Identification of the Immunogenic Outer Membrane Protein A Antigen of *Haemophilus parasuis* by a Proteomics Approach and Passive Immunization with Monoclonal Antibodies in Mice

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Monoclonal antibodies (MAbs) against *Haemophilus parasuis* were generated by fusing spleen cells from BALB/c mice immunized with whole bacterial cells with SP2/0 murine myeloma cells. Desirable hybridomas were screened by enzyme-linked immunosorbent assay (ELISA). Neutralizing MAb 1D8 was selected in protection assays. ELISA results demonstrated that 1D8 can react with all 15 serotypes of *H. parasuis* and field isolate *H. parasuis* HLJ-018. Passive immunization studies showed that mice inoculated intraperitoneally with 1D8 had significantly reduced prevalence of *H. parasuis* colonization in the lung, blood, spleen, and liver and had prolonged survival time compared to that of the control group. Furthermore, the passive transfer experiment indicated that MAb 1D8 can protect mice from both homologous and heterologous challenges with *H. parasuis*. Using two-dimensional gel electrophoresis (2-DE), the immunoreactive protein target for MAb 1D8 was identified. The data presented confirm the protective role of MAb 1D8 and identify OmpA as the target of the protective monoclonal antibody. The data suggest that OmpA is a promising candidate for a subunit vaccine against *H. parasuis*.

*Haemophilus parasuis* is a Gram-negative, nonhemolytic, NAD-dependent bacterium belonging to the *Pasteurellaceae* family. The organism is an important upper-respiratory-tract pathogen in swine and is the etiological agent of Glässer’s disease, which is characterized by fibrinous polyserositis, polyarthritis, meningitis, arthritis syndrome (22, 34), acute pneumonia without polyserositis, and acute septicemia (20). In recent years, the development of protein-based vaccines has been given much more attention, and several immunogenic OMPs of Gram-negative bacteria have been identified by immunoproteomic analysis and protection assays (9, 31, 33). The identification of novel and more efficient immunoprotective antigens is crucial for the development of a monovalent or multivalent subunit vaccine that can protect swine from *H. parasuis* infection.

OmpA, a major outer membrane protein of Gram-negative bacteria, is very highly conserved (7) and participates in biofilm formation, bacterial conjugation, bacteriophage binding, cell growth, and the invasion of mammalian cells (13). Several OmpA-like proteins have been identified in other Gram-negative bacteria, including *Riemerella anatipestifer*, *Pasteurella multocida*, and *Leptospira* (6, 12, 32). However, information regarding the *H. parasuis* OmpA-induced immune response is limited.

In our study, monoclonal antibodies (MAbs) against OmpA were generated and identified, and the neutralizing activities of MAbs were evaluated using *in vitro* and *in vivo* experiments. The results demonstrate the protective roles of MAbs raised against OmpA and indicate that OmpA is a promising candidate for a subunit vaccine against *H. parasuis*.

**MATERIALS AND METHODS**

*Bacterial strains and culture media.* The *H. parasuis* HLJ-018 strain was used for monoclonal antibody production. It was isolated from the nasopharyngeal swabs of a diseased piglet in Heilongjiang province, China, in 2009. Reference strains of *H. parasuis* (strains 1 to 15) were kindly supplied by X. Chen from Beijing Academy of Agriculture and Forestry Science, Beijing, China. *H. parasuis* was maintained on tryptic soy agar (TSA; BD) containing 10% bovine serum and...
Preparation of OMPs. OMPs were prepared as previously described, with modifications (4, 34). Briefly, field isolate HLJ-018 of *H. parasuis* was grown in TSA at 37°C for 14 h with shaking. The cells were harvested by centrifugation at 8,000 × g for 15 min. The supernatant was removed, and the pellets were washed three times with precooled phosphate-buffered saline (PBS). The harvested cells were resuspended in precooled Tris-HCl buffer (pH 7.2) containing protease inhibitor and then disrupted twice using a French pressure cell (Thermo) at 16,000 lb/in². Unbroken cells were removed by centrifugation (8,000 rpm, 30 min, 4°C). The supernatants were diluted 10-fold with ice-cold 0.1 M NaCO₃ (pH 11) and stirred slowly on ice for 1 h. The OMPs were collected by ultracentrifugation in a Beckman Optima Max ultracentrifuge (Beckman) at 100,000 × g for 1 h at 4°C, and then the supernatants were removed. The pellets were resuspended and washed in 50 mM Tris-EDTA (pH 8.0) and collected by centrifugation at 120,000 × g for 1 h at 4°C. The pellets were solubilized in lysis buffer (7 M urea, 2 M thiourea, 1% [wt/vol] ASB-14, 1% [vol/vol] Triton X-100, 40 mM Tris, and 2 mM tributylphosphine). Protein concentration was determined using a PlusOne 2-D Quant kit (GE Healthcare).

MAb production. MAbs were produced as previously described, with slight modifications (16). Briefly, five 6- to 8-week-old female BALB/c mice were immunized subcutaneously with 80 mg of *H. parasuis* mixed with Freund’s incomplete adjuvant (Sigma) on day 0 and then intraperitoneally on days 14 and 21. Blood was taken from each mouse, and antibody titers were measured by enzyme-linked immunosorbent assay (ELISA). The mouse with the highest antibody titer in its serum was given a booster injection of 50 mg of *H. parasuis* intravenously 3 days prior to fusion. Sera collected from the nonimmunized and immunized mice served as negative and positive controls, respectively.

SP2/0-Ag14 murine myeloma cells were grown in Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% bovine serum and 0.01% NAD at 37°C.

Production and purification of ascites. Hybridoma cells were harvested and washed twice in PBS (pH 7.2). Ten to 14 days after pristane injection, 8-week-old BALB/c mice were injected intraperitoneally with 10⁴ hybridoma cells suspended in 0.5 ml normal saline. Fluid was collected from the peritoneal cavity 6 to 9 days after the injection of the cells. Ascites fluid was kept at 4°C for 1 h and centrifuged at 3,000 × g for 20 min. Supernatant was collected and stored at −20°C until use. The purity of monoclonal antibodies was performed according to the manufacturer’s protocol (GE Healthcare, Sweden).

Polyclonal antibody production. Polyclonal antibodies against *H. parasuis* were prepared by following a method described by Kelly et al. and Kim et al., with slight modifications (14, 15). Briefly, 6- to 8-week-old BALB/c mice were injected intraperitoneally with 10⁴ *H. parasuis* cells suspended in 0.5 ml normal saline. Fluid was collected from the peritoneal cavity 6 to 9 days after the injection of the cells. Ascites fluid was kept at 4°C for 1 h and centrifuged at 3,000 × g for 20 min. Supernatant was collected and stored at −20°C until use. The purity of monoclonal antibodies was confirmed according to the manufacturer’s protocol (GE Healthcare, Sweden).

Characterization of monoclonal antibodies. (i) ELISA. Hybridoma culture supernatants were screened for antibodies by ELISA using OMPs, all 15 reference strains of *H. parasuis*, and field isolate HLJ-018 of *H. parasuis*. Polyclonal antibodies (PAb) and SP2/0 culture supernatant were used as positive and negative controls, respectively.

(ii) Identification of antibody isotypes. MAbs were determined using a mouse monoclonal subisotyping kit that contains rabbit anti-mouse immunoglobulin kappa (α) and lambda (λ) light chains and IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3. The procedure was performed by following the manual provided by the manufacturer (Southern Biotech).

Evaluation of the protective activities of antibodies using in vitro and in vivo experiments. (i) In vitro bactericidal assay. The bactericidal assay was modified from previously published protocols (10, 28). Briefly, *H. parasuis* was grown to logarithmic phase and then was diluted to approximately 10⁴ CFU/ml and mixed with 100 μl heat-inactivated MAb 1D8. The mixtures then were incubated in triplicate in sterile tissue culture microtiter plates (Costar) for 30 min at 37°C. The complement was guinea pig serum with undetected antibodies against *H. parasuis*. Fifty microtiters of fresh guinea pig serum was added to each well and incubated at 37°C with gentle rocking for a further 120 min. Heat-inactivated mouse polyclonal antibodies against *H. parasuis* were used as positive controls. The irrelevant MAb 1G7 (against porcine reproductive and respiratory syndrome virus [PRRSV]) and PBS were used as negative controls. At the end of the experiment, samples were removed from each well and plated onto TSA agar plates, and colony numbers were measured after growth overnight at 37°C.
A Student’s t test was applied to determine significant differences in numbers of H. parasuis in lungs among the groups of mice. The chi-square test was used to determine significant differences in survival time and prevalence of H. parasuis in nonrespiratory organs among the groups of mice. A Student’s t test was applied to determine significant differences in the numbers of H. parasuis in lungs of mice.

**RESULTS**

**Generation and characterization of a murine monoclonal antibody against H. parasuis.** The hybridoma ID8, generated by the fusion of murine myeloma SP2/0 cells with splenocytes from mice immunized with H. parasuis, secreted an IgG2b MAb with a κ light chain. By ELISA, MAb ID8 showed a strong positive reaction to OMPs, all 15 reference strains, and the field isolate HLJ-018 of H. parasuis (Fig. 1).

**In vitro and in vivo protective efficiency and cross-reactivity of monoclonal antibodies against H. parasuis.** To determine the in vitro bactericidal activity of MAb ID8 against H. parasuis, a complement-mediated killing assay was performed. The bactericidal activity of MAb ID8 was assessed by the differences of colony numbers between experimental and control groups (Table 1). The colony numbers of H. parasuis were reduced significantly (P < 0.01) from 181 (group 3) and 141 (group 4) to 51 (group 1), a 72 and 64% reduction, respectively. In addition, a hit was considered positive when at least two peptides were identified by MS/MS.

**Statistical analysis.** The analysis of variance was applied to determine significant differences in numbers of H. parasuis in lungs among the groups of mice. A Student’s t test was applied to determine significant differences in survival time and prevalence of H. parasuis in nonrespiratory organs among the groups of mice. A Student’s t test was applied to determine significant differences in the numbers of H. parasuis in lungs of mice.

**TABLE 1.** Complement-mediated bactericidal activity of MAb1D8 in vitro

| Group | Treatment | No. of bacteria (mean ± SEM) | P value |
|-------|-----------|-----------------------------|---------|
| 1     | 1D8 + HPS + C  | 31.33 ± 4.055               | <0.01   |
| 2     | PAb + HPS + C  | 18.07 ± 19.55               | <0.01   |
| 3     | 1G7 (PRRSV) + HPS + C  | 141.7 ± 10.37              |         |

**Note:** Each mouse was inoculated intraperitoneally with MAh 1D8, PBS, and polyclonal antibodies at 1 h prior to challenge with 3.0 × 10^7 CFU of the homologous H. parasuis HLJ-018 strain. MAh 1D8 is directed against HLJ-018. PBS and polyclonal antibodies served as negative and positive controls, respectively.

**TABLE 2.** Mortality and survival time of mice treated with MAh 1D8 and challenged with homologous H. parasuis HLJ-018

| Group | Treatment | No. of mice | Mortality | Survival time (mean ± SD) |
|-------|-----------|-------------|-----------|---------------------------|
| 1     | 1D8       | 6           | 6/6       | 31.60 ± 3.52B             |
| 2     | PAb       | 6           | 6/6       | 10.38 ± 2.28              |
| 3     | 1G7       | 6           | 6/6       | 43.00 ± 4.30B             |

**Note:** Each mouse was inoculated intraperitoneally with MAh 1D8, PBS, and polyclonal antibodies at 1 h prior to challenge with 3.0 × 10^7 CFU of the homologous H. parasuis HLJ-018 strain. MAh 1D8 is directed against HLJ-018. PBS and polyclonal antibodies served as negative and positive controls, respectively.

**Mean survival time was significantly longer than that for group 2.**
For the protection experiment, BALB/c mice were challenged with a lethal dose of *H. parasuis* 1 h after MAb 1D8 injection. All mice in the groups injected with MAb 1D8 and the control died, although the survival time of MAb-injected groups was longer than that of the control group. The survival times of mice were 31.6, 10.4, and 43.0 h, and the difference in survival time was significant (*P* < 0.01) between group 1 and group 2 (Table 2). These results demonstrate that MAb 1D8 is a protective MAb in mice.

For the bacteria elimination assay, because MAb 1D8 can react with all reference strains by ELISA, we sought to determine whether MAb 1D8 could protect mice against heterologous challenges with serotype 4 and 5 strains, which are the most widespread strains of *H. parasuis* in China. The results showed that bacterial counts in the blood were significantly lower in mice receiving MAb 1D8 than in the control group. Moreover, bacteria were completely eliminated after 7, 15, and 24 h of infection in mice challenged with SW124, HLJ-018, and HS80, respectively, whereas all of the control mice had positive blood cultures after 24 h (Fig. 2).

Mice were sacrificed and *H. parasuis* was isolated from organs, including lungs, livers, and spleens. The prevalence and numbers of *H. parasuis* in lungs, livers, and spleens were compared between the groups. The prevalences of *H. parasuis* in lungs of mice which were challenged with the homologous *H. parasuis* HLJ-018 were 17.8% (1/6), 100% (6/6), 0 (0/6), and 100% (6/6) for groups 1, 2, 3, and 4, respectively, and a significant (*P* < 0.01) difference among group 1, group 2, and group 4 was found. The number of *H. parasuis* cells in lungs was reduced by 6.7 × 10^2 (group 2) and 3.2 × 10^2 (group 4) to 7 (group 1), a 96- and 46-fold reduction (*P* < 0.01). The *H. parasuis* prevalences in nonrespiratory organs were 17.8% (2/12), 91.6% (11/12), 8.3% (1/12), and 75% (9/12) for groups 1 to 4, respectively. The *H. parasuis* prevalence had a significant reduction (*P* < 0.01) of 91.6, 75, and 17.8%. Significant protection was observed in mice immunized with MAb 1D8 and challenged with *H. parasuis* SW124 and HS80. The results are shown in Tables 3 and 4.

These results suggest that MAb 1D8 has a potent bacteria-neutralizing activity *in vitro* and protective activity *in vivo*.

**2-DE profile and immunoblot analysis of OMPs.** The OMPs of *H. parasuis* were separated by 2-DE. Many protein spots were detected using silver nitrate stain (Fig. 4A), and most of the OMP spots were distributed in the pH range of 4.0 to 9.0 with molecular masses ranging from 15 to 120 kDa. Four immunoreactive protein spots with molecular masses of approximately 35 kDa were detected with MAb 1D8 by Western blot analysis (Fig. 4B). These proteins matched with the protein spots that could be seen in the preparative 2-DE gel.

**Identification of immunogenic proteins.** The immunoreactive protein spots were excised from the 2-DE gel by in-gel digestion and identified by MALDI-TOF-MS on the basis of peptide mass matching. By searching the NCBI database, the protein (protein scores 152 greater than 82 are significant, *P* < 0.05) that reacted with MAb 1D8 was identified as OmpA, a protein with a molecular mass of 37 kDa. Figure 5B shows the identified sequence with a coverage of 38%.

**DISCUSSION**

*H. parasuis* is a widespread major pathogen that affects naive herds and causes immense production losses, including nursery mortality, decreased weight gain, and lower meat value at
slaughter (24). Although traditional vaccines are effective, they are not efficient for cross-protection. The identification of novel and conservative immunogenicity antigens is crucial for the development of an effective subunit vaccine. MAb 1D8 was generated in our study, and its protective role was investigated in mice. Additionally, the immunogenic protein target for MAb 1D8 was identified using 2-DE.

Several researchers have demonstrated that humoral immunity plays a major role in the protection of immunized mice against *H. parasuis* (11, 17, 19). MAbs, which are an important part of humoral immunity, were produced to protect mice against *H. parasuis* and were involved in in vitro assay, the results suggest that MAb 1D8 was involved in bactericidal activity against *H. parasuis* in nonrespiratory organs of mice treated with MAb 1D8 and challenged with homologous *H. parasuis* HLJ-018.

| Group | Treatment | No of mice | Prevalence (%) | Mean CFU/g ± SEM* | Load (CFU/g) | No of nonrespiratory organs/total no. tested (%) |
|-------|------------|------------|----------------|-------------------|-------------|-----------------------------------------------|
| 1     | 1D8        | 6          | 1/6 (17.8)     | 4.00 × 10^3 ± 0.400 | 7           | 2/12 (17.8)                                  |
| 2     | PBS        | 6          | 6/6 (100)      | 2.828 ± 0.125     | 6.7 × 10^2  | 11/12 (91.6)                                 |
| 3     | PAb        | 6          | 0/6 (0)        | 0                 | 0           | 1/12 (8.3)                                   |
| 4     | 1G7 (against PRRSV) | 6          | 6/6 (100)      | 2.505 ± 0.132     | 3.2 × 10^2  | 9/12 (75)                                    |

* Means of the log of geometric mean CFU per g of lung tissue. The geometric mean CFU per g of lung in each mouse was calculated and converted to the logarithmic number. The means and standard errors of the means then were calculated.

| Mean CFU numbers in lung were significantly less (P < 0.01) than those for groups 2 and 4. |

| Prevalence of *H. parasuis* in nonrespiratory organs (liver and spleen) was significantly less (P < 0.01) than that for groups 2 and 4. |

| Prevalence of *H. parasuis* in nonrespiratory organs was detected. |

Several researchers have demonstrated that humoral immunity plays a major role in the protection of immunized mice against *H. parasuis* (11, 17, 19). MAbs, which are an important part of humoral immunity, were produced to study their roles in protection. Using 2-DE, the immunogenic protein target for MAb 1D8 was identified using 2-DE.

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OmpA or OmpA-like proteins cannot confer passive immunoprotection (6, 10, 23). Huang et al. (12) deduced that neither recombinant OmpA nor cell-extracted protein elicits antibodies specific for the native proteins; additionally, they found that neither a single protein nor a subunit vaccination could afford effective protection and concluded that a combination of several kinds of proteins is necessary in a vaccine. However, a Croquet-Valdes et al. (5) study demonstrated that immunization with a portion of rickettsial OmpA stimulates protective immunity against a lethal *Rickettsia conorii* challenge in mice. OprF, an OmpA homolog in *Pseudomonas aeruginosa*, also has been used for vaccine development, as it possesses protective epitopes (3). Although the immunoprotective role of OmpA is controversial, the OmpA-specific MAb 1D8 showed strong *H. parasuis* neutralizing capacity in our study. On the basis of our present knowledge of outer membrane proteins regarding adhesion to and invasion of host cells, we hypothesize that MAb 1D8 impairs the biological functions of OmpA by disrupting the interaction between *H. parasuis* and the host cell.

In summary, MAb 1D8, raised against OmpA, demonstrated neutralizing and protective activities in vitro and in vivo. This

**FIG. 4.** 2-DE proteome map (A) and immunoblot analysis (B) of outer membrane proteins (OMPs) from *H. parasuis* HLJ-018. OMPs were separated in the first dimension by isoelectric focusing (IEF) in the pI range of 3 to 10 and by 10% SDS-PAGE in the second dimension. Arrows indicate immunogenic proteins recognized with MAb 1D8.

**FIG. 5.** Identification of the immunogenic OmpA by MALDI-TOF-MS. (A) Identification results of protein spots. Protein scores (n = 152 for MAb 1D8) that are greater than 82 are significant (P < 0.05). Protein scores are derived from ion scores as a nonprobabilistic basis for ranking protein hits. (B) The amino acid sequence of OmpA.
suggests that OmpA is a target for protective antibodies in mice. OmpA may be a desirable immunogen to stimulate immune protection; thus, it merits further study as a vaccine candidate against \textit{H. parasuis} infection.

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