Hue University Journal of Science: Agriculture and Rural Development
pISSN: 2588-1191; eISSN: 2615-9708
Vol. 132, No. 3C, 2023, P. 5–17, DOI: 10.26459/hueunijard.v132i3C.7060

CLONING AND EXPRESSION OF GENE ENCODING P46 ANTIGEN OF *MYCOPLASMA HYOPNEUMONIAE* IN *ESCHERICHIA COLI*

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(*Received: December 19, 2022; Accepted: March 13, 2023*)

Abstract. Enzootic pneumonia is a highly contagious chronic respiratory disease in pigs caused by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) worldwide. P46 of *M. hyopneumoniae* is considered one of the main surface antigen proteins. This study aims to successfully clone and express P46 of *M. hyopneumoniae* in *Escherichia coli* M15 cells. The gene encoding P46 of *M. hyopneumoniae* was isolated from lung samples of infected pigs raised in Thua Thien Hue province, Vietnam, and cloned into vector pGEM®-T Easy. The PCR product digested by KpnI and BamHI enzymes was cloned into the pQE30 and expressed in *E. coli* M15 cells. The results of sequencing show that the nucleotide sequence of the cloned gene encoding P46 antigen protein has a length of 456 bp, corresponding to 152 amino acid residues and 99.34% similarity to the polypeptide chain of recorded *M. hyopneumoniae* P46 protein in GenBank (accession number: AAZ53879.1). The results of sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) analysis show that the molecular mass of the expressed protein is approximately 20 kDa, and anti-histag ELISA confirms that the expressed protein is the 6xHis-P46 recombinant protein.

Keywords: *Mycoplasma hyopneumoniae*, porcine enzootic pneumonia, P46, cloning, expression of protein, recombinant protein

1 Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is a primary etiological agent of porcine enzootic pneumonia (PEP), a chronic respiratory disease that affects pigs of all ages worldwide [1]. The disease occurs in growing and adult pigs and is characterized by chronic non-productive coughing, poor growth rate, high morbidity and low mortality, thus causing considerable economic losses in the pig industries [2, 3]. In addition, pigs with PEP are predisposed to secondary infections caused by porcine reproductive and respiratory syndrome viruses [4], influenza viruses [5], and other pathogens [6, 3]. Porcine enzootic pneumonia prophylaxis comprises the use of antibiotics, management procedures, and vaccination [7, 8]. However, a

long-term use of antibiotics for disease treatment increases the risk of antibiotic-resistant bacteria and causes antibiotic residues to remain in products, thus affecting the health of consumers. On the other hand, the currently used commercial vaccines are mainly inactivated and attenuated live vaccines and provide only partial protection [4, 9]. Therefore, it is necessary to develop alternative bio-active products to more effectively prevent and treat the PEP caused by *M. hyopneumoniae* bacteria.

P46 is a membrane surface and species-specific immunogenic protein strongly recognized by convalescent pig sera [10–12] and considered a promising candidate for developing a vaccine [13, 12]. This protein is also an essential material source for producing bio-active products against PEP.

The objective of this study is to successfully clone and express P46 of *M. hyopneumoniae* in *Escherichia coli* M15 cells to create an initial material source for developing effective solutions for the prevention and treatment of the PEP caused by *M. hyopneumoniae* bacteria.

2 Materials and methods

Bacterial strains

Escherichia coli M15 strains (Qiagen) were used to express the recombinant P46 antigen protein. The *E. coli* TOP10 (Invitrogen, USA) strain was used for all of the cloning steps.

Sample collection, plasmids and culture media

The fresh lung tissue samples of pigs with PEP from herds that had not been vaccinated were taken. The pGEM®-T Easy vector (Promega, USA) and pQE30 (Qiagen) were used as cloning and expression systems. For plasmid DNA amplification and recombinant protein production, *E. coli* TOP10 (Invitrogen, USA) and *E. coli* M15 (Qiagen, USA) strains were utilized as hosts. The LB and YJ culture media were used for the incubation and expression of the target recombinant protein.

DNA isolation and PCR amplification of P46 gene

The genomic DNA of *M. hyopneumoniae* was isolated by using the QIAamp DNA Mini Kit (Qiagen) and used as a template in PCR amplification. The primer pairs were designed based on the coding DNA sequence of the P46 antigen available from the NCBI gene database (http://www.ncbi.nlm.nih.gov). The nucleotide composition of the gene-specific primer pairs was designed in the study by Virgino et al. [14], and the BamHI and KpnI restriction enzymes allowed the subsequent cloning into the pQE30 expression vector (Qiagen).

Table 1. Primer 16S rRNA and P46

Gene	Primer	Sequence	Length (bp)
16SrRNA	Mh.F	5′-GAGCCTTCAAGCTTCACCAAGA-3′	649
	Mh.R	5′-TGTGTTAGTGACTTTTGCCACC-3′	
P46	P46.F	5'- CGCGGATCCCTCACTCAGCAAGCTAATTT-3'	456
	P46.R	5'- GCGGTACCTCCTGGGACATAAACAGC -3	

* The underlined nucleotides were the restriction enzyme sites added to the primers for the subsequent digestion.

As components of the PCR reaction for the identification of *M. hyopneumoniae* positive samples and the isolation of the gene encoding the protein P46, 25 μL of the PCR reaction mixture consisting of 2 μL DNA template (50 ng/μL), 12.5 μL 2x PCR master mix (Promega), 1 μL each primer (10 pmol/μL), and 8.5 μL distilled water was prepared, and a thermocycler (iCycler thermocycler, Bio-Rad, Hercules, CA) was used. The PCR product was tested by using 1% (w/v) agarose gel electrophoresis and stained with Redsafe TM (20.000x) with a supply potential of 70 V in a TAE buffer (40 mM Tris, pH 7.6, 20 mM acetic acid, and 1 mM EDTA). The electrophoresis images were then analyzed by using a Gelsmart (DLAB-USA).

Cloning and sequencing of P46 gene

The PCR products were purified from agarose gels by using the TopPure® PCR/Gel DNA purification kit (ABT, Vietnam) and then inserted into the pGEM®-T Easy vector according to the TA cloning method. The reaction components that attached the gene encoding the P46 protein to the pGEM®-T Easy vector were incubated at 4 °C overnight and then transformed into *E. coli* TOP 10 cells with the heat-shock method. Then, the recombinant transformed cells were spread on an LB plate with 100 μg/mL ampicillin, and 20 mg/mL X-gal, and 0.1 M IPTG for the selection of recombinant clones [15]. Positive colonies were identified by using PCR with the P46 primer pairs and the M13 primer pairs (M13F: 5'-GTTTTCCCAGTCACGAC-3' and M13R: 5'-CAGGAAACAGCTATGAC-3') that were pre-designed on the pGEM®-T Easy vector.

The positive colony was cultured in 5 mL of a liquid LB medium with 100 μg/mL ampicillin at 37 °C overnight, and then the recombinant pGEM®-T Easy/P46 vector was extracted with the Isolate II Plasmid Mini Kit (Bioline). The recombinant plasmid was sequenced at The Institute of DNA Technology and Genetic Analysis (Vietnam). The results were analyzed with BioEdit software and compared with published sequences on the NCBI GenBank.

Expression of recombinant P46 protein

The recombinant plasmids from the recombinant pGEM®-T Easy vector were purified by using the TopPure® PCR/Gel DNA purification kit (ABT, Vietnam) and digested with *Bam*HI and *KpnI* restriction enzymes to obtain a P46 fragment that was cloned into the pQE30 expression vector harbouring promoter T5, previously digested with the same restriction enzymes, and incubated at 25 °C for 4 h and at 4 °C overnight. The recombinant pQE30 vector was transformed into *E. coli* M15 cells with the heat shock method (at 42 °C for 70 sec). The transformed product was cultured on LB plates with 100 μg/mL kanamycin and 100 μg/mL ampicillin and incubated at 37 °C overnight for the selection of recombinant clones.

The transformation results were confirmed by using PCR with P46-specific primers and T5 primers pre-designed on the pQE30 vector.

The positive colony was grown in 50 mL of the YJ culture medium at 250 rpm to an optical density of 0.8 measured at 600 nm (OD_{600}). IPTG was added to obtain a final concentration of 1 mM for induction, and the IPTG-induced culture was then incubated for another 8 h at 250 rpm and 40 °C. The cell biomass was centrifuged at 6000 rpm and 4 °C for 10 min, and the pellet was resuspended in an adequate volume of TNE buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4) containing 1% Triton X-100 and 0.008 mg/mL lysozyme and maintained on an ice-water bath for 1 h. After sonication for 5 min, the culture was centrifuged at 6000 rpm and 4 °C for 10 min, and the supernatant and pellets were collected for the identification of target protein localization. The inclusion body proteins in the pellets were solubilized with a urea buffer (8 M) in a rotary shaker at 150 rpm and 30 °C for 2 h, then centrifuged at 6000 rpm for 15 min to collect the supernatant. An equal volume from each sample was mixed with a sample loading buffer, heated at 100 °C for 5 min, and analyzed with 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant P46 protein was purified with the ProBond™ Purification System Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Protein expression levels were analyzed with the electrophoresis method on 15% SDS-PAGE, and the voltage was 60 V. The gel was then stained with Coomassie Blue R-250, and the image was analyzed with Gelsmart (DLAB-USA).

ELISA analysis

The enzyme-linked immunosorbent assay (ELISA) was used to confirm the presence of the recombinant P46 protein. In brief, 100 μL of purified recombinant P46 protein diluted in a coating buffer (Sigma-Aldrich, USA) (20 μg/mL) was transferred into wells and incubated at 4 °C overnight. After washing wells three times with 200 μL Wash Solution Buffer, we added 200 μL of Blocking solution (BSA 1%) to each well and incubated at room temperature for 2 h. After

washing all wells three times, we added 100 μL of HRP Anti-6X His-tag® antibody (Abcam, UK) (diluted 1:5000) to each well and incubated at room temperature for 2 h. Next, we washed with Wash Solution Buffer six times, added 100 μL/well TMB substrate (Abcam, UK), and incubated for 10 min. Finally, we added 50 μL of stop solution (HCl 3 M) to each well and read the results.

3 Results and discussion

3.1 Isolation of the gene encoding the P46 antigen of *M. Hyopneumoniae*

Polymerase chain reaction was performed by using the genomic DNA of fresh lung tissue samples from pigs suffering from PEP as a template. The specificity test shows that gene P46 encoding the antigen of *M. hyopneumoniae* on a 1% agarose gel is a prominent single, clear, high-concentration DNA band with a length of approximately 456 bp on the standard Bioline DNA scale. The PCR amplification of the peptide coding sequence of the P46 gene fragment is shown in Figure 1. No other amplification fragments are observed in the figure.

This demonstrates that the diagnosis, from clinical to lesions and extraction to primer design, is completely accurate and in accordance with the research process. Besides, the obtained PCR product results correspond to the original calculated size. It confirms that the P46 protein-coding gene was successfully isolated.

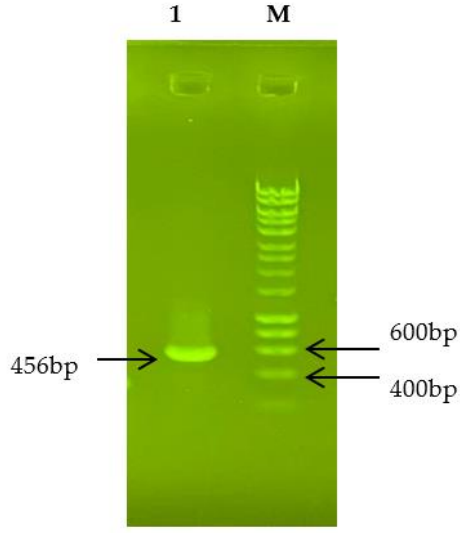

Figure 1. PCR amplification of the peptide-coding sequence of the P46 gene fragment from genomic DNA isolated from fresh lung tissue samples of pigs suffering from PEP in Thua Thien Hue province, Vietnam, M: hyperladder 1 Kb (Bioline); 1: PCR product of the P46 gene isolated from DNA obtained from infected pig lungs with specific primers

3.2 Cloning and sequencing of the P46 gene

The PCR product was purified from agarose gels and inserted into the pGEM®-T Easy vector. Then, it was transformed into chemically competent *E. coli* Top 10 cells. The presence of DNA bound to the vector and transformed into bacterial cells was determined by using PCR amplification with the M13 primer pairs of the pGEM®-T Easy vector and electrophoresis on a 1% agarose gel. The results of PCR show that the pGEM®-T Easy/P46 vector was successfully transformed into *E. coli* TOP 10 cells, and the DNA band is a single, bold, clear band with a length of 656 bp, corresponding to the expected length of the insert of 456 bp and 200 bp of primer pairs in the vector (Figure 2).

The results of sequencing the nucleotide sequence and analysis of the amino acid sequence of the P46 gene from *M. hyopneumoniae* isolated from Thua Thien Hue province, Vietnam, are shown in Figure 3. It can be seen that the P46 gene consists of 456 nucleotides (from nucleotide position 680575 to 681030) as 98.9% homologous to the gene sequence of *M. hyopneumoniae* 7448 on the NCBI (Code AE017244.1) (Figure 3).

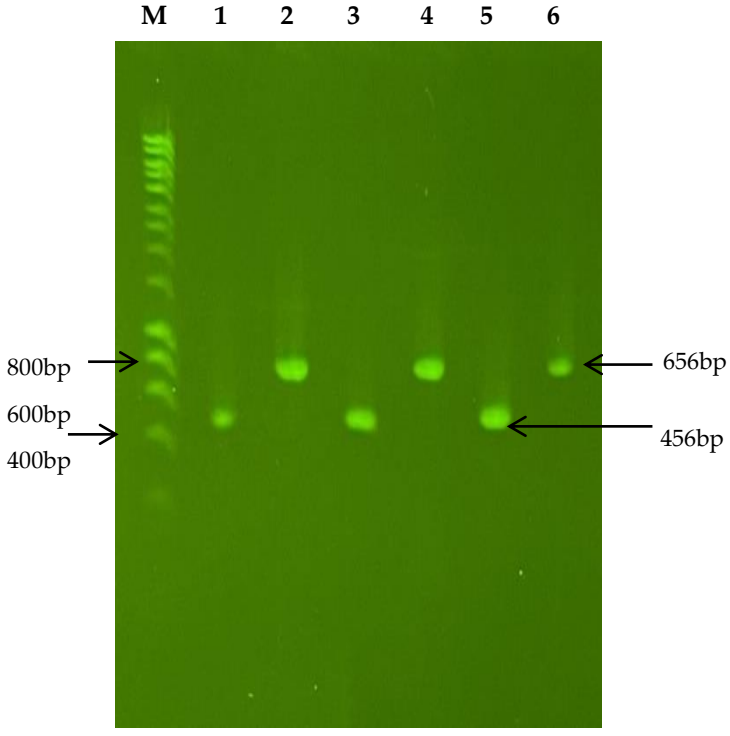

Figure 2. PCR results were used to identify colonies carrying the recombinant vector pGEM/P46. Lane M: hyperladder 1 Kb (bioline); Lanes 1, 3, 5: PCR products with P46 primer pairs; Lanes 2, 4, 6: PCR products with M13 primer pairs

Figure 3. Comparison of the nucleotide sequence of the P46 antigen coding segment with the gene sequence of M. hyopneumoniae 7448 (AE017244.1). The sequences of *M. hyopneumoniae* 7448 are shown on the top line and the P46 sequences are at the bottom. Vertical lines indicate identical nucleotides; the parts of dissimilar nucleotides are indicated by bolding in site 31, 111, 400, and 414 of the P46 sequence.

In this study, the simulated amino acid sequence from the P46 gene obtained above is a 152 amino acid long peptide fragment that exhibits a 99.34% similarity to the polypeptide chain of recorded *M. hyopneumoniae* P46 protein in GenBank (accession number: AAZ53879.1) (Figure 4).

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P46            1 LTQQANLSPAPKGFIIAPENGSGVGTAVNTIADKGIPIVAYDRLITGSDKYDWYVSFDNE  60
AAZ53879.1   102 LTQQANLSPAPKGFIIAPENGSGVGTAVNTIADKGIPIVAYDRLITGSDKYDWYVSFDNE 161
P46           61  KVGELQGLSLAAGLLGKEDGAFDSIDQMNEYLKSHMPQETISFYTIAGSQDDNNSQYFYN  120
AAZ53879.1   162 KVGELQGLSLAAGLLGKEDGAFDSIDQMNEYLKSHMPQETISFYTIAGSQDDNNSQYFYN  221
P46           121  GAMKVLKELMKNSQNKIIDLSPEGENAVYVPG  152
AAZ53879.1    222  GAMKVLKELMKNSGNKIIDLSPEGENAVYVPG  253
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Figure 4. Comparison of amino acid sequences derived from the P46 antigen coding gene with the amino acid sequence of the P46 of *M. hyopneumoniae* in GenBank (accession number: *AAZ53879.1*)

3.3 Expression and purification of recombinant P46 protein in *E. coli* M15

The PCR product was then attached to the pQE30 vector before transforming with a heat shock into *E. coli* M15 cells (at 42 °C for 70 s) cultured on a selective medium to find positive colonies, and the transformation results were confirmed by PCR.

The results presented in Figure 5 show that the tested colonies are all positive; the amplified DNA bands are about 456 bp in size, a PCR product of the P46 primer pair, and are about 656 bp with the T5 primer pair. This proves that the gene encoding the P46 antigen was successfully cloned into the pQE30 vector in *E. coli* M15 cells. The expression of recombinant

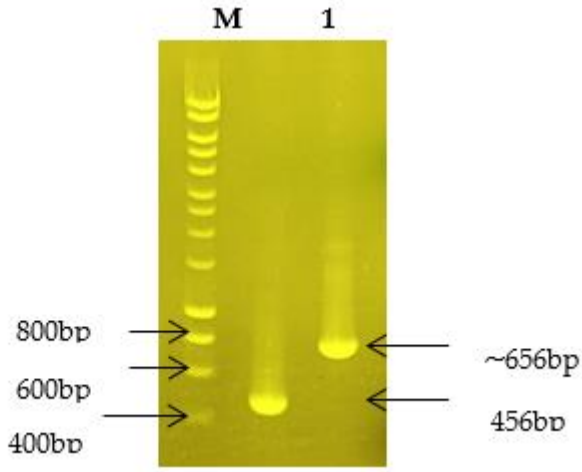

Figure 5. PCR product electrophoresis results determine the presence of recombinant plasmid pQE30/P46 in M15 cells. M: hyperladder 1 Kb (Bioline); Lane 1: PCR product with specific primers for P46 gene; Lane 2: PCR product with T5 primer pair

P46 protein in *E. coli* M15 cells on YJ culture with 1 mM IPTG induction was analyzed on 15% SDS-PAGE (Figure 6).

The SDS-PAGE analysis reveals that the expressed P46 protein is not present in the protein sample of transformed *E. coli* M15 cells without IPTG (Lane 1) and the supernatant protein induced by IPTG (Lane 2), but it was present in the inclusion body of bacterial cell lysates of *E. coli* M15 cells induced by IPTG induction (Lane 3). A prominent protein band of approximately 20 kDa corresponds to the expected size. The theoretically calculated size of the isolated P46 protein is about 16.49 kDa (according to the online software tool EXPASY), and the 6X His-tag of the vector pQE30 with a mass of about 3.7 kDa forms a recombinant P46 protein with a mass of about 20 kDa. The results indicate that the expressed protein is correct, and the urea buffer (8 M) is efficacious in solubilizing inclusion body protein. In addition, for IPTG uninduced transformed *E. coli* M15 cells, no dark protein bands of expected size are observed (Lane 1), suggesting that induction with IPTG is required for the expression of P46 protein.

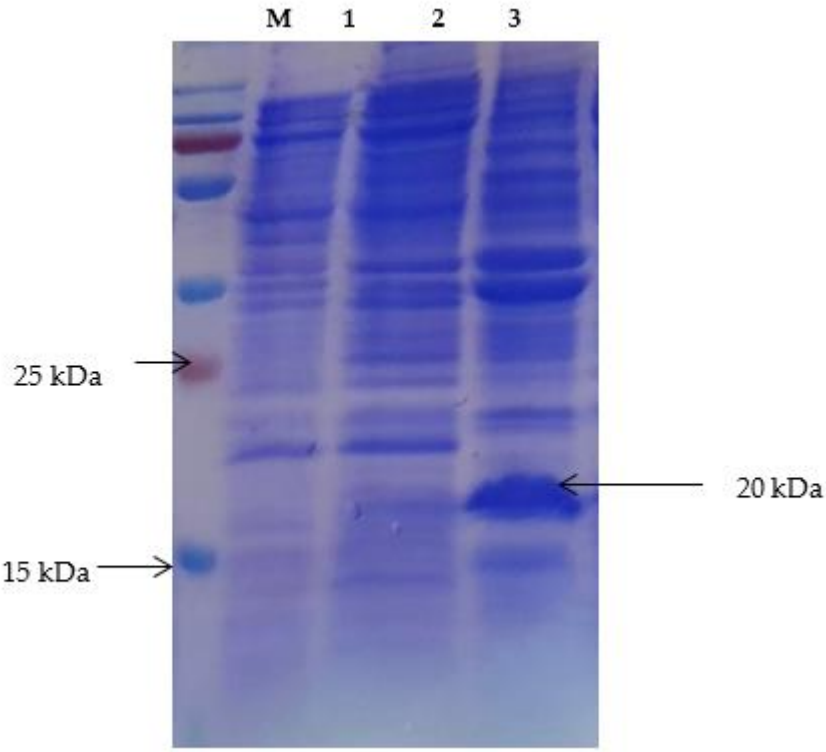

Figure 6. Expression of recombinant antigen 6xHis-P46 in *E. coli* M15. M: Marker 10-200 kDa (Thermo fisher). Lane 1: The protein P46 sample obtained from *E. coli* M15 cells carrying the recombinant vector pQE30/P46 was not induced by IPTG. Lane 2, 3: The extracted protein sample (supernatant and inclusion body) from *E. coli* M15 cells carrying the recombinant vector pQE30/P46 was induced by IPTG.

In Figure 7, the SDS-PAGE analysis also shows that the purification of the recombinant P46 protein was achieved by using the ProBondTM Purification System Kit (lanes 8, 9, and 10) in these lanes, and the pH of the denaturing elution buffer is stable, so there are not accompanying by-products (small bands above). The purified recombinant fusion protein with a size of approximately 20 kDa is recognized with an HRP Anti-6X His-tag® antibody, confirming that the expressed protein is a recombinant P46 protein.

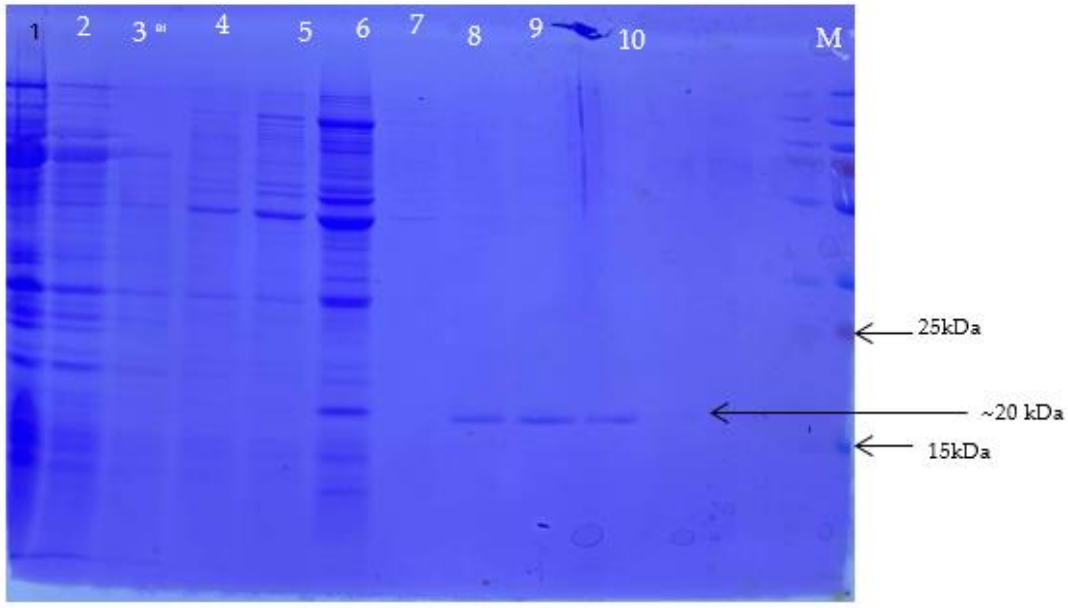

Figure 7. Purification of recombinant 6xHis-P46 antigen from inclusion bodies M: Marker 10–200 kDa (Thermo fisher); Lane 1: The fluid obtained after passing the recombinant protein solution through the gel. Lane 2, 3: The results of washing the gel with Denaturing Binding Buffer. Lanes 4–7: The solutions are obtained after washing the gel with Denaturing Wash Buffer. Lanes 8–10: Liquids obtained after washing gel with Denaturing Elution Buffer.

3.4 Recombinant protein P46 specificity test results

The results of testing the specificity of the recombinant protein P46 by direct ELISA are shown in Figure 8.

The results presented in Figure 8 show that wells 1–6, coated with the recombinant antigen P46, are positive for HRP anti-6X His-tag® antibody, but their concentrations are lower than that of the positive control. These results confirm that the recombinant P46 is successfully expressed in *E. coli* M15 cells and has specificity for the *Anti-6X His* antibody. Therefore, the recombinant

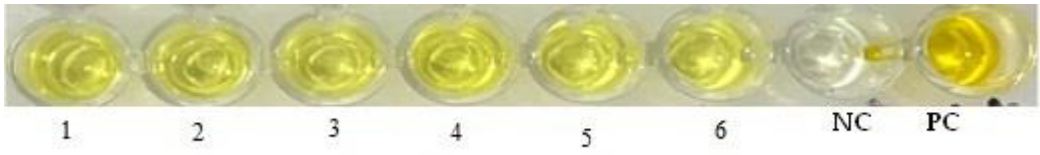

Figure 8. *The Elisa analysis of recombinant protein P46 result, Lane 1-6* coated with the recombinant antigen P46 (20 μg/mL); *NC: un*coated with the recombinant antigen P46*; PC: the pre-prepared anti-6X His positive control* (20 μg/mL)

P46 antigen can be used to develop bio-active products for preventing and treating PEP in coming trials.

4 Conclusions

We successfully cloned the gene encoding the P46 antigen protein of *M. hyopneumoniae* isolated from infected pigs and expressed the recombinant P46 protein in *E. coli* M15 cells. The nucleotide sequence of the gene encoding the P46 antigen protein is 456 bp in length, corresponding to 152 amino acid residues with a 99.34% similarity to the polypeptide chain of recorded *M. hyopneumoniae* P46 protein on GenBank (accession number: AAZ53879.1). The molecular mass of the P46 protein is approximately 20 kDa. The expressed protein is the 6xHis-P46 recombinant protein of *M. Hyopneumoniae*, confirmed by using anti-histag ELISA. This P46 antigen protein can be used to develop bio-active products for the prevention and treatment of PEP in coming trials.

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