Research Article

Cloning of the Gene Encoding *Mycoplasma hyopneumoniae* P36 Protein, Expression and Evaluating the Antigenicity of Recombinant Protein

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Abstract | This study aimed to clone the gene (432 bp) encoding *M. hyopneumoniae* P36 protein, express this protein in *E. coli* BL21(DE3), and evaluate its antigenicity as a base for further studies to against *M. hyopneumoniae*. The gene encoding *M. hyopneumoniae* P36 protein isolated from DNA of fresh lung tissue samples from PEP infected pigs was cloned into pGEM®-T Easy vector for sequencing, and then was inserted into pET28a vector to express the protein. The cloning result revealed that the gene encoding the *M. hyopneumoniae* P36 protein was 432 bp in length, and 99.07% similarity to the nucleotide sequence of P36 gene from strain *M. hyopneumoniae* 7448 recorded in GenBank (accession No. AE017244.1), corresponding to 144 amino acids. Expression of this protein in *E. coli* BL21 produced a fusion protein with molecular weight of approximately 20 kDa. Moreover, the antibodies against *M. hyopneumoniae* in sera taken from pigs that were immunized with the PEP inactivated vaccine (HYOGEN®) strong detected the recombinant *M. hyopneumoniae* P36 protein by ELISA, demonstrating that expressed recombinant P36 protein has the antigenicity and is specific for *M. hyopneumoniae*. In summary, we have successfully cloned the gene (432 bp) encoding the *M. hyopneumoniae* P36 protein. The expressed recombinant *M. hyopneumoniae* P36 protein in strain *E. coli* BL21 has the antigenicity and is specific for *M. hyopneumoniae*. Further experiments should be performed to evaluate the immunogenicity of *M. hyopneumoniae* P36 protein for use as an antigen to develop novel vaccines and bio-products (e.g., egg yolk antibody) against *M. hyopneumoniae*.

Keywords | *Mycoplasma hyopneumoniae*, porcine enzootic pneumonia, P36 protein, protein expression, recombinant protein, antigenicity

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INTRODUCTION

The porcine enzootic pneumonia (PEP), a chronic respiratory disease causing by the primary pathogen of *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) damages enormous economic losses to the pig production worldwide (Sheldrake et al., 1991; Thaker et al., 1998; Thaker, 2006). For control of the disease, the main strategies comprise of good management of herds and housing condition, vaccination and use of active antibiotics were recommended (Maes et al., 2007; Simionatto et al., 2012; Holst et al., 2015). However, according to the literatures, the currently used commercial PEP vaccines in the markets are largely inactivated and attenuated live vaccines that have still many drawbacks, and provide only partial protection (Tao et al., 2019; Maes et al., 2021). On the other hand, the use

of antibiotics for the disease treatment, especially for the long-term use will increase the risk of antibiotic-resistant bacteria and cause antibiotic residues in products that affect the health of consumers. Therefore, in addition to improvement of herds management quality and housing condition, it is needed to develop novel vaccines and antibiotic alternative bio-products (e.g., egg yolk antibody) to more effectively prevent and treat the PEP.

With the quick development in molecular biology and bioinformatics in last time, the genome of *M. hyopneumoniae* has been known to encode several important immunodominant proteins (Caron et al., 2000). One of which is the cytosolic protein (P36; L-Lactate dehydrogenase). It carried highly conserved species-specific antigenic determinants for *M. hyopneumoniae* (Mori et al., 1987; Strasser et al., 1991; Caron et al., 2000), and induced an early immune response in pigs (Frey et al., 1994). To our knowledge, few studies on cloning entire P36 gene (948 pb) of *M. hyopneumoniae,* expression, and use of recombinant P36 protein for diagnosis of *M. hyopneumoniae* have been performed (Strasser et al., 1991; Caron et al., 2000; Feng et al., 2014). More recently, Vo et al. (2018) reported that there is a similarity of genetic features between the *M. hyopneumoniae* strains isolated in Vietnam and *M. hyopneumoniae* strains isolated in various parts of the world, and a gene encoding *M. hyopneumoniae* P36 protein with 432 bp in length was determined. Theoretically, the identification of novel genes and creating novel immunogenic antigens from *M. hyopneumoniae* will contribute to the development of novel vaccines, diagnostic tests, as well as bio-products (for example egg yolk antibody) against *M. hyopneumoniae*. Therefore, this study aimed at *cloning the* gene (432 bp) encoding *M. hyopneumoniae p36 protein* from PEP infected pigs in Thua Thien Hue province, Vietnam, expressing the protein in *E. coli* BL21 and evaluating its antigenicity as a base for further studies of novel vaccines and bio-products development against *M. hyopneumoniae*.

MATERIALS AND METHODS

Plasmids, growth medium and serum samples

The pGEM®-T Easy (Promega, USA) and the pET28a vectors (Novagen) harboring the T7 promoter were used as cloning and expression systems of the recombinant P36 protein, respectively. *E. coli* TOP 10 (Invitrogen, USA) was used as host strain for plasmid DNA amplification, and *E. coli* BL21 (Invitrogen, USA) for production of recombinant P36 protein. The growth medium for *E. coli* strains was Luria broth (LB). Serum samples obtained from 4 pigs immunized with the PEP inactivated vaccine (HYOGEN®) were used to detect the recombinant *M. hyopneumoniae* P36 protein.

PCR amplification of the gene encoding *M. hyopneumoniae* P36 protein

The PCR was performed using genomic DNA of *M. hyopneumoniae* isolated from the fresh lung tissue from PEP infected pigs in Thua Thien Hue province, Vietnam as a template to amplify. The PCR components included 40 ng DNA (40 ng/µL) of *M. hyopneumoniae*, 5 pmol each primer (10 pmol/µL) of P36 specific primer pairs designed by Vo et al. (2018) with the minor modification by adding two restriction sites for BamHI and HindIII (underlined nucleotides) at the 5' ends of forward primer: 5'- GGATCCGCGGGAAGACCACAAAAACC-3' and reverse primer: 5'- GCAAGCTTTTCATAAGCCCGGCGAGAAA-3', respectively to insert into pET-28a, 6.25 µL MyTaq Red Mix, 2X (Bioline), and 4.25 µL of deionized water. The reaction volume of 12.5 µL was pre-denaturated at 95 °C for 5 min, followed by 35 cycles including 30 sec at 95 °C for denaturation, 30 sec at 60 °C for annealing, 30 sec at 72 °C for extension per each cycle. After the final cycle, the temperature of 72 °C was held for an additional 10 min. The amplified product (gene encoding *M. hyopneumoniae* P36 protein) was tested by 1% (w/v) agarose gel electrophoresis with 1 µL RedSafe™ nucleic acid staining solution (20.000X). Electrophoresis image was analyzed by Gelsmart (Dlab - USA).

Cloning of the gene encoding *M. hyopneumoniae* P36 protein

The gene encoding *M. hyopneumoniae* P36 protein from the 1% agarose gel was purified using TopPURE® PCR/GEL DNA PURIFICATION KIT (ABT, Vietnam) following the manufacture's instruction. Techniques for insertion of the target gene into pGEM®-T Easy vector, transformation of the recombinant plasmid into competent *E. coli* TOP 10 cells, selection and confirmation of positive colonies, biomass production, and extraction of recombinant pGEM®-T Easy/P36 vector were carried out as described by Phung et al. (2019). The gene encoding *M. hyopneumoniae* P36 protein was sequenced by Genlab Company (Vietnam). The nucleotide sequence of the target gene was compared with that of P36 gene (L-lactate dehydrogenase) from strain *M. hyopneumoniae* 7448 recorded in GenBank (accession No. AE017244.1) using the software of nucleotide BLAST online of NCBI.

Expression, purification and confirmation of the recombinant *M. hyopneumoniae* P36 protein

The pET28a expression vector and the purified gene encoding *M. hyopneumoniae* P36 protein were treated simultaneously with BamHI and HindIII restriction enzymes, then ligated by T4 ligase to form recombinant pET28a/P36 vector and transformed into strain *E. coli* Star™ BL21. The techniques in detailed for the ligation, transformation, expression, purification and analysis of

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recombinant P36 protein were conducted as described by Phung et al. (2019).

Enzyme-linked immunosorbent assay (ELISA) was used to confirm the presence of recombinant *M. hyopneumoniae* P36 protein in *E. coli* BL21. Briefly, a microtiter plate was coated with 20 ng/well (100 µL) of purified recombinant *M. hyopneumoniae* P36 protein in 0.1 M carbonate-bicarbonate coating buffer (Sigma-Aldrich, USA), and incubated at 4°C overnight. After washing wells three times with PBS (200 µL/well) containing 0.05% Tween 20 (PBS-T), 200 µL of blocking solution of BSA 1% was added each well, and incubated at room temperature for 2 h. Next, all wells were washed again three times with PBS-T, and 100 µL of Anti-6X His-tag® antibody (Abcam, UK) (diluted 1:5000) was added to each well, and incubated at room temperature for 2 h. Finally, wells were washed with PBS-T six times, incubated with 100 µL/well TMB substrate (Abcam, UK) for 10 min, stopped the reaction by HCl 3M (50 µL/ well) and read the results.

Evaluating antigenicity of the recombinant *M. hyopneumoniae* P36 protein by ELISA

To evaluate the antigenicity of recombinant *M. hyopneumoniae* P36 protein, an ELISA was carried out using sera taken from 4 pigs (n=4) immunized with a PEP inactivated vaccine (HYOGEN®) to detect recombinant *M. hyopneumoniae* P36 protein. The protocol for ELISA was performed as described above with a replacement of Anti-6X His-tag® antibody (Abcam, UK) by sera taken from 4 pigs (n=4) immunized with a PEP inactivated vaccine (HYOGEN®) diluted 1:100 in blocking buffer.

RESULTS AND DISCUSSION

Cloning the gene encoding *M. hyopneumoniae* P36 protein

The result of PCR amplification and electrophoresis of the gene encoding *M. hyopneumoniae* P36 protein from genomic DNA of *M. hyopneumoniae* has been shown in Figure 1. On 1 % agarose gel, the gene encoding *M. hyopneumoniae* P36 protein was observed as a bright band with a length approximately 432 bp (Lanes 1, 2). This size is similar to that reported in the study of Vo et al. (2018). The purified gene encoding *M. hyopneumoniae* P36 protein was cloned into pGEM®-T Easy vector, and transformed into strain *E. coli* TOP 10. The positive colonies were chosen and amplified by PCR with specific primer pairs for *M. hyopneumoniae* P36 gene (Vo et al., 2018) and M13 primer pairs of the pGEM®-T Easy vector to confirm the presence of the insert in transformed bacterial cells. The result of electrophoresis on 1% agarose gel showed that bands of gene encoding *M. hyopneumoniae* P36 protein were approximately 432 bp in length with specific primer pairs for *M. hyopneumoniae* P36 gene designed by Vo et al. (2018), and approximately 632 bp in length with M13 primer pairs of the pGEM®-T Easy vector (Figure 2), corresponding to the expected length of 432 bp of the insert plus 200 bp of M13 primer pairs on the vector.

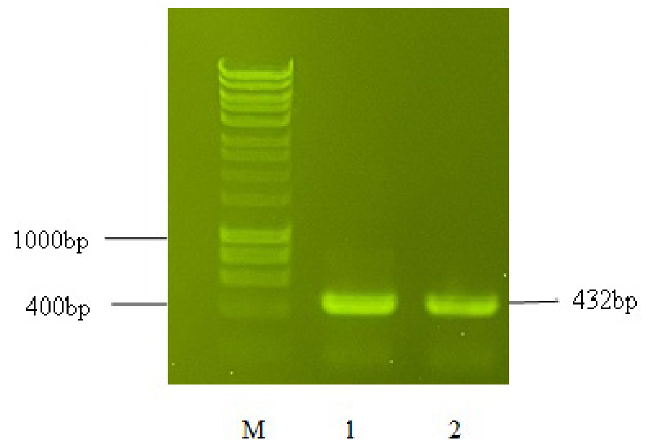

Figure 1: PCR amplification of the gene encoding *M. hyopneumoniae* P36 protein from genomic DNA isolated from fresh lung tissue samples of PEP infected pigs in Thua Thien Hue province, Vietnam with specific P36 primer pairs. Lane M: DNA size marker (100-1500 bp, Bio Basic); Lanes 1-2: PCR products of the *M. hyopneumoniae* P36 gene.

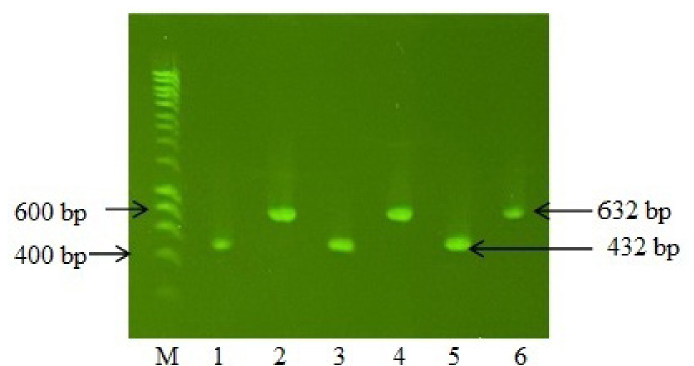

Figure 2: Electrophoresis results of PCR products on gel agarose 1% to identify the presence of the gene encoding *M. hyopneumoniae* P36 protein and the recombinant pGEM®-T Easy/P36 vector in *E. coli* TOP 10. Lane M: DNA size marker (100-1500 bp, Bio Basic), Lanes 1, 3, 5: PCR products with specific primer pairs for *M. hyopneumoniae* P36 gene; Lanes 2, 4, 6: PCR products with M13 primer pairs of the pGEM®-T Easy vector.

The results of sequencing the nucleotide sequence and analysis of amino acid sequence of *M. hyopneumoniae* P36 gene in this study have been shown in Figures 3 and 4, respectively. The result presented in Figure 3 showed that the nucleotide sequence of *M. hyopneumoniae* P36 gene had 432 nucleotides, 99.07% similarity to the nucleotide sequence of P36 gene of strain *M. hyopneumoniae* 7448 recorded in GenBank (accession No. AE017244.1). Deduced amino

acid sequence was 144 amino acids, 98.61% similarity to the amino acid sequence of L-lactate dehydrogenase (P36) of strain *M. hyopneumoniae* 7448 recorded in GenBank of NCBI (accession No. AAZ53511.1) (Figure 4).

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P36          1       GCGGGAAGACCACAAAAACCGGGTGAAACTCGGCTTGAATTAGTAGCTGATAACATCCGA  60
AE017244.1   176761  GCGGGAAGACCACAAAAACCGGGTGAAACTCGGCTTGAATTAGTAGCTGATAACATCCGA  176820

P36          61      ATTATCCGGGAAATTGCACTAAAAGTCAAAGAAAGTGGCTTTAGTGGAATAAGTATTATT  120
AE017244.1 176821    ATTATCCGGGAAATTGCACTAAAAGTCAAAGAAAGTGGCTTTAGTGGAATAAGTATTATT  176880

P36          121     GTTGCTAATCCCGTTGATATAATTACAAGGGCTTACCGGGATGCATCTGGATTTTCCGAT  180
AE017244.1   176881  GTTGCTAATCCTGTTGATATAATTACAAGGGCTTACCGGGATGCATCTGGATTTTCCGAT  176940

P36          181     CAAAAAGTTATCGGTAGTGGAACTGTTTTAGATACAGCAAGGCTTCAATTTGCAATCGCA  240
AE017244.1   176941  CAAAAAGTTATCGGTAGTGGAACTGTTTTAGATACAGCAAGGCTTCAATTTGCAATCGCA  177000

P36          241     AAAAGAGCAAAAGTATCGCCTAATTCGGTTCAGGCCTACGTAATGGGTGAACATGGTGAT  300
AE017244.1   177001  AAAAGAGCAAAAGTATCGCCTAATTCGGTTCAGGCCTACGTGATGGGTGAACATGGTGAT  177060

P36          301     CCATCTTTTGTTGCTTATTCAAATATTAAAATTGCCGGTGAATGTTTCTGTGCTTATTCT  360
AE017244.1   177061  TCATCTTTTGTTGCTTATTCAAATATTAAAATTGCCGGTGAATGTTTCTGTGCTTATTCT  177120

P36          361     AAACTAACCGGAATCAATAGCTCAAATTACGAAAAAGAACTTGAATATCCAGTTTCTCGC  420
AE017244.1   177121  AAACTAACCGGAATCGATAGCTCAAATTACGAAAAAGAACTTGAATATCCAGTTTCTCGC  177180

P36          421     CGGGCTTATGAA  432
AE017244.1 177181 CGGGCTTATGAA 177192
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Figure 3: Comparison of nucleotide sequence of gene encoding P36 protein from *M. hyopneumoniae* isolated from PEP infected pigs in Thua Thien Hue province, Vietnam with the nucleotide sequence of recorded P36 gene of *M. hyopneumoniae* 7448 in GenBank (accession No. AE017244.1).

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P36          1     AGRPQKPGETRLELVADNIRIIREIALKVKESGFSGISIIVANPVDIITRAYRDASGFSD  60
AAZ53511.1   79    AGRPQKPGETRLELVADNIRIIREIALKVKESGFSGISIIVANPVDIITRAYRDASGFSD  138

P36          61    QKVIGSGTVLDTARLQFAIAKRAKVSPNSVQAYVMGEHGDFSFVAYSNIKIAGECFCAYS  120
AAZ53511.1   139   QKVIGSGTVLDTARLQFAIAKRAKVSPNSVQAYVMGEHGDSSFVAYSNIKIAGECFCAYS  198

P36          121   KLTGINSSNYEKELEYPVSRRAYE  144
AAZ53511.1   199   KLTGIDSSNYEKELEYPVSRRAYE  222
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Figure 4: Comparison of amino acid sequence of recombinant P36 protein from *M. hyopneumoniae* with the amino acid sequence of recorded P36 protein of *M. hyopneumoniae* 7448 in GenBank (accession No. AAZ53511.1).

Together, these results demonstrated that the gene encoding the *M. hyopneumoniae* P36 protein was successfully isolated, cloned and transformed into strain *E. coli* top 10.

Expression and purification of the recombinant *M. hyopneumoniae* P36 protein in *E. coli* BL21(DE3)

Expression of the *M. hyopneumoniae* P36 protein in strain *E. coli* BL21 on LB culture with IPTG induction is expected to produce a recombinant protein with molecular weights of approximately 20 kDa. On 15% SDS-PAGE, the expressed recombinant *M. hyopneumoniae* P36 protein was not found in soluble fraction (supernatant) of transformed *E. coli* BL21 cells with 0.8 mM IPTG induction (Figure 5, Lane 1), but found to be inclusion body form in the pellet of bacterial cell lysates (Figure 5, Lane 2). A prominent band of fusion protein with a size of approximately 20 kDa could be seen clearly on the gel. This size of the expressed protein is equivalent to the calculated protein size of approximately 20 kDa including 15.73 kDa of the *M. hyopneumoniae* P36 protein and 3.7 kDa of 6x His-tag of the vector pET28a. These results indicated that the expression of recombinant *M. hyopneumoniae* P36 protein in *E coli* BL21 was successful, and expressed protein was in inclusion body form. The molecular weight of expressed *M. hyopneumoniae* P36 protein in this study was approximately 20 kDa, which is different from 36 kDa of P36 protein of *M. hyopneumoniae* expressed in *E. coli* according to a report of Strasser et al. (1991). The smaller molecular weight of expressed *M. hyopneumoniae* P36 protein in this study could be explained by the cloning of shorter *M. hyopneumoniae* P36 gene (432 bp). The analysis of purified recombinant protein on 15% SDS-PAGE obtained clear and sharp bands (Figure 5, Lanes 3-4), proving that the purification of recombinant *M. hyopneumoniae* P36 protein by ProBond™ Purification System Kit was successful. Furthermore, the purified recombinant fusion protein with a size of approximately 20 kDa was recognized by anti-6X His-tag® antibody (data not shown), confirming that the expressed protein was recombinant *M. hyopneumoniae* P36 protein.

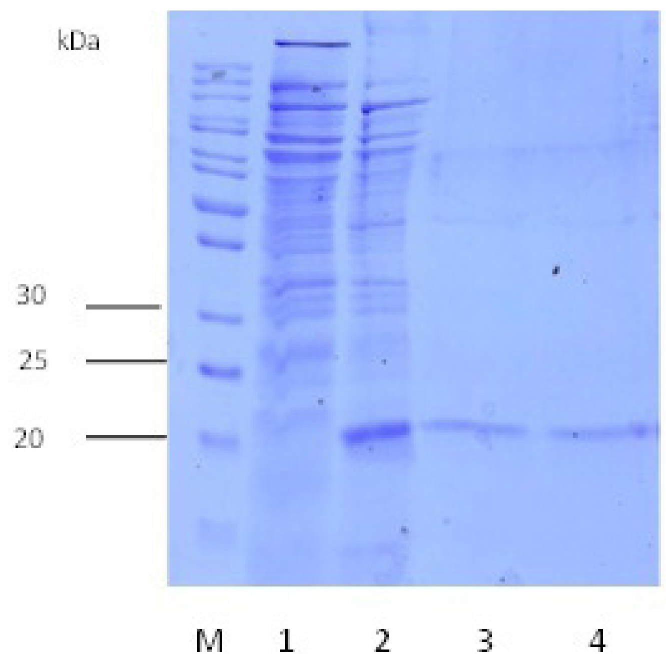

Figure 5: SDS-PAGE analysis of the expression of recombinant *M. hyopneumoniae* P36 protein in *E. coli* BL21(DE3) cells on LB medium. M: Protein weight marker (10-200 kDa, Bio Basic); Lane 1: Soluble P36 protein (in supernatant) of transformed *E. coli* BL21(DE3) cells induced with 0.8 mM IPTG when OD_{600} of the cultures reached 0.8 and incubated at 37°C, 150 rpm for 8 h post-induction; Lane 2: Inclusion body proteins of transformed *E. coli* BL21(DE3) cells in the same condition of expression; Lanes 3-4: Purified recombinant *M. hyopneumoniae* P36 protein.

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Evaluating antigenicity of the recombinant *M. hyopneumoniae* P36 protein by ELISA

The antigenicity of expressed recombinant *M. hyopneumoniae* P36 protein was evaluated by reacting the sera taken from 4 pigs that were immunized with the PEP inactivated vaccine (HYOGEN®) with the expressed recombinant P36 protein of *M. hyopneumoniae* by ELISA. The result presented in Figure 6 showed that wells 1-4 coated with the recombinant *M. hyopneumoniae* P36 protein were positive for antibodies in sera taken from 4 pigs immunized with a PEP inactivated vaccine (HYOGEN®), that mean one hundred percent (4 of 4 wells) of recombinant *M. hyopneumoniae* P36 protein samples tested by ELISA were strongly recognized by sera taken from 4 pigs immunized with a PEP inactivated vaccine (HYOGEN®). The above results determined that recombinant *M. hyopneumoniae* P36 protein has antigenicity and is specific to the *M. hyopneumoniae* antibodies. However, further experiments should be performed to evaluate the immunogenicity of the recombinant *M. hyopneumoniae* P36 protein in pigs as well as in laying hen to confirm potential for use as an antigen to develop novel vaccines and bio-products (e.g., egg yolk antibody) for prevention and treatment of the PEP.

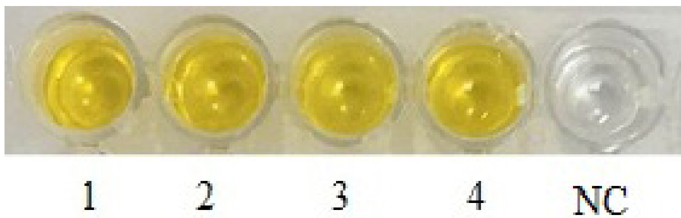

Figure 6: Evaluation of antigenicity of recombinant *M. hyopneumoniae* P36 protein by ELISA. Wells 1-4 coated with the recombinant *M. hyopneumoniae* P36 protein (20µg/mL); NC: uncoated with the recombinant *M. hyopneumoniae* P36 protein (coating buffer).

CONCLUSIONS AND RECOMMENDATIONS

In the current study, we have successfuly cloned the gene with 432 bp in length encoding the *M. hyopneumoniae* P36 protein and expressed this protein in *E. coli* BL21 cells. The expressed recombinant *M. hyopneumoniae* P36 protein has the antigenicity and is specific for *M. hyopneumoniae*. However, it is needed to perform further experiments to evaluate the immunogenicity of *M. hyopneumoniae* P36 protein for use as an antigen to develop novel vaccines and bio-products (e.g., egg yolk antibody) against *M. hyopneumoniae*.

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NOVELTY STATEMENT

The novelty of this research is to successfully clone and express in E. coli BL21 a novel gene of M. hyopneumoniae with 432 bp in length for the recombinant P36 protein with antigenicity and specificity for M. hyopneumoniae as a base for further studies to develop novel vaccines and bio-products against M. hyopneumoniae.

AUTHORS CONTRIBUTION

Nguyen Thi Thu Hien, Dong Huu Rin and Le Viet Tuan Khanh were responsible for the designation and performance of experiments, and for writing the draft of manuscript. Nguyen Xuan Hoa and Dinh Thi Bich Lan provided guidance and supported to carry out the experiments. Phung Thang Long interpreted the data, and edited the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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