Use of serological and mucosal immune responses to *Mycoplasma hyopneumoniae* antigens P97R1, P46 and P36 in the diagnosis of infection

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**ABSTRACT**

Currently available ELISAs used to diagnose *Mycoplasma hyopneumoniae* infection in pigs have high specificity but low sensitivity. To develop more sensitive assays, the kinetics of specific serum IgG and respiratory mucosal sIgA responses against three *M. hyopneumoniae* antigens, namely, P97R1 (an adhesin protein), P46 (a membrane protein), and P36 (a cytosolic protein), were characterised over 133 days following experimental infection.

Immunoglobulin G against the three proteins remained at high concentrations from 28 to 133 days post-infection (dpi), although IgG against P97R1 was detected earlier and was more reactive than the other two antigens under assessment. Mucosal sIgA appeared earlier than serum IgG but did not persist as long; sIgA concentrations against P97R1 were the highest. Serocconversion was detected 2 weeks earlier with the P97R1-based ELISA than with a commercially available ELISA. On analysis of serum samples from five pig farms that did not use a *M. hyopneumoniae* vaccine, the P97R1-based IgG ELISA demonstrated a 73.6% coincidence rate with the commercial kit. Moreover, this more specific P97R1-based ELISA detected more positive samples than the commercial kit (52.8% vs. 39.2%).

It was concluded that the systemic immune response to *M. hyopneumoniae* infection in pigs was delayed in onset but persistent whereas the mucosal response developed more rapidly but was less sustained. The P97R1 antigen was identified as a suitable serological marker for diagnosing *M. hyopneumoniae* infection in pigs, particularly early stage infection.

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**Introduction**

*Mycoplasma hyopneumoniae* plays a central role in the pathogenesis of porcine enzootic pneumonia (PEP), the chronic respiratory disease of pigs that manifests as coughing, retarded growth and poor feed conversion efficiency (Ross, 1999). Given that infection with *M. hyopneumoniae* also contributes to high morbidity rates through co-infections (Straw et al., 2006), its early and precise identification within herds is critical to their health and welfare, as well as to the economic viability of pig production. Currently available *M. hyopneumoniae* ELISA kits have limited sensitivity (Erlandson et al., 2005; Straw et al., 2006). One of the critical factors in optimising diagnostic tests is the identification of the most appropriate target antigens. Serocconversion is delayed in pigs infected with *M. hyopneumoniae* and is only detectable in a proportion of animals 28 days post-infection (dpi); the majority do not seroconvert until 90 dpi (Fano et al., 2005; Pieters et al., 2009).

Although the evidence suggests our capacity to detect seroconverting pigs varies when different protein antigens of *M. hyopneumoniae* are used (Young et al., 1990; Frey et al., 1994; Futo et al., 1995), the dynamics of antibody responses to such antigens remains unclear. Moreover, although mucosal immunity is...
directly responsible for clearance of *M. hyopneumoniae* (Thacker et al., 2000; Feng et al., 2010b), little is known about the timing and scale of this local response.

The *M. hyopneumoniae* genome codes for several immunodominant proteins, of which P46, P65, and P74 are membrane proteins, P36 is a cytosolic protein, and P97 an adhesin. Both P36 and P46 are highly species-specific, do not cross-react with sera from swine infected with *M. flocculare*, *M. hyorhinis*, or *M. hyosynoviae* (Mori et al., 1988; Stipkivs et al., 1991; Futo et al., 1995; Caron et al., 2000), and are therefore potentially suitable as diagnostic antigens (Mori et al., 1988; Stipkivs et al., 1991). The C-terminal of the R1 repeat region of P97 (P97R1) contains both cilia and antibody binding sites (Hsu et al., 1997; Hsu and Minion, 1998; Minion et al., 2000), and has been used in the development of a subunit vaccine (Conceicao et al., 2006; Chen et al., 2008). This antigen may also be useful in assessing humoral responses to vaccination and/or infection (Djordjevic et al., 1994; Caron et al., 2000; Jang and Kim, 2007).

The objectives of this study were to determine if the P36 (cytotoxic), P46 (membrane), and P97R1 (adhesive) antigens of *M. hyopneumoniae* are effective candidate antigens when used to diagnose infection. To this end, a longitudinal study of both serum and mucosal antibody responses to these proteins was performed in experimentally-infected pigs.

**Materials and methods**

**Antigen production**

The P36, P46, and P97R1 proteins of *M. hyopneumoniae* were expressed in Escherichia coli, purified using Ni NTa affinity columns (Genscript Laboratories), and identified by Western blot analysis (see Appendix: Supplementary material). The concentration of the purified proteins was determined with the BCA Protein Assay Kit (Beyotime Laboratories), and the proteins were stored at −70°C.

**Experimental design**

All animal research was approved by the Science and Technology Agency of Jiangsu Province (approval ID SYXK (SU) 2010–0005), and by the Ethics and Animal Welfare Committee of Jiangsu Academy of Agricultural Sciences (Reference 169). Twelve cross-bred 55–60 day old pigs were obtained from a commercial herd free of *M. hyopneumoniae* infection as assessed at slaughter. Before the experiments were initiated, sera and nasal swab samples were collected from each pig, and all samples were shown to be free of antibody against *M. hyopneumoniae*, swine influenza virus, porcine reproductive and respiratory syndrome virus, and porcine circovirus type-2 using commercially available ELISA kits (IDEXX Laboratories and Keqian Laboratories). The absence of *M. hyopneumoniae* infection was confirmed by screening for the presence of *M. hyopneumoniae* in nasal swabs using a nested PCR (Lu et al., 2010).

Ten pigs were challenged intratracheally with 5 ml of lung homogenate containing 5 × 10^7 colour changing units (CCU) of *M. hyopneumoniae* strain JS (Feng et al., 2010b). A further two pigs were inoculated with 5 ml of PBS by the same route and were housed separately (uninfected control group). Serum samples and nasal swabs were collected at predetermined timepoints following challenge (to be shown later). Concentrations of serum IgG and nasal S IgA against P36, P46 and P97R1 were monitored for 133 days after infection (dpi) using the ELISAs to be described later, and the results were compared to those obtained with a commercially available *M. hyopneumoniae* IgG ELISA (IDEXX Laboratories).

**Sample collection and analysis**

Serum samples were collected on 0, 7, 14, 21, 28, 52, 77, 91, 105, 119, and 133 dpi and stored at −70°C until assayed. Nasal swab samples were collected and treated as described previously (Feng et al., 2010a) on 0, 2, 4, 6, 8, 12, 14, 16, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, 98, 105, 112, 119, and 133 dpi. Briefly, the nasal swabs were collected by inserting cotton swabs deep into each nasal cavity. The suspensions were then centrifuged at 10,000 × g for 5 min and the supernatants were collected and stored at −70°C prior to analysis.

To detect serum IgG against P36, P46 and P97R1, three IgG-specific indirect ELISAs were developed under the same conditions, using the equivalent recombinant proteins as targets against P36. Briefly, high binding affinity 96-well microtitre plates (Costar Laboratories) were coated with 2 μg/mL P36, P46 or P97R1 in 0.05 M bicarbonate/carbonate buffer (pH 9.6). The plates were incubated at 37°C for 1 h, and then at 4°C overnight. Following three washes in PBS containing 0.05% Tween 20 (PBST), the plates were blocked with 1% casein for 2 h at 37°C, and then incubated with 100 μL of 1:20 dilution of each serum sample in duplicate at 37°C for 1 h. Following five washes with PBST, 100 μL of horseradish peroxidase (HRP)-labelled goat anti-pig IgG antibody at 1:10,000 dilution (Boster Laboratories) was added to each well and the plates were incubated for 30 min at 37°C. The plates were washed again, and 100 μL of substrate (0.1 mg tetramethylbenzidine [TMB, Sigma]/ml, 100 μM acetyl buffer, pH 5.6, 1 mM urea hydrogen peroxide) was added to each well and the plates were incubated for 5 min at room temperature. Colour development was halted on addition of 2 M H₂SO₄, and the optical density (OD) was measured at 450 nm (OD450) using a universal microplate reader (ELX800, Bio-Tek Instruments). Sera from the pigs free of *M. hyopneumoniae* infection and from animals repeatedly infected with *M. hyopneumoniae* to induce greater antibody concentrations were used as negative and positive controls, respectively. The S/P values were calculated using the formula: S/P = (OD sample − OD negative control)/(OD positive control − OD negative control). An S/P > 0.4 was considered positive, the same cut-off as that used in an equivalent commercial ELISA (IDEXX Laboratories). The rate of seroconversion of pigs to each protein was calculated for each sampling timepoint, and compared with the results from this commercial ELISA.

Three S IgA-specific indirect ELISAs were developed to detect mucosal responses to the P36, P46 and P97R1 antigens. In brief, the antigen coating and plate blocking components of these assays were similar to those described for the IgG-specific indirect ELISAs described earlier. The blocked plates were incubated with 100 μL of twofold serial dilutions of supernatants of the nasal swab samples in duplicate at 37°C for 1 h. The nasal samples from *M. hyopneumoniae*-free pigs were also added in duplicate without dilution as negative controls. Following five washes with PBST, 100 μL of HRP-labelled goat anti-pig IgA at a 1:10,000 dilution (BETHYL Laboratories) was added to each well and the plates were incubated for 0.5 h at 37°C. The plates were then washed and developed as IgG-specific indirect ELISAs. The endpoint titre was defined as the reciprocal of the sample dilution with an OD greater than the cut-off value (mean negative value plus 3SDs). Total protein concentration in the samples was determined using a BCA Protein Assay (Beyotime Laboratories), and expressed in mg/ml. The S IgA titres were normalised against the concentration of total protein in the nasal samples (expressed as concentration) / 0.1 mg of total protein. The proportion of pigs positive for antibody against each protein was calculated for each sampling timepoint.

**Serum IgG responses to P36, P46 and P97R1 antigens in clinically affected pigs**

A total of 125 serum samples were collected from 3–15 week old pigs with a history of sporadic coughing from five farms that did not use a *M. hyopneumoniae* vaccine. IgG against P36, P46 and P97R1 in serum samples was measured as described earlier, and samples were also assayed using a commercially available ELISA (IDEXX Laboratories). The coincidence rates among different methods were calculated based on the results of each sample, following the formula: number of samples with the same results (positive and negative) by the given method/total number of samples × 100.

**Statistical analysis**

The IgG and IgA titres against the three antigens at different timepoints were compared using two-way analysis of variance (ANOVA) with Bonferroni post-tests in Microsoft Excel (version 5), where P < 0.05 was considered significant. Values were graphed to show the mean and standard deviation of samples, which were tested in duplicate. The IgG response in the clinically affected pigs sampled were also tested in duplicate and compared by one-way ANOVA with the Tukey test, where P < 0.05 was considered significant.

**Results**

**Systemic immune response**

Seroconversion was detected in some pigs on 7 dpi using the anti-P97R1 (2/10), anti-P46 (2/10), and anti-P36 (1/10) ELISAs, respectively. Seroconversion rates continued to rise over the following 2 weeks: there was greater seroconversion to the P97R1 antigen by 14 dpi (8/10) and 21 dpi (9/10), than to P46 (2/10 and 5/10) or P36 (2/10 and 2/10). By 28 dpi, seroconversion had occurred in all pigs to all three proteins, except for three animals that did not have detectable responses to P36. On 42 dpi, all 10 pigs had seroconverted to all three proteins (Fig. 1A). Seroconversion was detected less rapidly by the commercial ELISA with 5/10 pigs seropositive at 21 dpi and 10/10 at 28 dpi (Fig. 1B). Seroconversion did not occur in the two uninfected controls (data not shown).
The S/P values rose with all three candidate recombinant ELISAs between 21 and 42 dpi (Fig. 2). The S/P values in the P97R1 ELISA continued rising until 77 dpi, and then decreased gradually after 105 dpi. In contrast, the S/P values in the P36 ELISA dropped slightly on 56 dpi, then rose slowly until 105 dpi, and were stable at the last sampling timepoint (133 dpi). There were significant differences between the S/P values in the P36 and P97R1 ELISAs on 56 (P < 0.05), 77 (P < 0.01), 91 (P < 0.05) and 119 (P < 0.05) dpi. The S/P value in the P46 ELISA remained high and stable from 42 to 133 dpi. No significant differences were observed between the S/P values in the P46 and P97R1 ELISAs at each sampling timepoint throughout the experiment (Fig. 2).

Mucosal immune response

The sIgA respiratory mucosal response to the three selected *M. hyopneumoniae* antigens was also monitored by ELISA. Due to marked variation in immunoglobulin titres, no significant differences were observed between the sIgA responses to P97R1 and P36 (Fig. 3). Significant differences (P < 0.01) were only detected in the responses to P97R1 and P46 on 12 and 16 dpi, respectively. Mucosal responses to all three proteins were identified by 6 dpi, and increased in subsequent days, peaking at 12–16 dpi (Fig. 3). Titres then gradually declined until 49 dpi at which point a slight increase was seen. The sIgA concentrations to all three proteins had returned to pre-immune levels in the majority of animals by 84 dpi, although a very weak increase was observed in the case of anti-P46 sIgA on 105 dpi. On calculating the proportion of pigs with a sIgA response against each protein for each sampling time point (Fig. 4), all animals had detectable titres against all three antigens by 12 dpi. Anti-P97R1 sIgA was first detected in pigs on 4 dpi (3/10), anti-P36 sIgA was no longer detectable in any pig after 105 dpi, but anti-P46 sIgA remained detectable in 2/10 by the end of the experiment.

Serum IgG responses to P36, P46 and P97R1 antigens in clinically affected pigs

The prevalence of serum IgG responses to P36, P46 and P97R1 was investigated in 125 samples from pigs which did exhibit clinical signs of *M. hyopneumoniae* infection but were from farms that did not vaccinate against this pathogen. Prevalences were determined as 52.8% (66/125) for P97R1, 41.6% (52/125) for P46, and 35.2% (44/125) for P36, respectively (Fig. 5). The prevalence of serum IgG against *M. hyopneumoniae* was 39.2% (49/125) as detected by a commercial ELISA kit. The proportion of sera testing positive differed significantly between the P97R1 and P36 ELISAs, as well as between the P97R1 and commercial ELISAs (Fig. 5). The coincidence in positive and negative samples between the P97R1, P46 and P36 ELISAs was higher (80.8%, 101/125) than that across all four assays (61.6%,

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The coincidence between the commercial ELISA and the P97R1, P46, and P36 ELISAs were 73.6% (92/125), 72.8% (91/125), and 77.6% (97/125), respectively (Table 1).

**Discussion**

We infected 8 week old pigs with *M. hyopneumoniae* in this experiment given that active immunity to this infection occurs around this age (Wallgren et al., 1998), and we then monitored their antibody responses for 19 weeks, by which time most commercial pigs have been slaughtered. Previously, using whole-cell, sodium dodecyl sulfate (SDS) or Tween 20 ELISAs, seroconversion was detected 2–6 weeks after infection with *M. hyopneumoniae*, peaking at 7–12 weeks (Suter et al., 1985; Messier et al., 1990; Sheldrake et al., 1990; Young et al., 1990; Kobisch et al., 1993; Frey et al., 1994; Morris et al., 1995). Differences in assay sensitivity and specificity may relate to the different components of the mycoplasmal cell used as well as differences in strain and dose used to infect animals (Nicolet et al., 1980; Frey et al., 1992; Vicca et al., 2003). In common with the present study, previous research has highlighted the delayed nature of the systemic antibody response to *M. hyopneumoniae*, possibly due to the fact that the pathogen remains adherent to the ciliated respiratory epithelium and does not invade the pulmonary tissue, potentially resulting in slower presentation of mycoplasmal antigen (Sibila et al., 2009).

In our study, variation in serum IgG kinetics to the three selected immunodominant proteins (P97R1, P46 and P36) was a feature. The anti-P46 and anti-P36 responses developed slowly, as previously described (Suter et al., 1985; Messier et al., 1990; Morris et al., 1995), whereas seroconversion to P97R1 occurred more rapidly. Interestingly, previous reports found that P97R1 was not or was only weakly recognised by sera of mice immunised with a commercial inactivated whole cell vaccine (Conceicao et al., 2006; Chen et al., 2008). This suggests the P97R1 antigen, along with Mhp366, may be useful in a DIVA assay, differentiating infected from vaccinated animals (Meens et al., 2010), although this would depend on the commercial vaccine used. The P36 antigen, L-lactate dehydrogenase (LDH), is cytoplasmic in nature, and is not secreted by *M. hyopneumoniae* in vitro (Frey et al., 1994). The anti-LDH response that develops slowly between 5 and 10 weeks post-infection is greater by week 12 post infection, and persists until 21 weeks (Frey et al., 1994). In our study, this ‘two-step’ response was not observed, although the anti-P36 IgG response was the slowest to develop.

**Fig. 3.** Kinetics of mucosal IgA response to *Mycoplasma hyopneumoniae* challenge. The sIgA titres were defined as the reciprocal of the sample dilution with an optical density (OD) greater than cut-off value (mean negative value + 3 SD), and were further normalised against the concentration of total protein in nasal swab samples expressed as concentration/0.1 mg of protein. Data represent means ± SD where n = 10. Significant differences in the response to the P97R1, P46, and P36 antigens were identified using a two-way ANOVA together with the Bonferroni post-tests. **P < 0.01.

**Fig. 4.** Cumulative incidence of sIgA antibody conversion against the P97R1, P46 and P36 antigens post-challenge with *Mycoplasma hyopneumoniae*. 

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The adherence of *M. hyopneumoniae* to respiratory ciliated epithelium is a prerequisite for the establishment of infection resulting in the activation of mucosal immunity and the production of IgA (Suter et al., 1985; Young et al., 1990). Our findings confirm that mucosal immunity precedes a systemic response following *M. hyopneumoniae* infection, and in fact occurred earlier than in previous studies (Suter et al., 1985; Young et al., 1990). Differences in sampling techniques (airway washings vs. nasal swabs), ELISA coating antigens (purified antigens vs. whole cell antigens), as well as infecting doses and strains probably account for this variation. Given that nasal swabs likely contain a greater concentration of sIgA than airway washings, nasal swab fluid seemed a practical and effective alternative sample to airway washings.

Specific mucosal sIgA did not persist, peaking at 12–16 dpi, and returning to pre-infection concentrations in most animals by 84 dpi. Young et al. (1990) reported P97-specific IgA and IgM in airway washings between 35 and 60 days prior to the development of antibody to other major *M. hyopneumoniae* antigens. However, in our study, the humoral response to all three candidate antigens exhibited similar kinetics, although anti-P97R1 and anti-P46 sIgA were significantly elevated at 12 and 16 dpi (P < 0.01), possibly the result of heightened stimulation from a highly infectious environment (Suter et al., 1985). The enhanced understanding of the kinetics of the antibody response to *M. hyopneumoniae* provided by this study will assist in the development of new detection methods. The P97R1 IgG ELISA facilitated the detection of infection two weeks earlier than with the commercial ELISA kit and also identified a significantly greater proportion of seropositive pigs (52.8%) on commercial farms than either the P36 (35.2%) or commercial (39.2%) ELISA. Thus the low sensitivity of currently available *M. hyopneumoniae* ELISAs (Straw et al., 2006) may be improved by using alternate antigens.

### Conclusions

In this study, the kinetics of serum IgG and respiratory mucosal sIgA of pigs to the *M. hyopneumoniae* antigens P97R1, P46 and P36 were monitored for 133 days following experimental infection with the pathogen. The systemic immune response was found to be delayed in onset but persistent, while the mucosal response developed more rapidly, but was less sustained. The P97R1 protein was found to be sensitive in both serological- and mucosal-based settings in diagnosing *M. hyopneumoniae* infection, especially at an early stage.
Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.tvjl.2014.06.019.

References


