplasma hyopneumoniae* by immunoblotting. Am. J. Vet. Res. 48, 651–656.

Zhang, Q., Young, T.F., Ross, R.F., 1995. Identification and characterization of a *Mycoplasma hyopneumoniae* adhesin. Infect. Immun. 63, 1013–1019.