Identification of Mycoplasma hyorhinis P37 protein-specific B cell linear epitopes using monoclonal antibodies against baculovirus-expressed P37 protein

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Abstract

Background

Mycoplasma hyorhinis (Mhr) is the etiologic agent of lameness and polyserositis in swine. Mhr P37 is a membrane protein that may play a critical role in immunity. It is a potential target for diagnostic development, but there is little information concerning its B cell epitopes. To investigate the epitopes of Mhr P37, a recombinant protein was developed in a baculovirus system using monoclonal antibodies (mAbs) prepared against P37 protein.

Results

Western blot and indirect immunofluorescence assays (IFA) confirmed that the expressed P37 protein was recognized by Mhr-positive porcine and mouse sera. Furthermore, the P37 protein was purified using affinity chromatography and used to immunize mice for hybridoma cell fusion. Four mAbs were found to be positive for Mhr. A panel of truncated P37 proteins was used to identify the minimal B cell linear epitopes of the protein based on these mAbs. The core epitope was determined to be 206KIKKAWNDKDWTFRNF222.

Conclusions

This study identified mAbs that could provide useful tools for investigating the antigenic structure and function of the Mhr P37 protein as well as its application to diagnostic techniques.

Background

Mycoplasma hyorhinis (Mhr) is first isolated in 1953 and found to lack a cell wall [1]. It is a commensal microorganism that inhabits the upper respiratory tract of swine [2]. Mhr infections in pigs can cause lameness and polyserositis, and severe infections can cause pneumonia [3]. Systemic infection caused by Mhr is found on pig farms worldwide and is characterized by high morbidity and low mortality rates [4, 5]. At present, Mhr infection
detection mainly depends on pathogen isolation and culture and polymerase chain reaction (PCR) methods, and there is no commercially available kit for serological detection [6]. Although Mhr is easily isolated from porcine alveolar lavage fluid and nasal swabs, the process of isolation and identification of Mhr is often time consuming [7]. Mhr has also been proven to be a zoonotic disease that promotes the malignancy of tumor cells, such as in gastric cancer, and antibodies against Mhr P37 protein can inhibit the migration of infected cells [8, 9].

Mycoplasma surface lipoproteins have been shown to perform a variety of functions during infection and interactions with the host [10]. P37 is an important membrane protein of Mhr and is part of the periplasmic binding protein-dependent transport system [11, 12]. P37 may play a role in tumor invasion, and detection of antibodies against P37 in human serum may help diagnose cancer [13, 14].

Previously, the P37 protein was used as a coating antigen to measure the immunoglobulin G (IgG) responses in swine vaccinated with an inactivated Mhr vaccine [15]. However, the mechanism by which host antibodies to P37 are produced, their function in the process of infection, and the precise epitope of P37 is still unknown.

In this study, the P37 protein was expressed using a baculovirus expression system. Specific antibodies were obtained from the expressed P37 protein, and these antibodies were used to analyze the key amino acids and core epitope of the antigens. Analysis of the antigenic epitope of the P37 protein will facilitate the development of effective tools for serological diagnostic techniques.

Results

Identification of recombinant plasmid and shuttle plasmid

The recombinant plasmid pFastBac™ 1-His-P37 was identified by dual-restriction
endonuclease digestion with *Bam*H I and *Xho* I, and the 4693 bp vector fragment and the 1140 bp target gene fragment were visualized by 1% agarose gel electrophoresis (Fig. 2A). pFastBac™1-His-P37 was specifically amplified using M13 primers, and a 3440 bp band was obtained on a 1% agarose gel. The negative control pFastBac™1 was observed as a 2300 bp fragment (Fig. 2B).

**Detection and purification of recombinant protein**

Using fluorescence microscopy, strong fluorescence was observed in insect cells infected with pFastBac™1-His-P37 (Fig. 3A), whereas no fluorescence was observed in uninfected cells (Fig. 3B), indicating that the P37 protein was successfully expressed in insect cells. Western blot analysis showed that the protein reacted with the prepared anti-Mhr mouse positive serum, and a specific reaction band appeared at approximately 43.3 kDa (Fig. 4). We speculate that the P37 protein was modified in eukaryotic cells.

**Characterization of P37 protein-specific mAbs**

The four positive mAbs were designated C6, C8, E1, and E6. Subtype identification results showed that the heavy chain subtype of all four mAbs was IgG1, and the light chain subtype of all mAbs was kappa (Table 2). Reactivity analysis of mAbs to Mhr showed that the Mhr strain reacted specifically with the four mAbs, and a specific reaction band appeared at a position of 43.3 kDa (Fig. 5).

**Identification of P37 protein B cell linear epitopes using P37-specific mAbs**

To determine the epitopes of the four generated mAbs, a series of overlapping peptides were analyzed by Western blot analysis. The results showed the presence of specific bands at aa128–254, aa171–254, and aa199–226 (Fig. 6). Because the four mAbs were identical in reactivity, only one result is shown here.

To confirm the epitopes of the mAbs, Western blot analysis was performed after mutating
Analysis of P37 protein from different Mhr strains

Analysis of the p37 sequence of seven Mhr strains demonstrated that the epitope $^{206}\text{KIKKAWNDKDWNTFRNF}^{222}$ was highly conserved (100% aa identity, Fig. 8).

Homology modeling of the P37 protein epitope

The spatial structure of the P37 protein from aa1–379 was predicted using three-dimensional homology modeling. Model analysis showed that the overall shape of P37 is an irregular prolate ellipsoid, and the core epitope domain consists of two $\alpha$-helices and a nonregular coil (Figs. 9A and 9B). The epitope region where the antigen-antibody reaction was detected was located on the surface of the P37 protein (Fig. 9C).

Discussion

Mhr is the most common mycoplasma in clinical farms [16]. Typical lesions found in infected animals include serum fibrotic pleurisy, otitis media, pericarditis and peritonitis, which may cause fibrous adhesions during the chronic phase [17, 18]. Most pigs infected with Mhr do not show significant clinical symptoms, which may contribute to several swine disease complexes. Therefore, accurate diagnosis is critical to establishing effective treatment and prevention measures in affected herds. A previous study reported an ELISA method using Tween-20-extracted membrane protein as an antigen for detecting serum antibodies in pigs after Mhr challenge; however, this method showed cross-reactivity between mycoplasmas [19]. The high-resolution structure of Mh-p37 solved using the new heavy-atom derivative I3C has contributed to better understanding of the biological significance of P37 in tumorigenesis and cancer progression [20]. P37 is the major
antigenic protein in Mhr, it is an ideal target for the development of diagnostic techniques. Therefore, we expressed the P37 protein in a baculovirus system. Western blot analysis revealed that the P37 protein was modified during expression; however, the mechanism of P37 protein modification is unknown and requires further study. A linear B cell epitope (aa206–222, 206KIKKAWNDKDWNTRNF222) was identified as specific to the P37 protein and was highly conserved among Mhr strains. Structural predictions revealed that the epitope consists of an α-helix, a nonregular coil, and a second α-helix. The functional part of the enzyme was often in the conformational region of the nonregular coil, although its specific function requires further study. Analysis of P37 protein sequence revealed that this epitope region may be one of the predominant domains of the P37 protein. Therefore, these mAbs could provide useful tools for investigating the antigenic structure and function of Mhr P37 protein as well as its application in diagnostic procedures.

Conclusions
This study identified the core epitope of P37 protein using a eukaryotic expression system. The epitope of P37 protein was 206KIKKAWNDKDWNTRNF222, and homology modeling analysis showed that this epitope consists of two α-helices and a nonregular coil. The epitope of P37 protein may be involved in the production of host antibodies, but further studies are required to develop new methods for the diagnosis and prevention of Mhr infection.

Methods

Pathogens, plasmids, and cells
The previously described Mhr-DL strain (CGMCC No. 11092) [21], used to amplify the p37 gene and for sequencing analysis, was grown in Friis medium [22], and the fifth
generation was used as the inoculant. Mhr was quantified using the color change unit (CCU) assay as previously described [23]. The baculovirus transfer vector pFastBac™1 (Invitrogen, Carlsbad, CA, USA) was used to construct the pFastBac™1-His-p37 plasmid, which expressed the complete P37 protein. The prokaryotic expression vector pGEX-6P-1 (GE Healthcare, Uppsala, Sweden) was used to express the truncated P37 protein. The insect cell line Spodoptera frugiperda (Sf21) (Invitrogen, Carlsbad, CA, USA) was cultured in Grace’s Insect Medium (Invitrogen) at 27°C. The SP2/0 myeloma cell line (ATCC® CRL-1581™) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Grand Island, NY, USA) with 10% inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, North Shore City, New Zealand) in a humidified incubator with 5% CO₂ at 37°C.

**Acquisition of the p37 gene and generation of recombinant baculovirus**

The p37 gene fragment was amplified by PCR with forward (5´-CGGGATCCATGCTGAAGAAGCTGAAG-3´) and reverse (5´-CCGCTCGAGTTACTTGATGGCCTTCTC-3´) primers designed using Premier 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA) based on the reference sequence (GenBank Accession No. X14140.1). The signal peptide sequence was removed from the p37 gene coding region and the p37 gene was optimized and synthesized by BGI Co. (Beijing, China). For protein purification, a 6× His-tag was fused to the NH₂-terminal end of the p37 gene. The gene sequence was optimized to obtain the highest possible level of expression, and the target gene (1140 bp) was subsequently cloned into the expression vector pFastBac™1 via two restriction sites (BamHI and XhoI).

The linearized baculovirus DNA was transformed into competent DH10Bac (Invitrogen) according to the manufacturer’s instructions. Identification of recombinant baculovirus was carried out using universal M13 primers (forward primer: 5´-GTTTTCCAGTCACGAC-3´,
reverse primer: 5'-CAGGAAACAGCTATGAC-3'). pFastBac™ 1-His-P37 was transfected into logarithmic phase Sf21 insect cells according to the Bac-to-Bac® Baculovirus Expression System instructions (Invitrogen). When the cells had obvious lesions, the cell supernatant was collected as the first generation recombinant baculovirus, designated P1-pFastBac™ 1-His-P37. A representative recombinant virus was used for expression studies.

Expression and purification of P37 protein

For P37 protein expression, three generations of high titer seed virus stocks were prepared by infecting Sf21 cells at a multiplicity of infection (MOI) of 0.1 plaque forming units (PFUs). Cells in 24-well plates were infected with pFastBac™ 1-His-P37 baculovirus, and uninfected Sf21 cells were used as mock control. The cell culture medium was aspirated after 60 h and fixed with 10% paraformaldehyde (500 μL per well) at room temperature for 15 min. The Sf21 cells were then fixed with 0.2% TritonX-100 for 10 min, washed three times with phosphate buffered saline (PBS), and incubated with mouse anti-Mhr serum (diluted 1:500 in PBS) at 37°C for 1 h. The cells were then washed three times with PBS, incubated with DyLight 488 AffiniPure Goat Anti-Mouse IgG (H+L) (diluted 1:500 in PBS; Pierce, Rockford, IL, USA) at 37°C for 1 h, and washed three times with PBS. The fluorescent signal was visualized with an EVOS inverted fluorescence microscope (Life Technologies, Carlsbad, CA, USA).

Sf21 cells infected with 5 MOI pFastBac™ 1-His-P37 virus were harvested 72 h post infection. The cells were suspended in PBS (1% of the original volume), lysed by ultrasonic lysis, centrifuged to remove the precipitate, and purified using Ni-NTA affinity chromatography (Genscript, Nanjing, China). The purified sample was mixed with 5× loading buffer at a ratio of 4:1 and boiled for 10 min. The expressed protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim
milk powder in PBS solution overnight at 4°C, incubated with anti-Mhr mouse serum (1:100) at room temperature for 1 h, and washed three times with PBS containing 0.5% Tween 20 (PBST). After incubation for 1 h at room temperature with DyLight™ 680-Labeled Antibody To Mouse IgG (H +L) (1:10000) (KPL, Gaithersburg, MD, USA), washed three times with PBST, and scanned using an Odyssey infrared imaging system (Licor Odyssey, Lincoln, NE, USA).

Preparation of mAbs against P37 protein

Four 6-week-old female BALB/c mice were purchased from the Laboratory Animal Center of Harbin Veterinary Research Institute, CAAS (Harbin, China). The mice were immunized with 80 μg of the purified P37 protein. Protein emulsification and immunization were performed as previously described [24]. Following the booster immunization, mice were intraperitoneally administered 80 μg of P37 protein without adjuvant at a 2-week interval. Mice were then euthanized with cervical dislocation 3 days later, and spleen cells were fused with SP2/0 cells as previously described [25]. The fused cells were mixed with DMEM medium containing hypoxanthine-aminopurine-thymidine (HAT) (Sigma-Aldrich, New York, NY, USA) and 20% FBS. Cells were cultured together and the media were replaced with selection medium containing hypoxanthine-thymidine (HT) (Sigma-Aldrich) and 10% FBS after 5 d [26]. Hybridoma supernatants were collected after 7 d and screened for the presence of Mhr-specific antibodies by indirect enzyme-linked immunosorbent assay (ELISA). The purified P37 protein was used as the coating antigen at a concentration of 5 ng/μL as previously described [27]. Positive hybridoma cells were cloned three times by limiting dilution and stored in liquid nitrogen.

Characterization of mAbs against P37 protein

The subtypes of monoclonal antibodies (mAbs) produced were determined using the SBA Clonotyping System-HRP kit (Southern Biotech, Birmingham, AL, USA) according to the
manufacturer’s instructions. The hybridoma supernatant was added as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody was used as the secondary antibody. Color development and screening were performed as described above. The reactivity of mAbs to Mhr was determined by Western blot analysis as described above.

**Preliminary identification of P37 protein B cell line epitopes using P37-specific mAbs**

To identify the epitopes of the mAbs produced against the P37 protein, a series of amino acid (aa) nucleotide sequences were cloned into the BamH I and Xho I sites of pGEX-6P-1 (Fig. 1). After sequencing, *Escherichia coli* BL-21 cells were transformed with the recombinant plasmids, which were induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 37°C with shaking for 6 h. Cultures were harvested and lysed, and lysates were analyzed by SDS-PAGE and Western blot. Lysate from induced pGEX-6p-1 *E. coli* BL-21 cells was used as the negative control. The prepared mAbs against the P37 protein were used as the primary antibody, and DyLight™ 680-Labeled Antibody to Mouse IgG (H + L) (1:10,000) was used as the secondary antibody. The plate was incubated for 1 h at room temperature in the dark, washed three times with PBST, and analyzed using the Odyssey infrared imaging system (Licor Odyssey).

**Precise localization of the P37 protein B cell epitope**

To define the minimal linear epitope of the P37 protein, aa199–226 was deleted one by one (listed in Table 1) and their corresponding peptides were assessed by Western blot as described above. The immunoreactivity of the core epitope to corresponding mAbs was then analyzed.

**Multiple alignment of P37 amino acid sequences**

Multiple alignments of aa sequences of the P37 protein of seven Mhr isolates (GenBank
accession Nos. CP002170.1, CP002669.1, CP003231.1, CP016817.1, NC_019552.1, NC_022807.1, and NZ_LS991950.1) were performed using the Clustal W method within DNASTAR software version 7.0 (https://www.dnastar.com/software/).

**Homology modeling of P37 epitopes**

Homology modeling of aa1–379 of the P37 protein was performed using SWISS-MODEL (https://www.swissmodel.expasy.org/interactive). The spatial locations of the identified P37 protein epitopes were determined by mapping the epitopes to a three-dimensional model of the P37 protein using Chimera 1.11.2 software (https://www.cgl.ucsf.edu/chimera/).

**Abbreviations**

Mhr: *Mycoplasma hyorhinis*; mAbs: monoclonal antibodies; IFA: Indirect immunofluorescence assays; PCR: Polymerase chain reaction; IgG: Immunoglobulin G; kDa: kilodalton; K: kappa; aa: amino acid; Lys: Lysine; Phe: Phenylalanine; CCU: color change unit; Sf21: *Spodoptera frugiperda*; DMEM: Dulbecco’s modified Eagle’s medium; FBS: fetal bovine serum; MOI: multiplicity of infection; PFUs: plaque forming units; PBS: phosphate buffered saline; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBST: PBS containing Tween 20; HAT hypoxanthine-aminopurine-thymidine; HT: hypoxanthine-thymidine; ELISA: Enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; IPTG: Isopropyl β-D-1-thiogalactopyranoside.

**Declarations**

**Ethics approval and consent to participate**

The experimental procedures used in this study were approved by the Animal Ethics Committee of the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (Heilongjiang-SYXX-2017-009) and were performed in accordance with the relevant animal ethics principles.
Consent for publication
Not applicable.

Availability of data and material
All data generated or analysed during this study are included in this published article.

Competing interests
The authors declare that they have no competing interests.

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Author contributions
HZZ carried out all studies, participated in study design, and drafted the manuscript. YWW contributed equally to this work. CML and LF conceived and designed the experiments and analyzed the results. LPH, DL, YXX, DLX, and HQB performed the experiments. All authors read and approved the final version of the manuscript.

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**Tables**

Table 1. Peptides used in this study and their corresponding amino acid sequences
| Peptide | Peptide sequence            |
|---------|----------------------------|
| B1      | NDETLAKIKKAWNDKDWNTFRNFGILH |
| B2      | DETLAKIKKAWNDKDWNTFRNFGILH  |
| B3      | ETLAKIKKAWNDKDWNTFRNFGILH   |
| B4      | TLAKIKKAWNDKDWNTFRNFGILH    |
| B5      | LAKIKKAWNDKDWNTFRNFGILH     |
| B6      | AKIKKAWNDKDWNTFRNFGILH      |
| B7      | KIKKAWNDKDWNTFRNFGILH       |
| B8      | IKKAWNDKDWNTFRNFGILH        |
| B9      | GNDETLAKIKKAWNDKDWNTFRNFGIL |
| B10     | GNDETLAKIKKAWNDKDWNTFRNFGI  |
| B11     | GNDETLAKIKKAWNDKDWNTFRNFG   |
| B12     | GNDETLAKIKKAWNDKDWNTFRNF    |
| B13     | GNDETLAKIKKAWNDKDWNTFRN     |
| B14     | GNDETLAKIKKAWNDKDWNTRF      |
| B15     | GNDETLAKIKKAWNDKDWNTR       |
| B16     | GNDETLAKIKKAWNDKDWN         |

Table 2. Identification of subclasses of mAbs in hybridoma cell supernatants

| Hybridoma | C6 | C8 | E1 | E6 |
|-----------|----|----|----|----|
| Ig subclass | IgG1 | IgG1 | IgG1 | IgG1 |
| Light chain type | K | K | K | K |

Figures
Figure 1

P37 protein fragment truncation protocol.

Schematic representation of the protocol for P37 protein epitope identification by fragment truncation. The black arrow represents regions (aa128–254, aa171–254, and aa199–226) that reacted with the four prepared mAbs.

Figure 2

Identification of recombinant plasmid and shuttle plasmid.

(a) Double enzyme digestion (BamH I and Xho I) revealed specific bands at 4693 bp and 1140 bp. Lane M indicates the DNA molecular quality standard. Lanes 1, 2, and 3 represent plasmids extracted after single-colony expansion of randomly selected colonies. (b) The pFastBac™1-His-P37 shuttle plasmid was identified using M13 primers, and a specific band appeared at 3440 bp, whereas pFastBac1 showed a specific band at 2300 bp. Lane M indicates the DNA molecular quality standard. Lane pFastBac1 indicates the pFastBac™1 plasmid negative control. Lanes 1, 2, and 3 represent plasmids extracted after single-colony expansion of randomly selected colonies.

Figure 3

Detection of recombinant P37 protein by indirect immunofluorescence assay (IFA).

(a) Detection of Sf21 cells infected with pFastBac™1-His-P37 by IFA. Infected insect cells showed strong fluorescence. (b) Detection of uninfected Sf21 cells by IFA. No fluorescence was detected from negative cells.
Figure 4

P37 protein identified by Western blot analysis.

Lane M, PageRuler™ Prestained Protein Ladder (Thermo, Vilnius, Lithuania); lane 1, cell lysate from normal Sf21 cells; lane 2, recombinant P37 protein expressed in the baculovirus expression system; lane 3, recombinant P37 protein purified from the baculovirus expression system.

Figure 5

Western blot analysis of four mAbs to Mhr.

Western blot analysis revealed four mAbs (C6, C8, E1, and E6). A specific Mhr band was detected at 43.3 kDa, indicating that the P37 protein expressed by the eukaryotic expression system had good immunogenicity. Lane M, PageRuler™ Prestained Protein Ladder; lane Mhr, Mhr lysate.

Figure 6

Western blot analysis of mAbs that reacted specifically with the P37 protein fragment.

Western blot analysis showed that mAbs reacted specifically with aa128–254, aa171–254, and aa199–226, and the results were consistent for the four mAbs. Lane M, marker; lane 6P-1, vector lysate after IPTG induction.
Western blot analysis verification of key amino acids.

In order to identify key amino acids, peptides were deleted one by one. B1–B8 indicates deleted peptides aa209–206, respectively. B9–B16 indicates deleted peptides aa226–219, respectively. (a) No specific band was detected when 206Lys was deleted. (b) No specific band was detected when 222Phe was deleted.

Alignment of sequences with Mhr P37 epitopes.

A total of seven Mhr strains were analyzed. The sequence motif recognized by mAbs is shown in the red box.

Structural analysis of the P37 protein.

Structural analysis and identification of B cell epitopes in the predicted three-dimensional structure of the P37 protein. Red areas represent α-helix regions, blue areas represent nonregular coil regions, and yellow areas represent strand regions. (a) The location of epitope 206KIKKAWNDKDWTFRNF222 is shown in a gray box. (b) Areas where antigen reacted to antibodies. The epitope was located on the surface of the P37 protein (c).

Supplementary Files

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