

Development of an ELISA Using a Recombinant P46-Like Lipoprotein for Diagnosis of *Mycoplasma pulmonis* Infection in Rodents

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(Received 13 June 2013/Accepted 7 September 2013/Published online in J-STAGE 20 September 2013)

ABSTRACT. *Mycoplasma pulmonis* is one of the most prevalent bacterial pathogens that infects laboratory mice and rats. To develop an *M. pulmonis*-specific antigen for serological diagnosis, we cloned the cDNA of P46-like lipoprotein (P46L), an *M. pulmonis* putative periplasmic protein. P46L is a homolog of P46, an *M. hyopneumoniae* antigen. We produced recombinant P46L fused to glutathione S-transferase (GST) in *Escherichia coli*. Immunoblot analysis revealed that sera from *Mycoplasma*-infected mice and rats contained anti-P46L antibodies. We developed an ELISA using the recombinant P46L-GST protein as an antigen. Thirteen of the 14 samples from rats naturally infected with *M. pulmonis* were determined to be positive according to the commercial ELISA (MONILISA Myco) and positive by our ELISA. Furthermore, 18/19 samples from mice experimentally infected with *M. pulmonis* were positive using our P46L-GST ELISA. In contrast, only 8/19 samples from infected mice were positive by the commercial ELISA. Our results indicate that P46L-GST was an appropriate antigen for developing a serological test to determine *M. pulmonis* infection in laboratory mice and rats.

KEY WORDS: ELISA, *Mycoplasma pulmonis*, P46L.

doi: 10.1292/jvms.13-0308; *J. Vet. Med. Sci.* 76(2): 151–157, 2014

Mycoplasma pulmonis is a Gram-negative pathogenic bacterium that lacks a cell wall. Infection in rats and mice by this organism is known to cause pneumonia. The bacterium belongs to the class *Mollicutes*, order *Mycoplasmatales*, within the family *Mycoplasmataceae*. It is one of the most prevalent pathogenic agents in laboratory colonies of mice and rats [16, 32]. Transmission is thought to be through intrauterine and aerosol routes between cage-mates and between adjacent cages [14, 31]. Murine respiratory mycoplasmosis (MRM) is caused by *M. pulmonis* infections. MRM is usually subclinical, however, when clinical signs are occasionally observed, they can include dyspnea, snuffling, chattering, weight loss and reduced fertility [2, 23, 24, 30]. The severity of lesions in respiratory tissues and mortality due to MRM are dependent upon environmental factors and the strains of the host or organism [2, 3, 7–9]. As an example, C57BL/6 mice are resistant to *M. pulmonis* infection with the severity of lung lesions in these mice much lower than those observed in C3H/He and DBA/2 mice [3, 8]. With respect to rats, Lewis rats are more susceptible to *M. pulmonis* than F344 rats [9]. Additionally, it has been found that *M. pulmonis* infection enhances the metastatic ability of melanoma cells in mice by inducing the release of proinflammatory cytokines [26]. To monitor *M. pulmonis*

infections in laboratories and animal facilities, an accurate and sensitive diagnostic test is necessary.

Serological assays that detect antibodies against pathogens are the most popular screening tests for diagnosing infections [5, 13] with ELISAs the most commonly used serological assays for detecting *M. pulmonis* infections [5, 13, 15]. To date, purified organisms have generally been used as antigens in serological tests for detecting antibodies against *M. pulmonis* [11]. However, obtaining a useful yield of *Mycoplasma* at the required purity requires time. In addition, cross reactivity between anti-*M. pulmonis* antibodies present in sera and antigens derived from other *Mycoplasma* was reported previously [20, 22]. Therefore, the use of purified *M. pulmonis*-specific antigens, such as recombinant proteins or synthesized peptides, should result in more accurate, specific and sensitive serological assays. Membrane-associated lipoproteins derived from *Mycoplasma* have been reported to be antigenic and applicable to serological diagnosis. Recombinant P48 lipoproteins from both *M. bovis* and *M. agalactiae* have been used as ELISA antigens for the detection of anti-P48 antibodies [25, 27]. The P46 and P65 lipoproteins from *M. hyopneumoniae* are known to be antigenic in swine [4], and an ELISA system using recombinant P46 was developed and used to detect *M. hyopneumoniae* infection [10].

In our study, we used a homolog of the *M. hyopneumoniae* P46 protein, P46-like lipoprotein (P46L), to assist in the development of an ELISA to determine *M. pulmonis* infection in laboratory rats and mice. Our protein of interest, P46L, consists of 460 amino acids and contains a periplasmic binding protein domain. We attempted to produce a recombinant P46L protein fused to glutathione S-transferase (GST; GST-P46L) in *Escherichia coli*, which could then be applied as an ELISA antigen.

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MATERIALS AND METHODS

Animals: C57BL/6N and DBA/2N mice were maintained in the animal facility of the Graduate School of Veterinary Medicine (Hokkaido University) until inoculation with *M. pulmonis*. Our animal facility was maintained at $23 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity with a 12-hr light-dark cycle. 8 weeks old mice (C57BL/6N: n=12, DBA/2N: n=7) were anesthetized with ketamine/xylazine, collected blood samples from the tail vein and inoculated intranasally with 1×10^2 or 1×10^5 CFUs of *M. pulmonis* m53 strain (a gift from Dr. Akira Takakura from the Central Institute for Experimental Animals, Japan). After 2 weeks, blood samples were obtained from the tail veins of inoculated mice anesthetized with ketamine/xylazine. Blood samples before inoculation (C57BL/6N: n=3, DBA/2N: n=3), confirmed negative for *M. pulmonis* infection by analysis with the commercial ELISA (described below), were used as controls. Inoculated mice were maintained in isolator cages with ventilation. The sera from rats (a congenic strain derived from Long-Evans, LEH/Hkv-Ednr^b^{sl}, 8–30 weeks old) that were normal (n=6) or naturally infected with *M. pulmonis* (n=14), confirmed whether negative or positive for *M. pulmonis* infection by analysis with the commercial ELISA, were obtained for routine screening according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Graduate School of Veterinary Medicine (Hokkaido University). Animal experiments were conducted according to the Regulations for the Care and Use of Laboratory Animals of Hokkaido University. Our experimental protocol was approved by the IACUC of Hokkaido University.

cDNA cloning of P46L proteins: The *M. pulmonis* m53 strain was grown in PPLO broth (BD, Franklin Lakes, NJ, U.S.A.) with 20% heat-inactivated calf serum (Life Technologies, Carlsbad, CA, U.S.A.), 2.5% fresh yeast extract, 0.05% thallium acetate (Wako, Tokyo, Japan) and 1,000 U/ml penicillin G (Wako) at 37°C for 7 days and then harvested by centrifugation. Total RNA was isolated by TRIzol reagent (Life Technologies). Synthesis of first-strand cDNA was performed in a $10 \mu\text{l}$ reaction mixture containing $0.5 \mu\text{g}$ of *M. pulmonis* total RNA, 50 U of reverse transcriptase (ReverTra

Ace, Toyobo, Tokyo, Japan), $1 \mu\text{l}$ of 2.5 mM deoxyribonucleoside triphosphates (dNTPs) and $1 \mu\text{l}$ of 10 μM random hexamer oligonucleotides. Regions 1–5 of P46L cDNA (Fig. 2A) were amplified by PCR in a $25 \mu\text{l}$ reaction containing $1 \mu\text{l}$ of reaction solution of reverse transcription, 2.5 U of *Taq* polymerase (*ExTaq*, Takara, Tokyo, Japan), $1 \mu\text{l}$ of 2.5 mM dNTPs and $1 \mu\text{l}$ of PCR primers (10 μM ; Fig. 2A). The thermal cycling profile was 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec over 30 cycles. Overlap extension PCR for amplification of cDNA encoding the full-length P46L protein was performed in a $25 \mu\text{l}$ reaction containing $1 \mu\text{l}$ of PCR primers (10 μM ; Fig. 2B), 0.1 ng of each purified PCR product (Fig. 2A) and 2.5 U of *ExTaq* polymerase. The thermal cycling profile was 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min over 30 cycles. The nucleotide sequence of all primers and their binding positions are based on the sequence of *M. pulmonis* (GenBank Accession Number NC_002771) as listed in Table 1. The lowercase small letters in the sequences represent mutated nucleotides. DNA fragments obtained by PCR were cloned into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and confirmed by sequencing with an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Expression and purification of recombinant GST-P46L proteins: P46L cDNA, containing TGG codons that had been substituted for TGA, was cloned into pGEX-6P-2 (GE Healthcare Bio-Science, Piscataway, NJ, U.S.A.) between the *Bam*HI and *Eco*RI recognition sites. *E. coli* BL21 (GE Healthcare, Buckinghamshire, U.K.) was used for transformation of the cloned plasmids with transformed cells grown in LB broth. Expression of proteins was induced through the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Cultures were then left to grow for 4 hr, before they were harvested by centrifugation. The resulting cell pellet was resuspended in PBS containing 1% Triton X-100 and 1% Tween 20, sonicated and centrifuged. Recombinant GST-P46L in the supernatant was isolated by batch purification with glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer's protocol. Lysates of transformed *E. coli* treated with IPTG and purified recombinant proteins were analyzed by SDS-PAGE and visualized by

Table 1. Oligonucleotide primers used in the cloning of *M. pulmonis* P46L

Primer name	Sequence (5'→3')*	Nucleotide position in the <i>M. pulmonis</i> sequence (NC_002771)
MP-p46L-F1	CTG GGA TCC TTG AAA TGg GTT TAT ATG	401976–402093
MP-p46L-Tr	CTG GGA TCC TCG GGT TCA ACA GCA TCT	402096–402113
MP-p46L-m01F	AAT CAA AGA TGg GTA GAG AC	402192–402211
MP-p46L-m01R	GTC TCT ACc CAT CTT TGA TT	402211–402192
MP-p46L-m02F	ACA ATT GgT ATG TTG CCT TC	402463–402482
MP-p46L-m02R	GAA GGC AAC ATA cCA ATT GT	402482–402463
MP-p46L-m03F	AGC AAA CTG gAA CTA TTC AC	402836–402855
MP-p46L-m03R	GTG AAT AGT TcC AGT TTG CT	402855–402855
MP-p46L-m04F	GAA GAC TGg AAA ATA GCA GC	403089–403108
MP-p46L-m04R	GCT GCT ATT TtC CAG TCT TC	403108–403089
MP-p46L-R1	CTA TGA TTC TTT CTT GAA AA	403358–403339

*Lowercase letters in the primer sequences indicate substituted nucleotides for site-directed mutagenesis.

Coomassie brilliant blue R-250-staining.

Immunoblot analysis of P46L-GST: Transformed *E. coli* lysate (10 μ g) and recombinant protein (1 μ g) were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, U.S.A.) with a semi-dry blotter (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Membranes were incubated in blocking buffer {5% skim milk in PBS-T (137 mM NaCl, 81 mM Na₂HPO₄, 15 mM KH₂PO₄, 27 mM KCl and 0.1% Tween 20)} at room temperature for 1 hr, rinsed with PBS-T and then incubated at room temperature for 1 hr in blocking buffer with diluted serum (1:1,000) from *M. pulmonis*-positive mice diagnosed with a commercial ELISA kit (MONILISA Myco, Wakamoto Pharmaceutical Co., Ltd., Tokyo, Japan). Membranes were washed with PBS-T and incubated in blocking buffer containing horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:10,000; GE Healthcare Bio-Science) at room temperature for 1 hr. They were then washed with PBS-T and treated with ECL Plus reagents (GE Healthcare Bio-Science). Detection of chemiluminescence was conducted with an LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

ELISA: The commercial MONILISA Myco ELISA kit was used to detect antibodies against *M. pulmonis* in serum samples according to the manufacturer's instructions. Serum samples (200 μ l) were diluted 1:40, before they were applied to the ELISA. Determination of positive and negative samples was conducted by macroscopically comparing the color reaction in wells with known positive control samples within the assay. Positive control sera within the commercial ELISA kit yielded OD values of 0.3–0.4 at 492 nm [18]. For the ELISA we developed, a 200 μ l aliquot of 1 μ g/ml affinity-purified P46L-GST in 50 mM sodium carbonate-bicarbonate buffer (pH 9.6) was added to each well of a 96-well plate and incubated at 4°C overnight. Wells were washed three times with PBS-T and incubated with 200 μ l of ELISA blocking buffer (1% bovine serum albumin in PBS-T) at 37°C for 1 hr. Wells were washed a further three times with PBS-T, before 200 μ l of mouse sera (diluted 1:160 in ELISA blocking buffer) or 200 μ l of rat sera (diluted 1:200 in ELISA blocking buffer) was added and incubated at 37°C for 1 hr. Wells were again washed three times with PBS-T, and an aliquot (200 μ l) of HRP-conjugated anti-mouse IgG (1:10,000; GE Healthcare Bio-Science) in ELISA blocking buffer or HRP-conjugated anti-rat IgG (1:20,000; Sigma-Aldrich, St. Louis, MO, U.S.A.) was added to each well and incubated at 37°C for 1 hr. Wells were again washed three times with PBS-T, before 1.5 mg/ml *o*-phenylenediamine with 0.01% H₂O₂ (200 μ l) was added to each well and incubated at 37°C for 10 min. The reaction was stopped through the addition of 3 M H₂SO₄ (50 μ l), and the OD at 450 nm for each well was measured with a Model 680 microplate reader (Bio-Rad Laboratories).

Statistics: For statistical analyses, Student's *t*-test, one-way analysis of variance (ANOVA) with Tukey's post-hoc test and Fisher's exact test were performed using GraphPad Prism 5.0 (GraphPad software, La Jolla, CA, U.S.A.).

RESULTS

Cloning P46L from *M. pulmonis*: The P46L protein is known to be encoded by the MYPU_3430 gene (NCBI Gene ID: 911636). The protein comprises 460 amino acids with a predicted molecular weight of 50.6 kDa. The amino acid sequence of P46L was found to be 28% identical to that for *M. hyopneumoniae* P46 (Fig. 1). Protein motif scanning using InterProScan [33] revealed that P46L was predicted to contain a prokaryotic membrane lipid attachment site [12] at positions 1–31 (Fig. 1) and a periplasmic binding protein domain at positions 64–390 (Fig. 1) [21].

For the entire coding region of the P46L gene (nucleotides 401,976–403,358; GenBank Accession No. NC_002711), there were five TGA codons that corresponded to tryptophan in *Mycoplasma*, however, these are termination codons in mammals and other bacteria (Fig. 2A). These five TGA codons were altered to TGG. Cloning of mutated P46L cDNA was conducted using site-directed mutagenesis and overlap extension PCR. The P46L coding sequence was divided into five fragments (Fig. 2A), and each cDNA fragment was amplified by RT-PCR using the primers presented in Table 1. We then amplified the entire coding sequence by overlap extension PCR using all five amplicons as templates (Fig. 2B). This PCR product was cloned, and we confirmed by sequencing that it corresponded to the entire P46L coding sequence with five TGG codons in place of the TGA codons (data not shown).

Expression and purification of P46L-GST: The P46L cDNA was successfully cloned into pGEX-6P between the *Bam*HI and *Eco*RI sites and then transformed into *E. coli* to enable expression of a recombinant protein. Protein samples

1	MKVVYMKINKLLEVLSSLSLATLSPPLVAISCGVSGSIASTYKLVSSPEQPAKAVSEKIS	60
1	---MKMLRKKFLYSATYATSLASIAFVAAGCGGTESGSTDSEK--QAE TLKHKV	54
61	DGSLITVITDPEINQRVETKQDLDAYSKTNGFHEISLNHWKQAEONSFVDAELAKTGNN	120
55	NDSTRITATDPPRIVISAQKDIISY--VDETEAATSTITKQDAONNWLTTQQ--ANLSP	110
121	KPKVVLGMAADSGNATQAIETSTTNAQAQFIAVDRFTHKISNNYVNYVAENNYTVGQGL	180
111	APKGFIIAPENSGVGTAVNTIADKGIPIVAYDRLITG--SDKYDWWYSFENKVKGLQGL	169
181	ALTSCTYKGGEPKLTLEEAKTYVMANKLASEKGFVALAGAPEDNNSHLFFKGAMPVILTA	240
170	SLAAGLLGKEDGAFDSIDQMNEYLKSHMPQETISFYTIAGSQDDNNSQVFEYNGAMKVLKE	229
241	IMKIDSNLKYFGELEGADVFNRLSGLSKPTDQELTSAITSHFRVVAQANWNYSQKQFLD	300
230	LMK--NSQNKIIDLSPGENAVYVPCWNYGTAGQ---RTQS--ELTINKDP---AGCNK--IK	280
301	TLFAKSTFDSRKNLAAVLPANDEMAQAQATISTEQKGLDPKTYITIGODSNQPSLRSITD	360
281	AVGSKPA-----SIFKGLAPNDGMAEQALITKCLKLEGFDTQKTEVITGODYNDKAKTFIK	334
361	NETGQNMITSKEDWKIAAISTAAYYIVKINPKQSETDHYYNKVAYLKEQYPIIPFQI	420
335	DGQNMITYKPKVKGKVAVEVIRVLTARKNKASRSEVENELKAKLPNISFKYDQNTYK	394
421	SRTEYVAKESESNKKEINSVLLTPTLVTKANFKVFKKES	460
395	QKGNITILVSPVIVTKANVDPDA-----	419

Fig. 1. Amino acid sequence alignment for *M. pulmonis* P46L (upper sequence) and *M. pneumoniae* P46 (lower sequence). Identical amino acid residues are highlighted with black boxes. The dashed lines indicate gaps in the sequence alignment. The putative prokaryotic membrane lipid attachment site is indicated by the dotted line. A putative periplasmic binding protein domain is indicated by the solid line. P46 accession number, BAA04085; P46L, NP_326174.

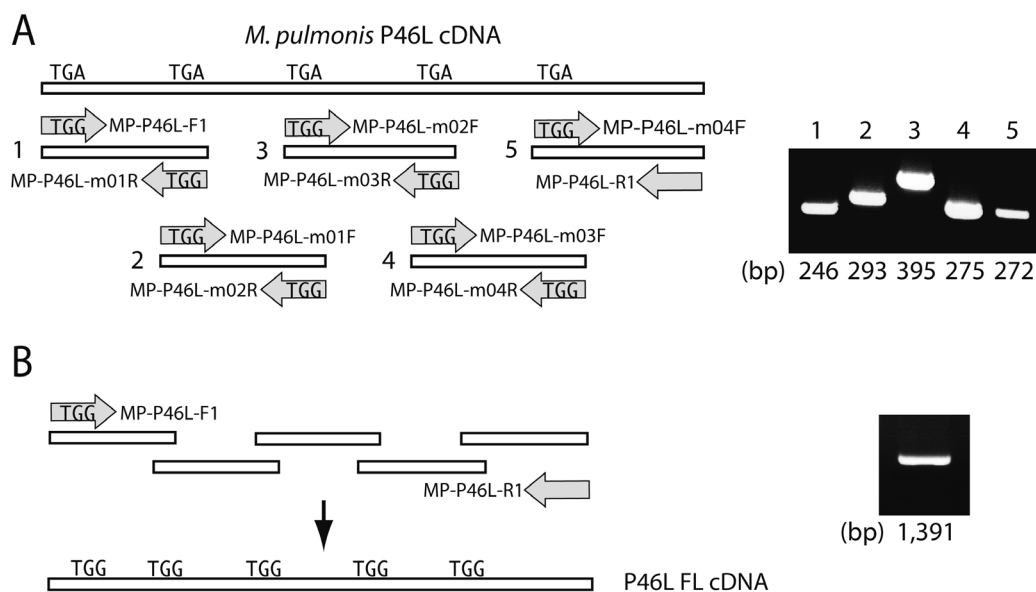


Fig. 2. Amplification of mutated P46L cDNA using site-directed mutagenesis and overlap extension PCR. (A) Overlapping primers containing TGG mutations (left panel and Table 1) were used for amplification of P46L cDNA fragments 1–5. Amplicons were electrophoresed on agarose gels, stained with ethidium bromide and visualized using UV light (right panel). (B) P46L cDNA was amplified by overlapping PCR using purified PCR products obtained in (A) and the primers shown in the left panel. The P46L cDNA PCR product (1,391 bp) can be seen in the right panel.

from the lysates of transformed *E. coli* and purified P46L-GST were analyzed by SDS-PAGE (Fig. 3A). A strong band consistent with 77.5 kDa was observed; this was around the expected size for P46L-GST. We performed immunoblot analysis of protein samples using mouse sera from *M. pulmonis*-infected mice (Fig. 3B). An immunoreactive band was observed at the same position as that for the purified P46L-GST seen in Fig. 3A. We also observed the same band when sera from *M. pulmonis*-infected rats were employed (data not shown).

ELISA detection of anti-*M. pulmonis* antibodies: When we analyzed *M. pulmonis*-positive mouse serum by ELISA using P46L-GST, the OD at 450 nm for samples diluted 1:80 was higher than the ODs at other dilutions. In contrast, OD values for control serum were similar to those when serum was absent and did not vary across dilutions (Fig. 4A). Similar ELISA results were obtained using rat sera (Fig. 4B). The OD values were almost constant among samples diluted 1:100–1:800 and then decreased at higher dilutions. We analyzed the same rat serum samples from Fig. 4B using a commercial ELISA (data not shown). In contrast to our ELISA, OD values for samples diluted 1:320 decreased to one-third the level of that observed for the 1:40 dilution.

Evaluation of the developed ELISA as a suitable diagnostic tool for *M. pulmonis* infection: The range in OD values for control rat sera was 0.075–0.152 (Fig. 5A), whereas that for sera from rats naturally infected with *M. pulmonis* rats was 0.109–0.620 (Fig. 5A). Our determined means and standard deviations (SDs) were 0.114 and 0.028, respectively, for control rats and 0.444 and 0.147 for infected rats. The

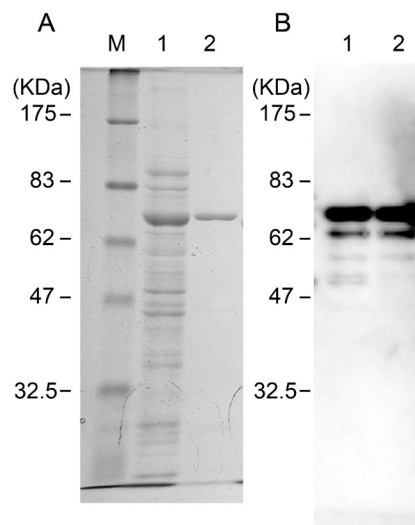


Fig. 3. Recombinant P46L-GST expressed in *E. coli* and its antigenicity. (A) SDS-PAGE stained with Coomassie brilliant blue R-250. Lane M, size marker; 1, sample of *E. coli* cell lysate after induction with IPTG (10 μ g); 2, affinity-purified P46L-GST (1 μ g). (B) Immunoblot analysis of recombinant P46L using serum from experimentally *M. pulmonis*-infected C57BL/6N mice. A typical blot taken from five independent experiments. Lane 1, sample of *E. coli* cell lysate following IPTG induction (10 μ g); 2, affinity-purified P46L-GST (1 μ g).

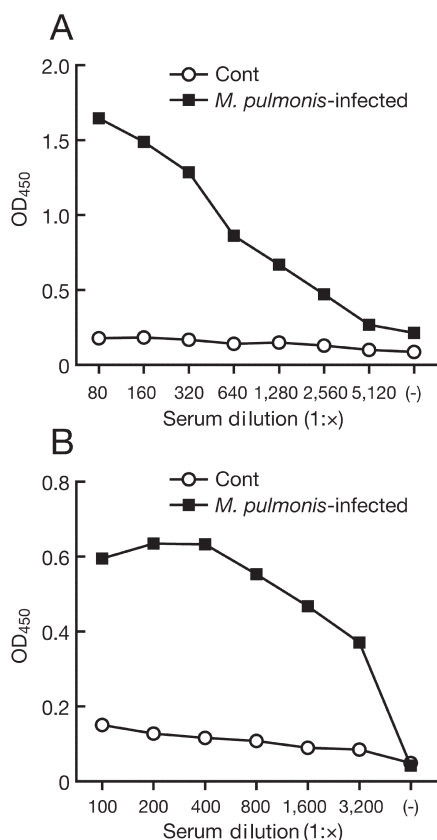


Fig. 4. ELISA using P46L-GST as the antigen. Sera from mice (A) and rats (B), used in Fig. 5, were diluted and used to test the ELISA we developed. Open circles indicate serum samples from control animals, and shaded squares are indicative of serum samples from *M. pulmonis*-infected animals. (-) indicates a non-serum sample. The plot is representative of data from two independent experiments.

mean OD value for infected rats was significantly higher than that for control rats using Student's *t*-test.

For mice, the range in OD values was 0.141–0.236 for control sera (Fig. 5B). For mice experimentally infected with *M. pulmonis* but where antibodies against *M. pulmonis* were not detected in sera using a commercial ELISA, the range was 0.194–0.437 (Fig. 5B). The range in OD values was 0.287–0.634 for sera from mice that were experimentally

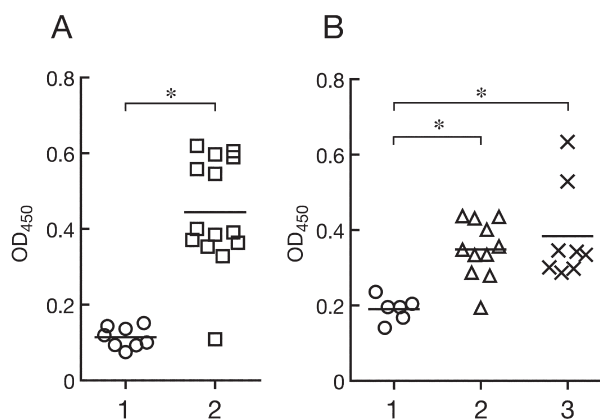


Fig. 5. Evaluation of the P46L-GST ELISA. The solid lines indicate the mean OD values. (A) 1, sera from control (uninfected) rats; 2, sera from rats naturally infected with *M. pulmonis* and diagnosed as *Mycoplasma*-positive using the commercial ELISA. **P*<0.05 vs. control using Student's *t*-test. (B) 1, sera from control (uninfected) mice; 2, sera from mice experimentally infected with *M. pulmonis* and diagnosed as negative for *M. pulmonis* infection using the commercial ELISA; 3, sera from mice experimentally infected with *M. pulmonis* and diagnosed as positive for *M. pulmonis* infection using the commercial ELISA. **P*<0.05 vs. control using ANOVA with Tukey's post-hoc test.

infected with *M. pulmonis* and where antibodies could be detected using the commercial ELISA (Fig. 5B). The determined means and SDs were 0.190 and 0.033, respectively, for controls, 0.349 and 0.076 for infected but ELISA-negative samples and 0.384 and 0.127 for infected and ELISA-positive samples. The mean OD values for infected mice were significantly higher than those for control mice.

For our P46L-GST ELISA, we determined the cutoff OD value (mean + 2SD of controls) for an *M. pulmonis*-positive rat sample to be 0.170 and for an *M. pulmonis*-positive mouse sample to be 0.255. Based on these cutoff values, we evaluated serum samples used in Fig. 5 (Table 2). All control samples were diagnosed as negative for *M. pulmonis* infection. Thirteen of the fourteen samples from rats naturally infected with *M. pulmonis* were determined to be positive according to the commercial ELISA and positive by our ELISA. Furthermore, 18/19 samples from mice experimentally infected with *M. pulmonis* were positive using our P46L-GST ELISA. In contrast, only 8/19 samples from infected mice were positive by the commercial ELISA.

Table 2. Evaluation of serum samples using the P46L-GST ELISA

	Positive samples/total samples	
	Commercial ELISA	P46L-GST ELISA
Control rats	0/8	0/8
Rats naturally infected with <i>M. pulmonis</i>	14/14	13/14
Control mice	0/6	0/6
Mice experimentally infected with <i>M. pulmonis</i>	8/19	18/19 *

**P*<0.05 vs. commercial ELISA using Fisher's exact test.

For the serum samples from infected mice, the frequency of positives was significantly different between the ELISAs.

DISCUSSION

M. pulmonis is a prevalent pathogenic microorganism in laboratory colonies of mice and rats and is known to cause murine respiratory mycoplasmosis [2, 32]. It is important to monitor for *M. pulmonis* infections so that the health of rodent colonies at important animal facilities can be maintained at the highest levels. Serological tests, such as ELISAs, are the most common methods for diagnosing *Mycoplasma* infections [5, 13]. In the present study, we developed an ELISA using a recombinant P46L protein as the antigen and found it to be sufficient for diagnosis of *M. pulmonis* infection comparing with a commercially available ELISA.

P46 is an antigenic protein of *M. hyopneumoniae* [4] with a recombinant form of P46 previously used as an ELISA antigen [10]. We hypothesized that antigenic proteins derived from *M. pulmonis* would also be useful for serological diagnosis in rodents. Using a BLAST search against a protein sequence database, we identified that P46L, a homolog of P46 in *M. pulmonis*, might be an appropriate protein that could be used as an ELISA antigen. We were unable to determine any other *M. pulmonis* proteins that were homologous to P46 (data not shown). P46L is predicted to be a lipoprotein, as it contains motifs similar to those found in prokaryotic membrane lipid attachment sites (amino acids 1–31). Additionally, P46L has a putative periplasmic binding domain at positions 64–390. The periplasmic binding domain is frequently seen in the component of the ATP binding cassette-type sugar transport systems in various bacteria [1, 6, 28]. Therefore, P46L may act as a component of a sugar transporter complex in *M. pulmonis*.

Our results show that P46L-GST was highly expressed in *E. coli* (Fig. 3) and could be successfully purified by affinity chromatography. Furthermore, this protein was strongly reactive to sera from *M. pulmonis*-infected mice and rats; therefore, we believe that P46L-GST is an appropriate antigen for serological diagnosis of *M. pulmonis* infections. Using purified P46L-GST, we developed an ELISA and compared it with a commercial ELISA. In the commercial ELISA we tested, the antigens used were untreated but cultured bacterial cells [11]. Our findings show that the P46L-GST ELISA was able to detect antibodies against P46L-GST (Fig. 4) in sera from infected mice and rats. The mean OD values attributed to sera from *M. pulmonis*-infected mice and rats were significantly higher than those from respective controls (Fig. 5). The commercial ELISA we used in this study is a standard test for diagnosis of *M. pulmonis* infection in laboratory mice and rats by International Council for Laboratory Animal Science Monitoring Center in Japan [11, 13]. Our results indicate that the P46L-GST ELISA we have developed is appropriate for determining the serological state of sera from *M. pulmonis*-infected mice and rats. However, we did observe one discrepancy involving a serum sample from a rat. According to the commercial ELISA used in this study, the sample was considered *Mycoplasma*-positive, yet

our P46L-GST ELISA indicated that the sample should be classed as *Mycoplasma*-negative. (Fig. 5A and Table 2). This is a strong indicator that our ELISA requires further validation and improvement. The expression of V-1 lipoproteins is regulated by site-specific genomic DNA inversions of the *vsa* genes that cause the phase-variable production of different V-1 proteins in individual isolates of *M. pulmonis* [29]. Furthermore, polymorphisms of the variable lipoprotein and hemagglutinin gene (*vlhA*) are seen among the strains of *M. synoviae* [17]. P46L gene was found in *M. pulmonis* strains UAB CTIP (MYPU_3430 gene in NCBI genome database) and m53 (this study). To validate our ELISA, it is important to examine whether P46L gene exists and expresses in various strains of *M. pulmonis*, and the gene product is antigenic in mice and rats. In addition, cross-reactivity of GST-P46L to sera from rats and mice infected with various strains of *M. pulmonis* or *Mycoplasma* spp. is necessary to investigate. A previous report revealed that *M. pulmonis* antigen had cross-reactivity against *M. arthritis* antisera [22]. By analysis of protein BLAST search, we found P46L-like proteins showing 30–36% homology in *M. hyopneumoniae*, *M. alligatoris*, *M. crocodyli*, *M. flocculare*, *M. mobile* and *M. hyorhina*, but not in *M. arthritis* and other *Mycoplasma* (data not shown). Therefore, it is expected that GST-P46L protein does not cross-react with *M. arthritis* antisera. Moreover, PCR-based method may be helpful to detect *M. pulmonis*-specific infection [19]. As stated previously, the antigen used in the commercial ELISA we tested was the *M. pulmonis* organism itself [11]. Propagation and purification of a sufficient amount of *Mycoplasma* for this purpose are time-consuming. In contrast, recombinant proteins, such as P46L-GST, can be mass-produced, demonstrate greater stability and are easier to use. This point gives our ELISA an advantage over the commercial ELISA used in this study.

In summary, we expressed and purified *M. pulmonis* P46L, a putative periplasmic lipoprotein homologous to P46 from *M. hyopneumoniae* antigenic protein. The recombinant protein we expressed was fused to GST, which allowed for easy purification. The antigenicity of the protein was confirmed in mice and rats infected with *M. pulmonis*. Application of the recombinant fusion protein as an ELISA antigen was found to be appropriate in the detection of anti-P46L antibodies. We believe that the ELISA we have developed can, and should be, successfully deployed for the serological diagnosis of *M. pulmonis* infection in mice and rats colonies at animal facilities.

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