Production and characterization of murine monoclonal antibodies against *Haemophilus parasuis* and study of their protective role in mice

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Monoclonal antibodies (MAbs) against *Haemophilus parasuis* were obtained by the fusion of SP2/0-Ag14 murine myeloma cells and spleen cells from BALB/c mice immunized with a whole-bacterial-cell suspension (WC) of *H. parasuis* strain SW124 (serotype 4). Two MAbs showing strong reactivity in ELISA were further characterized using SDS-PAGE and Western-blot assays. Different treatments of the WC indicated that MAbs 4D5 and 4G9 identified epitopes of proteinic and polysaccharidic nature, respectively. Electron microscopic examination revealed that, unlike the proteinic epitopes, the lipopolysaccharidic epitopes were exposed on the surface of the cell. Using coagglutination, Western-blot and dot-blot assays it was found that both MAbs recognized common epitopes of all the reference strains and field isolates of *H. parasuis*. None of the other bacteria tested reacted with the MAbs. These results indicated that both the proteinic and polysaccharidic antigens carried species-specific epitopes. It is suggested that these MAbs may potentially be useful for identification of *H. parasuis* isolates as well as for developing serological diagnostic tools. MAbs 4D5 and 4G9 were unable to kill *H. parasuis* *in vitro* in the presence of complement. However, an enhanced bacterial clearance from blood was observed in mice inoculated with either of the MAbs. Highly significant protection was observed in mice using MAb 4G9. This is believed to be the first report of MAbs capable of identifying common species-specific antigens of *H. parasuis* and of their implication in protection against challenge infection in mice.

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INTRODUCTION

Haemophilus parasuis has recently re-emerged as one of the major causes of nursery mortality and can cause severe acute disease when introduced into naive herds (Oliveira *et al.*, 2002). The exact factors that have contributed to the increase in the incidence of *H. parasuis* infections in the nursery are not clear. However, some hypotheses have been proposed, one of which is co-infection with the PRRS (porcine reproductive and respiratory) virus (Oliveira & Pijoan, 2002).

The association between capsule expression, whole-cell protein profile, serotype and virulence of *H. parasuis* is controversial and the situation remains unclear (Kielstein *et al.*, 1991; Rapp-Gabrielson *et al.*, 1986; Morozumi & Nicolet, 1986a, b; Nicolet *et al.*, 1980). Miniats *et al.* (1991) reported that the antibodies detected in the sera of

vaccinated pigs were only against outer-membrane proteins (OMPs) of *H. parasuis*, suggesting that the OMPs are more immunogenic than other components of bacteria. Virulent strains were found to be non-capsulated and these strains were considered for vaccine production by Kielstein *et al.* (1991). The precise cellular localization of type-specific antigens of *H. parasuis* has not been well defined, although studies by different investigators have indicated that they may be polysaccharides associated with either the capsule or outer membrane components (Morozumi & Nicolet, 1986b; Kielstein, 1991).

Relatively little is known about the constituents of the *H. parasuis* outer membrane. One major outer-membrane protein (MOMP) of 42 kDa has been reported (Hartmann *et al.*, 1995), and an N-terminal homology study suggested that this protein was related to the porin family. Although another MOMP called heat-modifiable protein (OmpA) has been reported in various Gram-negative bacteria (Vasfi Marandi & Mittal, 1996; Spinola *et al.*, 1993; Tagawa *et al.*, 1993; Beck & Bremer, 1980), nothing is known about it in *H. parasuis*. Several functions have been attributed to OmpA, such as maintenance of structural integrity of the

Abbreviations: BC, boiled cell suspension; CoA, coagglutination; LPS, lipopolysaccharide; MAb, monoclonal antibody; MOMP, major outer-membrane protein; OMP, outer-membrane protein; WC, whole-cell suspension.

cell envelope (Koebnik *et al.*, 2000), bacterial conjugation (Schweizer & Henning, 1977), bacteriophage attachment (Datta *et al.*, 1977), porin activity (Sugawara & Nikaido, 1992) and resistance to complement-mediated serum killing (Weiser & Gotschlich, 1991). Lipopolysaccharide (LPS) is another essential structural component of all Gram-negative bacteria and it is considered as an important virulence factor involved directly in adherence to various target cells, leading to colonization, which may be the first step in the initiation of pathogenesis (Jacques & Paradis, 1998).

The purpose of this study was the production and characterization of monoclonal antibodies against heat-modifiable OMP and lipopolysaccharidic epitopes of *H. parasuis* as well as the study of their possible implications in protection against *H. parasuis* infections in mice.

METHODS

Bacterial strains and culture media. Reference strains representing serotypes 1 to 15 of *H. parasuis* were kindly supplied by Dr Ross from ISU, College of Veterinary Medicine, Ames, Iowa and by Dr Astrid Raßbach from Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Jena, Germany (Table 1). Bacteria were grown on pleuropneumonia-like organisms medium (PPLO, Difco) overnight at 37 °C. Cultures were harvested and washed three times with 0·01 M PBS (pH 7·2) and centrifuged at 10 000 g for 20 min. The bacterial pellet was suspended and adjusted with PBS to an optical density (OD) of 1·0 at 640 nm and was referred to as whole-cell suspension (WC). The WC was boiled in a water bath for 20 min and referred to as boiled cell suspension (BC). Sonicated antigen was obtained when the bacterial pellet of WC was suspended in 10 mM of HEPES (pH 7·4). Reference strain SW124 of *H. parasuis* (serotype 4) was used for production of monoclonal antibodies, and systemic field strain 03-0177 of *H. parasuis* (serotype 13) was selected for protection, bacterial elimination and bactericidal assays. Reference strain 405 of *Actinobacillus pleuropneumoniae* (serotype 8) was used as a negative control in all the tests.

A total of 21 other bacterial strains representing different bacterial species were used for specificity studies (Table 1). In addition, 500 North American field isolates of *H. parasuis* representing several serotypes obtained from our stock culture were tested with two MABs (Table 1).

Antigen preparation. Outer-membrane proteins (OMPs) were produced by the method described by Carlone *et al.* (1986). Protein concentration was determined by Bio-Rad assay, based on the method of Bradford (1976). LPS was purified by the hot phenol/water procedure as described by Rebers *et al.* (1980).

Immunization procedure and production of monoclonal antibodies. Four 6-week-old BALB/c female mice were immunized intraperitoneally with 0·3 ml WC of *H. parasuis* strain SW124 mixed with Freund's incomplete adjuvant (Difco) followed by three intraperitoneal injections of WC on days 14, 21 and 28. Blood was taken from each mouse and the antibody response was measured by ELISA. The mouse with the highest serum antibody titre was selected as the spleen donor and was given an intraperitoneal booster injection of 0·3 ml WC in PBS 3 days before fusion. Sera collected from non-immunized and immunized mice served as negative and positive controls.

SP2/0-Ag 14 murine myeloma cells were grown in Dulbecco's modified

Eagle medium (DMEM; Gibco) supplemented with 10 % heat-inactivated bovine fetal serum, 100 U gentamicin ml⁻¹ and 2 mM L-glutamine (Gibco). The fusion of spleen cells from the selected mouse with SP2/0-Ag myeloma cells was carried out as described by Köhler & Milstein (1975), by using 50 % (w/v) of polyethylene glycol (molecular mass, 3000–3700 Da; Sigma). The fused cells were cultured in five 96-well microtitre plates in the presence of hypoxanthine, aminopterin and thymidine (HAT; Sigma) and incubated at 37 °C in a humid atmosphere of 5 % CO₂. Hybridoma culture supernatants were examined for the presence of antibodies by ELISA. Hybridoma cells producing antibodies were cloned twice by limiting dilution. Polyclonal hyperimmune sera against reference strains of serotypes 4 and 13 were produced in two rabbits and five mice (Tadjine *et al.*, 2004).

ELISA. Hybridoma culture supernatants were screened for antibodies by ELISA using WC, BC and sonicated cell suspension as antigens. A 96-well microtitre plate (Linbro) was coated with 2 µg of OMP, 1 µg of LPS and an optimally diluted antigen suspension of WC, BC or sonicated antigens per well in carbonate buffer (pH 9·6) and kept overnight at 4 °C. The plate was washed three times with PBS containing 0·05 % Tween-20 (PBS-T). Hybridoma culture supernatants and optimal dilutions of sera from immunized and non-immunized mice were added to the wells (100 µl per well). The goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma) was optimally diluted in PBS-T and added to each well after washing three times with PBS-T; the plate was incubated at 37 °C for 1 h and washed. ABTS colour development reagent was added thereafter. The absorbance of the peroxidase reaction product in the ELISA was read on an automated microplate reader (Bio-Rad model 450) at 405 nm. All the hybridomas showing at least 30 % of the OD value of the positive control were considered as positive and selected for further characterization.

Dot-ELISA. Dot-ELISA was carried out as described by Achacha & Mittal (1996). Two micrograms of OMP, 1 µg of LPS and an optimally diluted antigen suspension of WC, BC or sonicated antigens was placed on nitrocellulose membranes and allowed to dry at room temperature for 15 min. Membranes were incubated with 5 % skimmed milk in PBS-T before incubation with MAB supernatants for 1 h at room temperature. The blots were washed and incubated with goat anti-mouse (IgG) horseradish peroxidase conjugate (Bio-Rad) for 1 h at room temperature and washed. The membranes were treated with 4-chloro-1-naphthol substrates (Bio-Rad) for 15 min, and the colour reaction was stopped by flooding the membranes with distilled water.

Isotype determination. The isotypes of MABs were determined by an ELISA with a mouse monoclonal subtyping kit containing rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA, following the procedure provided by the manufacturer (Bio-Rad).

Enzymic and chemical treatments of antigens. Plates coated as described earlier with 100 µl of OMP (2 µg), LPS (1 µg), WC, BC or sonicated antigens (1/10 of OD 1 at 640 nm) of *H. parasuis* strain SW124 were treated with different concentrations of proteinase K, chymotrypsin and trypsin (all from Sigma) in PBS (pH 7·2) at 37 °C for 4 h. Following four washes, ELISA was performed as described above. Non-treated OMP and sonicated WC were used as controls. In addition, a plate coated with various antigens of *H. parasuis* strain SW124 of serotype 4 was washed with PBS-T and then rinsed with 50 mM sodium acetate buffer (pH 4·5) before treatment with different concentrations of sodium periodate (1–20 mM) (Sigma) in sodium acetate buffer for 1 h in a dark room. After a short rinse with sodium acetate, the plate was incubated in 50 mM sodium borohydride in PBS for 30 min. ELISA was performed as described above after washing the plate with PBS-T. Non-treated BC and OMP were used as controls (Woodward *et al.*, 1985).

Table 1. Reactivity of reference strains and field isolates of *H. parasuis* and other Gram-negative bacteria with MAbs 4D5 and 4G9 in ELISA, dot-ELISA and Western blot

Strain	Reactivity in ELISA, dot-ELISA and Western blot with MAbs	
	4D5	4G9
<i>Haemophilus parasuis</i> reference serotype strains		
1 (No. 4)	+	+
2 (SW140)	+	+
3 (SW114)	+	+
4 (SW124)	+	+
5 (Nagasaki)	+	+
6 (131)	+	+
7 (174)	+	+
8 (C5)	+	+
9 (D74)	+	+
10 (H555)	+	+
11 (H465)	+	+
12 (H425)	+	+
13 (84-17975)	+	+
14 (84-22113)	+	+
15 (84-15995)	+	+
Field isolates of <i>Haemophilus parasuis</i>		
Five hundred strains representing serotypes 1, 2, 3, 4, 5, 7, 9, 12, 13, 14, 15 and nontypable	+	+
Other species		
<i>Haemophilus influenzae</i> ATCC 9006	—	—
<i>Haemophilus parainfluenzae</i> ATCC 7901	—	—
<i>Histophilus somni</i> ATCC 700025	—	—
<i>Histophilus somni</i> 91-0334	—	—
<i>Haemophilus felis</i> 92-7957	—	—
<i>Haemophilus</i> sp. G555	—	—
<i>Pasteurella multocida</i> type F (P1436)	—	—
<i>Pasteurella multocida</i> type D (P210)	—	—
<i>Streptococcus suis</i> serotype 1/2	—	—
<i>Streptococcus suis</i> serotype 2	—	—
<i>Actinobacillus pleuropneumoniae</i> (App) serotypes 1 to 15 (reference strains)	—	—
App-like 99-536-55H	—	—
App-like RFO 0347	—	—
<i>Actinobacillus lignieresii</i> ATCC 19393	—	—
<i>Actinobacillus minor</i> 01-F821	—	—
<i>Actinobacillus porcini</i> 99-0088	—	—
<i>Actinobacillus suis</i> ATCC 15557	—	—
<i>Escherichia coli</i> ATCC 25922	—	—
<i>Escherichia coli</i> K-12 RNA (B)	—	—
<i>Bordetella bronchiseptica</i> ATCC 19395	—	—
<i>Klebsiella pneumoniae</i> ATCC 13883	—	—
<i>Pseudomonas aeruginosa</i> ATCC 27853	—	—
<i>Salmonella arizonae</i> ATCC 13314	—	—
<i>Yersinia enterocolitica</i> ATCC 23715	—	—
<i>Shigella sonnei</i> ATCC 29930	—	—

SDS-PAGE and Western blot. SDS-PAGE was performed according to the method of Laemmli (1970) by using 25 µg of OMP, 1.5 µg of LPS and 2 mg of WC of *H. parasuis* reference strain SW124 of serotype 4 as well as WC of 30 *H. parasuis* field isolates

and 21 strains of other Gram-negative bacteria. The antigens were mixed with an equal volume of solubilization buffer, heated for 20 min at 37 °C, 65 °C or 100 °C or for 5 min at 100 °C, treated with proteolytic enzymes and sodium periodate as described earlier and

then separated on 12% polyacrylamide vertical slab gels. Antigens separated by SDS-PAGE were stained either with silver nitrate as described by Tsai & Frasch (1982) to detect lipopolysaccharide antigen or 0.1% (w/v) Coomassie brilliant blue (R-250; Sigma) to detect separated proteins. Western blotting was performed as described by Towbin *et al.* (1979).

The Western blot was used for detecting antibodies against OMP and LPS antigens in pig sera (1 in 200 dilution) from specific-pathogen-free herds, uninfected pigs from conventional herds (not showing any signs of Glässer's disease but not free of *H. parasuis* infection) and naturally infected herds showing clinical signs of Glässer's disease and infected with multiple serotypes (mainly serotypes 4, 5 and 13) as well as from hyperimmunized rabbits and mice.

N-terminal amino acid sequencing. The samples containing OMPs were loaded onto mini-gels according to Laemmli (1970) and electroblotted onto a PVDF transfer membrane (problott 400994 from Applied Biosystems) using the method of Matsudaira (1987) with some modification. Automated Edman degradation was performed with a gas-phase sequencer (model 470A, Applied Biosystems) equipped with an on-line phenylthiohydantoin analyser (model 120A, Applied Biosystems) by using the general protocol of Hewick *et al.* (1981). The standard 03RPTH program was used for sequencing.

Colony blot. A colony-blotting assay was performed using the method of Mutharia & Hancock (1985) with some modification. Bacterial colonies were transferred from an agar plate onto a nitrocellulose membrane by direct contact at 37 °C for 30 min. The membrane was carefully removed and dried at room temperature for 20 min. The colony blot was blocked with PBS containing 3% skimmed milk (w/v) and incubated with hybridoma culture supernatants containing MAb 4D5 or 4G9. The membrane was then washed in PBS-T and bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) by using 4-chloro-1-naphthol as the chromogenic substrate.

Immunoelectron microscopy. Immunogold labelling was performed as described by Li *et al.* (1992). One drop of overnight culture of *H. parasuis* SW124 cell suspension was placed on Formvar-coated grids and blocked for 5 min with 1% bovine albumin. MAb diluted to 1 in 1000 in PBS was incubated for 30 min with grids and rinsed five times with distilled water. The grids were then incubated for 30 min with goat anti-mouse IgG conjugated with 10 nm gold particles (Sigma), rinsed and negatively stained with 1% phosphotungstate for 10 s. The grids were observed under an electron microscope.

Production of ascites fluids. Hybridoma cells producing IgG MAbs 4G9 and 4D5 were grown in DMEM supplemented with 10% fetal bovine serum, harvested and washed twice in PBS (pH 7.2). Ten to 14 days after pristane injection, 6- to 8-week-old BALB/c mice were injected intraperitoneally with 10^6 hybridoma cells suspended in 0.5 ml PBS (pH 7.2). 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 5000 g for 15 min. Supernatant was collected and stored at -20 °C until used.

Protection and bacterial elimination assays. The protection and bacterial elimination assays were carried out in the BALB/c mouse model. The tests were repeated in three independent experiments.

For the protection assay, bacterial cells from an overnight culture of *H. parasuis* on PPLO agar were harvested in PBS and adjusted to an OD of 1.0 at 640 nm, which corresponded to approximately 7×10^9 c.f.u. ml⁻¹ as determined using a Petroff-Hausser counting chamber. This concentration was used as the minimum lethal dose for challenge infection. Six groups of 6-week-old male mice were inoculated intraperitoneally with 0.2 ml of bacterial suspension

mixed with either 0.2 ml of rabbit polyclonal antiserum diluted 1 in 5 in PBS or ascites fluids containing MAbs 4D5 or 4G9. They were cared for in accordance with the principles of the Canadian Council on Animal Care. Mice were observed for a period of 7 days for mortality, if any.

For the bacterial elimination assay, a washed overnight culture of *H. parasuis* was adjusted to a concentration of 10^8 c.f.u. ml⁻¹ and injected as described for the protection assay. A higher volume of the ascites fluid MAb (0.3 ml) was used. Blood samples were taken in duplicate from the femoral vein at various time intervals after injection and viable counts were performed on PPLO agar to determine the number of c.f.u. ml⁻¹ of blood. Mice were killed, and lung, liver, heart and spleen tissues were removed from each mouse. One gram of each tissue was homogenized in 2 ml PBS for 5 min and 100 µl of each tissue homogenate was plated on PPLO agar in duplicate to determine the number of c.f.u. per g of tissue.

Bactericidal assay. The bactericidal assay was carried out as described by Mittal & Ingram (1975). *H. parasuis* grown to exponential phase in PPLO was diluted to 5×10^4 c.f.u. ml⁻¹. Fifty microlitres of heat-inactivated ascitic fluid of MAbs 4D5 or 4G9 was incubated with 100 µl of a live suspension of *H. parasuis* field strain 03-0177 of serotype 13 for 10 min at room temperature in microtitre plates. Fifty microlitres of an optimal dilution of fresh guinea pig serum was added to each well as a source of complement. The plates were incubated for a further 120 min at 37 °C. The colony counts were performed at 0, 60 and 120 min by plating samples onto PPLO agar plates in triplicate. The plates were incubated overnight at 37 °C. A heat-inactivated rabbit antiserum produced against reference strain of *H. parasuis* serotype 13 was used as a positive control, and negative controls consisted of ascitic fluid of MAb 4D5 and bacteria without added complement as well as bacteria and complement with no added MAb 4D5.

Coagglutination (CoA) test. A CoA test was used to detect *H. parasuis*-specific antigen in different tissues. The details of the preparation of the CoA reagents and the CoA test have been described previously (Mittal *et al.*, 1983). Briefly, MAb 4G9 produced in mouse and *Staphylococcus aureus* strain Cowan I (NCTC 8530) capable of producing a large amount of protein A were used for the preparation of CoA reagents. One gram each of mouse lung, spleen and liver were homogenized in 2 ml saline in mortar with help of a 60 mesh Norton Alundum RR (Fisher Scientific). The tissue suspension was kept in a small glass tube, boiled in a water bath for 20 min and centrifuged at 8000 g for 30 min to remove the particulate material. The clear supernatant was examined for the presence of *H. parasuis* antigen. One drop of the CoA reagent was mixed on a glass slide with an equal volume of supernatant of tissue homogenate. The CoA reaction was recorded within 4 min and was scored on a 0 to 4+ scale depending on the rapidity and intensity of the reaction.

RESULTS

Production of MAbs

A total of 65 hybridomas were tested in ELISA, out of which two hybridomas, namely 4D5 and 4G9, showing a positive reaction in ELISA were selected for further characterization. The immunoglobulin classes of MAbs 4D5 and 4G9 were IgG2b and IgG3, respectively. In the Western blot, using 12% separating gel, MAb 4D5 reacted with a major band of an estimated molecular mass of 35 kDa (Fig. 1, lane 3), whereas MAb 4G9 reacted with a

Fig. 1. SDS-PAGE and Western-blot analysis of *H. parasuis* SW124 whole-cell suspension (WC). Coomassie brilliant blue- and silver-stained WC (lanes 2 and 4, respectively). Reactivity of MAbs 4D5 and 4G9 with WC (lanes 3 and 5, respectively). Lane 1 contains molecular markers.

single diffuse band in the lower molecular mass range (Fig. 1, lane 5). Both monoclonal antibodies recognized common epitopes shared by all 15 reference strains (Figs 2 and 3).

Characterization of epitopes recognized by the MAbs

Treatment of the OMP preparation of *H. parasuis* SW124 with proteinase K, trypsin and chymotrypsin showed that the epitopes recognized by MAb 4D5 (Fig. 4, lanes 2–4) were completely sensitive to treatment with proteolytic enzymes. SDS-PAGE and Western-blot analysis of OMP showed that no effect was observed when the OMP preparation of *H. parasuis* SW124 was solubilized at 37 °C and 65 °C (Fig. 4, lanes 5 and 6). However, two major bands with molecular masses of 35 and 43 kDa were observed when OMP was solubilized at 100 °C for 5 min (Fig. 4, lane 7), and in aliquots solubilized at 100 °C for 20 min the molecular mass of the band changed from 35 kDa to approximately 43 kDa (Fig. 4, lane 8). The four last lanes of Fig. 4 showed that the epitope was not affected when treated with different concentrations of sodium periodate. On the other hand, the epitope recognized by MAb 4G9 was not affected by either proteolytic enzymes (Fig. 5, lanes 2–4) or heat treatment (Fig. 5, lanes 5–8), but marked, although not complete, sensitivity was observed after treatment with different concentrations of sodium periodate when WC was used as the antigen (Fig. 5, lanes 9–11). The epitope recognized by MAb 4G9 was completely destroyed when purified LPS was used as the antigen (data not shown).

Fig. 2. Reactivity of MAb 4D5 with OMP preparations of *H. parasuis* reference strains of serotypes 1 to 15 (A), *A. pleuropneumoniae* reference strain 405 of serotype 8 (B1) and *H. parasuis* reference strain SW124 of serotype 4 (B2) in Western blot and dot blot.

Fig. 3. Reactivity of MAb 4G9 with LPS preparations of *H. parasuis* reference strains of serotypes 1 to 15 (A), *A. pleuropneumoniae* reference strain 405 of serotype 8 (B1) and *H. parasuis* reference strains SW124 of serotype 4 (B2) in Western blot and dot blot.

Fig. 4. Immunoblot using MAb 4D5 and the OMP preparation of *H. parasuis* SW124 using different treatments. Lane 1, untreated OMP; lanes 2–4, proteinase K-, trypsin- and chymotrypsin-treated OMP, respectively; lanes 5, 6 and 8, heat treatment for 20 min at 37 °C, 65 °C and 100 °C, respectively; lane 7, heat treatment for 5 min at 100 °C; lanes 9–12, treatment with 2.5, 5, 10 and 20 mM sodium periodate, respectively.

Fig. 5. Immunoblot using MAb 4G9 and the LPS preparation of *H. parasuis* SW124 using different treatments. Lane 1, untreated LPS; lanes 2–4, proteinase K-, trypsin- and chymotrypsin-treated LPS, respectively; lanes 5–8, heat treatment at 37 °C, 65 °C, 100 °C for 5 min and 100 °C for 20 min, respectively; lanes 9–11, treatment with 5, 10 and 20 mM sodium periodate, respectively.

The results obtained in the ELISA also showed that the epitopes reactive with MAb 4D5 were sensitive to treatments with all the proteolytic enzymes used in this study, whereas those reactive with MAb 4G9 were not affected by these treatments (Fig. 6a). The treatment of OMP and WC with different concentrations of sodium periodate and the results obtained in the ELISA showed that the binding of MAb 4D5 to OMP was not affected by the sodium periodate, whereas more than 80 % loss of binding of MAb 4G9 occurred at a concentration of 2.5 mM of sodium periodate and complete loss of binding occurred at 5, 10 and 20 mM (Fig. 6b).

There was good concordance between the results obtained in the ELISA and Western blot.

Surface localization of the MAbs

The MAb 4D5 against a heat-modifiable epitope did not show any staining on the cell surface of *H. parasuis* in either

immunoelectron microscopy or colony-blotting assays, whereas MAb 4G9 showed a strong reactivity on the surface of *H. parasuis* (Fig. 7).

Protein sequence determination and homology

The first 12 amino acids of the N-terminal sequence of the 35 kDa MOMP of *H. parasuis* were Ala-Pro-Gln-Ala-Asp-Ser-Phe-Tyr-Val-Gly-Ala-Lys (Table 2). A comparative study of this sequence with known sequences of other Gram-negative bacteria revealed that the 35 kDa MOMP of *H. parasuis* exhibited 92 % homology with the sequence of OmpA from *Haemophilus ducreyi* (Spinola *et al.*, 1993), 76 % homology with the sequence of OmpA from *Pasteurella multocida* (Vasfi Marandi & Mittal, 1996), *Histophilus somni* (Tagawa *et al.*, 1993) and *Actinobacillus actinomycetemcomitans* (Wilson, 1991), and 50 % homology with OmpA of *Salmonella typhimurium* (Freudl & Cole, 1983) and *Escherichia coli* (Beck & Bremer, 1980). Comparison of the N-terminal amino acid sequences of OmpA proteins of *Pasteurellaceae* with those of OmpA proteins of *Enterobacteriaceae* demonstrated that the major differences were seen at residues 3 and 7.

Fig. 6. The effect of proteinase (a) and sodium periodate (b) treatments of WC and OMP of *H. parasuis* on their binding with MAbs 4D5 and 4G9 in ELISA. In (a): filled bars, MAb 4D5; open bars, MAb 4G9. In (b): squares, MAb 4D5; diamonds, MAb 4G9. Chy-try, chymotrypsin treatment; NT, no treatment; Prot K, proteinase K treatment; Tryp, trypsin treatment.

Fig. 7. Immunoelectron microscopy of *H. parasuis* SW124 (a, b) and *A. pleuropneumoniae* 405 (c, d). The figures show labelling of *H. parasuis* SW124 with MAbs 4D5 and 4G9 (a and b, respectively) and of *A. pleuropneumoniae* 405 by MAbs 4D5 and 4G9 (c and d, respectively).

Table 2. Comparison of the N-terminal amino acid sequence of the 35 kDa major heat-modifiable protein of *H. parasuis* with that of the OmpA proteins of other bacterial species

Bacterial species	Amino acid sequence*
<i>H. parasuis</i>	APQADSFYVGAK
<i>H. ducreyi</i>	APQADTFYVGAK
<i>P. multocida</i>	APQPNTFYVGAK
<i>H. somni</i>	APQANTFYAGAK
<i>A. actinomycetemcomitans</i>	APQANTFYAGAK
<i>S. typhimurium</i>	APKDNTWYAGAK
<i>E. coli</i>	APKDNTWYTGAK

*Bold type indicates an amino acid residue different from the equivalent one in *H. parasuis*.

Reactivity of MAbs with reference strains and field isolates of *H. parasuis*

Results obtained in ELISA and dot ELISA (Table 1) using different antigens of *H. parasuis* serotypes 1 to 15 showed that MAbs 4D5 and 4G9 reacted with all the reference strains of *H. parasuis*. A total of 500 *H. parasuis* field strains representing several serotypes isolated from various organs (clinical cases), serotyped by indirect haemagglutination test using rabbit polyclonal antibodies (Tadjine *et al.*, 2004), were tested by dot ELISA using the two MAbs (Table 1). When using sonicated antigen, both MAbs (4D5 and 4G9) reacted with all the 500 field isolates. However, when using WC as the antigen, MAb 4G9 reacted with 81 % of field isolates, in contrast to MAb 4D5, which reacted with only 30 % of field isolates.

Detection of antibodies against OmpA and LPS antigens of *H. parasuis* in the sera of naturally infected pigs

As shown in Fig. 8, epitopes of OmpA reacting with MAb 4D5 (lane 1) and those reacting with MAb 4G9 (lane 2) were also recognized by sera from a hyperimmunized rabbit and hyperimmunized mouse (lanes 3 and 4). Epitopes reacting with MAb 4D5 were recognized by two sera from naturally infected pigs (lanes 5 and 6). Sera from specific-pathogen-free herds as well as those from uninfected pigs did not show any reactivity with these epitopes.

Reactivity of MAbs with other bacterial species

A total of 21 strains of other bacteria did not show any reactivity with either of the MAbs in ELISAs, Western-blot assays and dot-blot assays (Table 1).

Involvement of MAbs 4D5 and 4G9 in bactericidal, protection and clearance activities against *H. parasuis*

MAbs 4D5 and 4G9 were not involved in bactericidal activity against *H. parasuis* as they were unable to activate mouse and guinea pig complement.

Fig. 8. Western-blot analysis of OmpA of *H. parasuis* SW124 with MAb 4D5 (lane 1), rabbit polyclonal antiserum produced against SW124 (lane 3), mouse polyclonal antiserum produced against OmpA of SW124 (lane 4), sera from two pigs naturally infected with *H. parasuis* (lanes 5, 6) and serum from a specific-pathogen-free pig (lane 7). Lane 2 represents MAb 4G9 with LPS of *H. parasuis*.

As early as 2 h after infection, MAbs 4D5 and 4G9 completely eliminated bacteria from blood, whereas in mice injected with PBS or normal serum, bacteria could still be recovered 24 h after infection, indicating that bacterial clearance was antibody-specific (Fig. 9).

Results obtained in the protection assay (Table 3), showed that all the mice in the negative control groups and in the group injected with MAb 4D5 were seriously ill or died. None of the mice in the group treated with MAb 4G9 died.

H. parasuis was not isolated from liver, spleen, heart or lung tissues of mice infected with bacteria with or without MAbs when they died or were killed 7 days after infection. However, a species-specific antigen was detected in the tissues of mice infected with *H. parasuis* in PBS or in normal serum using the MAb-based CoA test. The antigen

Fig. 9. Clearance of circulating *H. parasuis* in BALB/c mice injected with rabbit polyclonal and murine monoclonal antibodies 4D5 and 4G9, normal mouse serum and PBS.

Table 3. Protective activity of rabbit polyclonal and murine monoclonal antibodies 4G9 and 4D5 in mice against challenge infection with *H. parasuis* strain

No. of bacteria injected (c.f.u. ml ⁻¹)	Substance injected	No. of mice that died/ No. of mice inoculated	Protection (%)
10 ⁹	Rabbit antiserum	1/6	83
10 ⁹	MAb 4G9	0/6	100
10 ⁹	MAb 4D5	4/6	33
10 ⁹	PBS	6/6	0
10 ⁹	Normal serum	6/6	0

was not detected in tissues from mice treated with MAbs 4D5 or 4G9.

DISCUSSION

Not much is known about the pathogenesis, virulence factors and immunogenicity of *H. parasuis*, which makes control of systemic infections difficult. Knowledge of the composition and structural determination of the major antigens involved in virulence may provide crucial information that could lead to the development of specific serodiagnostic tools as well as effective vaccines. In this study, two MAbs against *H. parasuis* were produced and characterized, and their protective role was investigated in mice.

MAb 4D5 was directed against a proteinic epitope as shown by sensitivity to proteolytic enzymes and resistance to periodate oxidation. An OMP of 35 kDa from *H. parasuis* showed heat-modifiability after solubilization at 100 °C. Heat-modifiable properties of MOMP have been reported within both porin and OmpA proteins of *Enterobacteriaceae* (Nikaido & Vaara, 1985) and in *P. multocida* (Lugtenberg *et al.*, 1986). Hartmann *et al.* (1995) reported a MOMP of about 42 kDa of *H. parasuis* which did not display any heat-modifiability after solubilization at 37 °C. N-terminal homology suggested that this MOMP was related to porin protein and that our 35 kDa heat-modifiable OMP of *H. parasuis* was related to the OmpA family.

Prasadarao *et al.* (1999, 1996) examined OmpA of *E. coli* K1, a highly conserved 35 kDa protein, for its role in invasion of brain microvascular endothelial cells (BMEC). The invasive capability of the OmpA⁺ strains was 25- to 50-fold greater than that of OmpA⁻ strains. Invasiveness of the OmpA⁻ strains was restored to the level of the OmpA⁺ strain by complementation with the *ompA* gene. These results suggest that OmpA is one of the factors required for *E. coli* invasion of BMEC. The current understanding of the pathogenic mechanism and the precise role of OmpA in *E. coli* translocation of blood-brain barrier (BBB) were reported by Kim (2002). Besides, OmpA plays a structural role in the integrity of the bacterial cell surface (Koebnik *et al.*, 2000). OmpA is known to be involved in bacterial conjugation, to act as a receptor for bacteriophage, to mediate virulence and pathogenicity, and to form an

integral part of the membrane structure (Pautsch & Schulz, 2000, 1998; Koebnik, 1995). Thus, OmpA appears as a new type of pathogen-associated molecular pattern (PAMP) usable as a vector to provoke immunity (Jeannin *et al.*, 2002).

The second MAb, 4G9, was directed against an LPS epitope, as shown by sensitivity to periodate oxidation and resistance to proteolytic enzymes and heat treatment. It appeared to react with a core oligosaccharidic part of the LPS. Colony-blotting assay and electron microscopy analysis indicated that, unlike MAb 4D5, MAb 4G9 recognized epitopes exposed on the cell surface. Western-blot analysis indicated that both MAb 4D5 and MAb 4G9 recognized epitopes shared by all the reference strains. MAb 4G9 reacted with a core oligosaccharidic part of LPS and not with the O chain of LPS (Fig. 1, lane 5). It is speculated that the epitopes recognized by MAb 4G9 are partially exposed on the bacterial surface as shown in Fig. 7(b). Treatment of WC with sodium periodate destroyed all the exposed epitopes, which explains the negative reaction in the ELISA (Fig. 6b). Using the same antigen in SDS-PAGE, hidden epitopes were exposed and reacted with MAb 4G9 in Western blot (Fig. 5, lanes 9–11). Identical results were obtained in both ELISA and Western blot when purified LPS was used in place of WC as the antigen (data not shown).

Adherence to the surface of epithelial cells is important for colonization and pathogenicity of numerous bacterial species. LPSs are essential structural components of outer membranes of all the Gram-negative bacteria. LPS is an important virulence factor of *A. pleuropneumoniae* (Haesebrouck *et al.*, 1997; Tascon *et al.*, 1996) and a major adhesin involved in adherence to porcine respiratory tract cells (Jacques *et al.*, 1991; Bélanger *et al.*, 1990), mucus (Bélanger *et al.*, 1994, 1992) and to host glycosphingolipids (Abul-Milh *et al.*, 1999). Paradis *et al.* (1996) demonstrated that LPS can traverse the thick capsular material and reach the outer-most region of the cell. This may explain the positive reaction obtained in the colony-blotting assay and electron microscopy with MAb 4G9 using whole cells in our studies (Fig. 7). The development of a diagnostic tool or a vaccine should be based on molecules that are easily accessible to the host's immunological response cells and antibodies during the infection process.

The MAbs 4D5 and 4G9 reacted only with *H. parasuis* species and not with other species from the group *Haemophilus* or other members of *Pasteurellaceae* family, as shown in Table 1. These results indicated that the two MAbs may be directed against species-specific epitopes and could be used for identification of *H. parasuis* species. However, *Actinobacillus indolicus* is the most closely related organism to *H. parasuis* (Møller *et al.*, 1996) and is also a commensal organism that can be isolated from the upper respiratory tract of healthy pigs (Oliveira *et al.*, 2001). The taxonomic classification of *H. parasuis* and *A. indolicus* is still controversial, and the MAbs may help to better define whether these organisms are different, particularly in view of the fact that they are 96% similar regarding the 16S rRNA gene sequence (Møller *et al.*, 1996). We envisage that we will study the antigenic relationship between *A. indolicus* and *H. parasuis* using MAbs. Antibodies against both OmpA and LPS were present in sera from experimentally infected mice and rabbits as well as in pigs naturally infected with *H. parasuis*, suggesting that these two epitopes are recognized by the immune system. These epitopes may be involved in protection against *H. parasuis* infection in mice (mostly endotoxic shock), but the same may not be true in pigs. Rabbits and mice were hyperimmunized (at least 6 inoculations) and sera obtained from these animals may contain antibodies against both OMP and LPS antigens (Fig. 8). Sera from naturally infected pigs showed antibodies only against OMP, as shown in Fig. 8. However, a few sera also reacted with both OMP and LPS epitopes (data not shown). In view of the fact that protein antigens are more potent immunogens than LPSs, pigs exposed recently to *H. parasuis* may show antibodies only against OMP and antibodies against LPS may be produced later. Thus the detection of antibodies against OMP or both OMP and LPS may depend on the early or late stage of infection in pigs.

The results of bacterial clearance and protection assays demonstrated that the MAb against the LPS was able to diminish the bacterial invasion from the peritoneal cavity and later completely eliminate the bacteria from the blood (Fig. 9; Table 3). Although this MAb was unable to activate complement *in vitro*, it conferred complete protection in mice against challenge infection. It is speculated that this protection was mainly due to its ability to neutralize endotoxin in blood released by *H. parasuis*. These results are in agreement with those reported by Amano *et al.* (1994). They detected endotoxin in the plasma of inoculated pigs in the acute stage of infection and reported that septicaemia caused by *H. parasuis* induced disseminated intravascular coagulation and endotoxin shock, resulting in the aggravation of clinical signs and death in the affected pigs.

The MAb against OmpA was also able to induce bacterial clearance from the blood when inoculated with a sublethal dose, but failed to completely protect mice against a massive lethal dose resulting in endotoxin shock followed by

death. Based on the findings of Amano *et al.* (1994) and our results in this study, it is suggested that mortality in mice may be primarily due to endotoxemia resulting from overwhelming infection. Unlike the MAb against LPS, the MAb against OmpA was not able to neutralize the endotoxin released.

In conclusion, two MAbs were produced and characterized; MAb 4D5 recognized a major heat-modifiable OMP of *H. parasuis*, which was shown to be structurally related to the OmpA family, and MAb 4G9 recognized an epitope of LPS nature. The detection of antibodies to the OmpA protein and LPS in the sera of pigs naturally infected with *H. parasuis* suggested that these components may potentially be important in pathogenesis. MAbs against OmpA and LPS epitopes were involved in protecting mice against lethal challenge infection.

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