Cross Protection Among *Haemophilus parasuis* Strains in Immunized Gnotobiotic Pigs

O. Paul Miniats, Nonie L. Smart and Søren Rosendal

ABSTRACT

In an attempt to establish if cross protection can be induced by different strains of *Haemophilus parasuis*, three groups of 12 gnotobiotic pigs were immunized each with an aluminum hydroxide adsorbed whole cell bacterin of one of three *H. parasuis* strains. Two weeks later, four pigs within each vaccinated group were challenged with aerosols of live cultures of each of the three test strains and observed for response. Two virulent strains V1 and V2 protected all the vaccinated pigs, while all non-vaccinated controls succumbed to Glasser's disease when challenged with these strains. Vaccination with strain LV (of low virulence) protected the pigs against challenge with strain V2, but not against strain V1. Strain LV did not cause disease in the immunized animals and only in one of ten non-immunized pigs upon second challenge. The results suggest that strains may differ in antigenicity and that virulence and immunoprotection are positively related. Strains to be used in commercial vaccines should therefore be selected carefully. Antibodies detected in the sera of vaccinated pigs were to outer membrane proteins of the bacteria, but not to lipopolysaccharides or capsular polysaccharides. This would suggest that for gnotobiotic pigs outer membrane proteins are more immunogenic than lipopolysaccharide or capsular antigens. Further work is needed to determine if outer membrane proteins also contribute protective immunogens.

RÉSUMÉ

Dans le but d'établir si une protection croisée peut être induite par différentes souches d'*Haemophilus parasuis*, trois groupes de 12 porcs gnotobiotiques furent immunisés. Trois bactérines adsorbées à l'hydroxyde d'aluminium provenant d'autant de souches d'*H. parasuis* furent utilisées. Deux semaines plus tard, quatre porcs appartenant à chacun des groupes vaccinés furent exposés, par aérosol, aux cultures vivantes de chacune des trois souches testées. Tous les porcs vaccinés furent protégés contre l'infection par les deux souches virulentes V1 et V2 alors que les témoins non-vaccinés ont été atteints de la maladie de Glasser lors de l'épreuve avec ces mêmes souches. La vaccination avec la souche LV (peu virulente) a protégé les porcs contre la souche V2 mais non contre la souche V1. La souche LV n'a pas causé de maladie chez les animaux immunisés et seulement chez un des dix porcs non-immunisés après la deuxième épreuve. Les résultats suggèrent que les souches diffèrent antigéniquement et que la virulence et l'immunoprotection sont reliées. Les souches à inclure dans les vaccins commerciaux doivent donc être sélectionnés minutieusement. Les anticorps détectés dans le sérum des porcs vaccinés étaient dirigés contre les protéines de la membrane externe et non contre les lipopolysacharides ou les polysaccharides capsulaires. Pour les porcs gnotobiotiques les protéines de la membrane externe seraient plus immunogènes que les lipopolysaccharides ou les antigènes capsulaires. D'autres expériences seront nécessaires afin de déterminer si les protéines de la membrane externe représentent des immunogènes pouvant contribuer à la protection chez l'animal. *(Traduit par Dr Daniel Dubreuil)*

INTRODUCTION

We previously reported that all conventional and most specific pathogen free (SPF) swine herds tested in Ontario contained pigs carrying the causative organism of Glasser's disease, *Haemophilus parasuis*, in nasal passages without signs of clinical disease (1,2). Many strains of the organism are present in the Ontario pig population (1,3). It has been noticed that pigs originating from SPF herds frequently succumb to Glasser's disease following exposure to conventional pigs (1). A trivalent vaccine was developed which protected weaned pigs (1,4) and early vaccinated gnotobiotic primary SPF pigs (5,6) against challenge with homologous strains of *H parasuis*. This vaccine has also protected farm reared SPF pigs against Glasser's disease under field conditions (Smart, unpublished data). However, it has not been established experimentally if cross protection among different *H. parasuis* strains actually exists. Furthermore, in our previous work we have not found a serological method whereby resistance to *H. parasuis* could be predicted in immunized,

Department of Population Medicine (Miniats, Smart) and Department of Veterinary Microbiology and Immunology (Rosendal), Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1. Present address of Dr. N.L. Smart: Agriculture Canada, Animal Diseases Research Institute, P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9.

Submitted April 16, 1990.

colostrum-deprived, gnotobiotic pigs. This series of experiments was aimed at exploring these two aspects — cross protection among *H. parasuis* strains in vaccinated pigs and serological evidence of immunity.

MATERIALS AND METHODS

TEST STRAINS

Three strains of *H. parasuis* (V1, V2 and LV), previously isolated from pigs in Ontario and characterized by endonuclease fingerprinting (1,3), were selected for the cross protection studies. Strains V1 and V2 originated from pigs affected with Glasser's disease (1) and have been found experimentally to be pathogenic for both farm raised SPF and gnotobiotic pigs (1,5,6). Strain LV was an isolate from the nasal passage of a clinically healthy conventional pig (1).

TEST FOR VIRULENCE

Initially strain LV was tested for pathogenicity by exposing four ten day old gnotobiotic pigs to an aerosol of viable bacteria as described previously (1,4,6). Fourteen days later, three of these animals were challenged again in a similar manner with strain V1.

VACCINES AND CHALLENGE MATERIAL

A monovalent formalin killed whole cell, aluminum hydroxide adsorbed bacterin of each of the three *H. parasuis* strains was prepared. The material for challenge was a suspension in buffered saline of a fresh 18 h culture of each strain containing approximately 10^9 colony-forming units per mL. The preparation of the bacterin and the challenge material have been described previously (1,4,6).

EXPERIMENTAL ANIMALS

The experimental animals were 43 cesarean derived colostrum deprived gnotobiotic Yorkshire piglets reared free of detectable live microbial associates (7) until purposely infected with one of the three *H. parasuis* strains. Four of the piglets were used for a pathogenicity test of strain LV and infected initially at ten days of age. The remaining 39 pigs were used for the cross protection studies and exposed to one of the three *H. parasuis* strains when seven weeks old. The animals were treated in accordance with Canadian Council on Animal Care guidelines throughout the experiments.

EXPERIMENTAL DESIGN

Three cross protection trials were conducted. In each, 13 pigs were distributed among three isolators. The first two isolators in each trial housed four pigs each and the third isolator housed five pigs. At three and five weeks of age, three pigs in each isolator were vaccinated with one of the bacterins. The remaining pigs were nonimmmunized controls. In trial A, pigs in all three isolators were vaccinated with *H. parasuis* strain V1, in trial B, with strain V2, and in trial C, with strain LV. The dose of the bacterin was 4 mL, 2 mL injected subcutaneously behind each ear.

Two weeks following the second vaccination, pigs in each of the three isolators were challenged with one of the test strains by administering 8 mL of the *H. parasuis* suspension as an aerosol over 30 min as previously described (6). The pigs in isolator 1 were challenged with strain V1, in isolator 2 with strain V2, and in isolator 3 with strain LV.

The response to the challenge was evaluated on the basis of clinical signs, survival time, gross pathological lesions, and isolation of *H. parasuis* from the pigs as described (6). Blood samples for serology were collected 2 wk after the second immunization and from survivors prior to euthanasia.

DETECTION OF ANTIBODIES IN SERA OF GNOTOBIOTIC PIGS VACCINATED WITH *HAEMOPHILUS PARASUIS*

Antibodies against outer membrane proteins — An outer membrane protein (OMP) enriched fraction was prepared from *H. parasuis* strain V1 according to Barenkamp *et al* (9). Briefly, the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes (8). The membrane was treated with a buffered skim milk solution for 2 h before it was cut into 7 mm wide strips which were treated individually with the sera listed in Table III. The sera were diluted 1:100 and strips incubated at room temperature for 1 h. This was followed by three saline-Tris (pH 7.4) washes of 10 min each. All strips were then incubated for 1 h at room temperature with protein — A peroxidase conjugate (Miles Scientific, Rexdale, Ontario) diluted 1:2000 and again washed. The presence of antibody was detected by the application of H_2O_2 and 4-chloro-naphthol (Fisher Scientific, Don Mills, Ontario) for 20 min at room temperature.

Search for antibodies against lipopolysaccharide — Lipopolysaccharide (LPS) was extracted from *H. parasuis* V1 following the procedure described by Darveau and Hancock (10), subjected to SDS-PAGE, and elec-

TABLE I. Response of gnotobiotic pigs to immunization and challenge with *Haemophilus parasuis* strains V1, V2, and LV

Trial	*Haemophilus parasuis* strain Vaccine	Challenge	Vaccinated pigs n	Vaccinated pigs n sick/died	Nonvaccinated pigs n	Nonvaccinated pigs n sick/died
A	V1	V1	3	0	1	1
	V1	V2	3	0	1	1
	V1	LV	3	0	2[a]	0
B	V2	V1	3	0	1	1
	V2	V2	3	0	1	1
	V2	LV	3	0	2[a]	0
C	LV	V1	3	2	1	1
	LV	V2	3	0	1	1
	LV	LV	3	0	2[a]	1[b]

[a]Two challenges
[b]One of the nonimmunized pigs developed arthritis after two challenges

troblotted onto nitrocellulose as described for the OMP. Strips of the nitrocellulose membrane were stained with the sera in Table III diluted 1:100.

Search for antibodies to capsular polysaccharide — Strain *H. parasuis* V1 was cultured in 2 L of dialyzed tryptone yeast extract broth until late logarithmic growth phase. The bacteria were sedimented by centrifugation and washed once in phosphate-buffered saline (PBS) pH 7.4. The supernatant from the culture and the wash were combined and the polysaccharides extracted by hexadecyltrimethylammonium bromides, Kodak (Canadawide Scientific Ltd., Toronto, Ontario) as described (11). Proteins were digested with proteinase K and the LPS was removed by ultra centrifugation. The capsular polysaccharide was used as antigen in an enzyme-linked immunosorbent assay (ELISA) using 25 μg polysaccharide per well. The sera in Table III were tested at a dilution of 1:50 (11).

RESULTS

The four piglets subjected to the pathogenicity test of *H. parasuis* strain LV showed no clinical signs of disease. When three of the same piglets were rechallenged 14 days later with strain V1, one died with septicemia four days later, the second had a severe purulent polyarthritis and the third animal showed no clinical signs of Glasser's disease, but had a mild arthritis in the tarsal and carpal joints when euthanized and necropsied eight days post-infection.

In the cross protection tests (Table I), none of the pigs immunized with bacterin of the virulent *H. parasuis* strains V1 and V2 showed clinical signs of Glasser's disease following challenge with these two strains or strain LV. Two of the three pigs vaccinated with strain LV became clinically sick and the third had arthritic lesions at necropsy following challenge with strain V1, but none after challenge with strain V2 or with the homologous strain LV.

When challenged with strains V1 or V2, all six of the nonimmunized control pigs exposed to these strains developed Glasser's disease. Of the nonimmunized pigs exposed to *H. parasuis* strain LV, none became sick after the initial challenge but one did succumb to the disease five days after second challenge with the same strain and had lesions of polyserositis at necropsy.

TABLE II. Disease patterns in gnotobiotic pigs affected with Glasser's disease following challenge with *Haemophilus parasuis*

Pig no.	Vaccination status	Challenge strains	Day pi[a] Sick	Day pi Dead or moribound	Pathological lesions	*H. parasuis* isolation
1	Control	V1	3	4	Polyserositis	+
2	Control	V1	4	8	Polyserositis	+
3	Control	V1	3	3	Polyserositis	+
4	Control	V2	3	4	Polyserositis	+
5	Control	V2	4	6	Polyserositis	+
6	Control	V2	4	4	Polyserositis	+
7	Vacc LV	V1	3	7	Polyserositis	+
8	Vacc LV	V1	3	7	Polyserositis	+
9	Vacc LV	V1	0	–	Mild arthritis[b]	–
10	Control	LV	5	–	Polyserositis[c]	+

[a]Postinoculation
[b]Pig euthanized on day 10 PI
[c]Pig euthanized on day 5 PI

The pattern of the disease in affected pigs is presented in Table II. The results of serology are shown in Table III and Fig. 1. All vaccinated pigs had antibodies against OMP (lanes 3-12, Fig. 1), but the nonvaccinated pigs and the one exposed to an aerosol of viable strain LV organisms did not (lanes 13-18, Fig. 1). The two rabbits hyperimmunized with strains V1 and LV, respectively had antibodies to the major OMP of 37 kD molecular size and also to material of lower molecular weight interpreted as LPS (lanes 19 and 20, Fig. 1). It was not possible to determine whether antibodies to particular OMPs were correlated with protective immunity. One pig vaccinated with strain V1 was immune although its serum reacted only weakly with OMPs of 67 and 37 kD (lane 4, Fig. 1).

Only the hyperimmune rabbit sera recognized antigens in the blots of the LPS (example in lane 22, Fig. 1). None of the gnotobiotic pigs had antibodies to the LPS in the 1:100 serum dilution (examples in lane 21, Fig. 1).

When the sera were analyzed for antibodies to capsular polysaccharides the rabbit hyperimmune sera

TABLE III. History and antibody activity of sera from gnotobiotic pigs and rabbits vaccinated with *Haemophilus parasuis* strains V1 and LV

Serum no.	Vaccine strain	Time of sampling	Protective immune status	Antibodies detected against OMP	LPS	CPS
1 pig	V1 bacterin	prechallenge	+	+	–	–
2 pig	V1 bacterin	prechallenge	+	+	–	–
3 pig	V1 bacterin	postchallenge (V1)	+	+	–	–
4 pig	V1 bacterin	postchallenge (V1)	+	+	–	–
5 pig	LV bacterin	prechallenge	–	+	–	–
6 pig	LV bacterin	postchallenge (LV)	–	+	–	–
7 pig	LV bacterin	postchallenge (LV)	–	+	–	–
8 pig	LV bacterin	postchallenge (LV)	–	+	–	–
9 pig	LV bacterin	postchallenge (V1)	–	+	–	–
10 pig	live LV aerosol	postchallenge (V1)	–	+	–	–
11 pig	live LV aerosol	—	–	–	–	–
12 pig	—	postchallenge (V1)	–	–	–	–
13 pig	—	postchallenge (LV)	–	–	–	–
14 pig	—	—	–	–	–	–
15 pig	—	—	–	–	–	–
16 pig	—	—	–	–	–	–
17 rabbit	V1	postimmunization[a]	NA	+	+	+
18 rabbit	LV	postimmunization[a]	NA	+	+	+

OMP = outer membrane proteins, LPS = lipopolysaccharides, CPS = capsular polysaccharides
NA = not applicable
[a]Immunized intravenously six times over a three week period with formalin killed bacteria

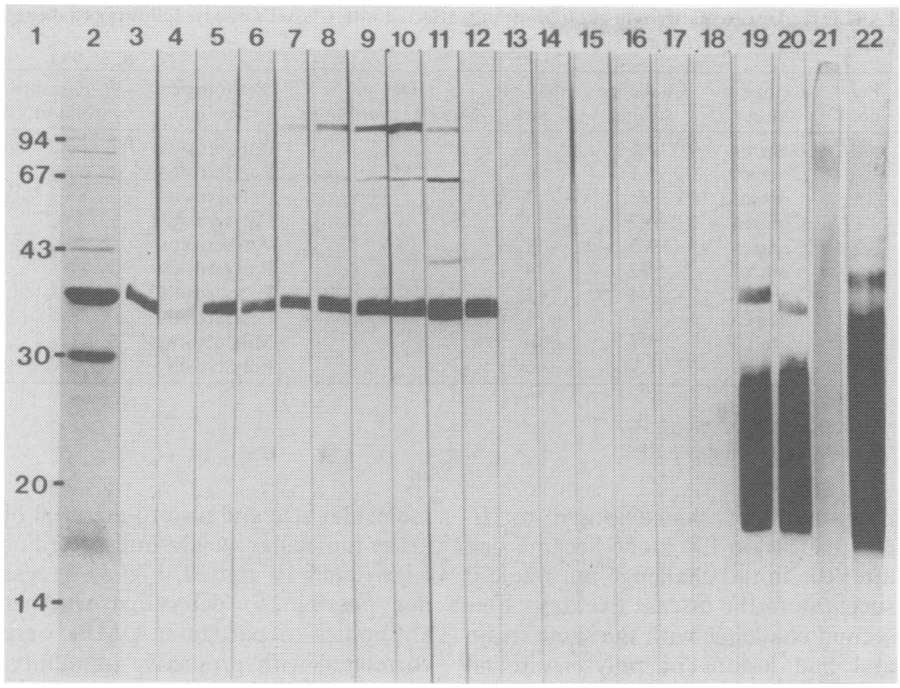

Fig. 1. Polyacrylamide gel electrophoresis of outer membrane proteins, immunoblotting of outer membrane proteins and lipopolysaccharide of *Haemophilus parasuis*, strain V1 using sera from gnotobiotic pigs and rabbits vaccinated with *H. parasuis* strains V1 and LV.
Lane 1 — Molecular weight references.
Lane 2 — Outer membrane proteins stained with coomassie blue.
Lane 3-20 — Immunoblots of outer membrane proteins using serum 1-18 (Table III).
Lane 21-22 — Immunoblots of lipopolysaccharide using serum 10 and 17 respectively (Table III).

gave readings of optical density (OD) > 0.800 in dilutions of 1:50 whereas the gnotobiotic pig sera consistently gave zero readings in dilutions of 1:50.

It was concluded that the gnotobiotic pigs vaccinated with *H. parasuis* bacterin had antibodies to OMP, but no detectable antibodies to LPS or capsular polysaccharide.

DISCUSSION

Using a limited number of *H. parasuis* strains and gnotobiotic pigs, this work suggests that there is a difference in the virulence and protective immunizing ability among different strains, and that there may be a positive relationship between virulence and protective immunogenicity of a given strain. Gnotobiotic pigs were chosen for these experiments because of the omnipresence of *H. parasuis* (2) in the Ontario pig population. It would have been difficult to find animals other than gnotobiotes which would be free of previous exposure to *H. parasuis* and suitable for this type of study.

Vaccination with the two virulent strains induced protection against each other, whereas vaccination with the low virulent strain resulted in protection against one but not the other of the two virulent strains tested. The lack of cross protection may also be due to antigenic differences among various *H. parasuis* strains, although this has not been studied.

The failure of strain LV to cause disease in very young pigs in the pathogenicity test seemed to indicate that this strain was of low virulence. Yet, following repeated challenge of pigs of the same genetic background at an older age, one out of ten pigs became severely ill. This would suggest that virulence of these organisms is relative and dependent on the dose of infectious organisms at challenge and the susceptibility of individual pigs. The age of gnotobiotic pigs is probably of minor significance, as they receive minimal microbial stimulation of their immunological systems in their environment.

In view of the heterogenicity of different strains of *H. parasuis* (3), the variations in their virulence (12) and since cross protection among all the strains does not necessarily exist it is important that vaccines be carefully designed and incorporate appropriate strains. However, in the field, both in Denmark (13) and in Ontario (Smart, unpublished data), where vaccination of SPF pigs against Glasser's disease has been practised for several years, experiences suggest that the vaccine is effective against multiple strains and that breaks are unlikely to occur.

The serological results showed that gnotobiotic pigs vaccinated with *H. parasuis* bacterin had serum antibodies to OMP, but no detectable antibodies to LPS or capsule. Whether the antibodies to OMP protected the pigs surviving challenge is not known. The failure of some pigs with antibodies to the low virulence strain LV to survive challenge with the virulent strain V1 would suggest that these antibodies are not associated with protection, at least not against that particular strain.

As the pigs vaccinated with bacterin of *H. parasuis* strains V1 and V2 were resistant to challenge with these strains, but had no detectable serum antibodies against either the LPS or capsular polysaccharide antigens, it appears that these antibodies do not play an essential role in the protective immunity against *H. parasuis*. The role of OMP, LPS and capsular polysaccharides in eliciting protective immunity needs to be studied further.

ACKNOWLEDGMENTS

The authors thank Mr. B. Bloomfield and the late Mrs. M. Nadvornik for rearing the gnotobiotic pigs and assisting with the experiments, Mr. E. Ewert for the microbiology, and Ms. J.T. Bossé for the ELISAs.

The project was supported financially by the Ontario Pork Producers Marketing Board and by the Ontario Ministry of Agriculture and Food.

REFERENCES

1. **SMART NL.** *Haemophilus parasuis*: Subclinical infection and disease in southern Ontario swine; restriction endonuclease analysis of field isolates and prevention of Glasser's disease through vaccination. MSc Thesis, University of Guelph, 1987.

2. **SMART NL, MINIATS OP, ROSENDAL S, FRIENDSHIP RM.** Glasser's disease and prevalence of subclinical infection with *Haemophilus parasuis* in swine in southern Ontario. Can Vet J 1989; 30: 339-343.
3. **SMART NL, MINIATS OP, MacINNES J.** Analysis of *Haemophilus parasuis* isolates from southern Ontario swine by restriction endonuclease fingerprinting. Can J Vet Res 1988; 52: 319-324.
4. **SMART NL, MINIATS OP.** Preliminary assessment of a *Haemophilus parasuis* bacterin for use in specific pathogen free swine. Can J Vet Res 1989; 53: 390-393.
5. **MINIATS OP, SMART NL.** Immunization of primary SPF pigs against Glasser's disease. Proc 10th Int Pig Vet Soc Congr, Rio De Janeiro, Brazil, 1988: 157.
6. **MINIATS OP, SMART NL, EWERT E.** Vaccination of gnotobiotic primary specific pathogen-free pigs against *Haemophilus parasuis*. Can J Vet Res 1991; 55: 33-36.
7. **MINIATS OP, JOL D.** Gnotobiotic pigs — derivation and rearing. Can J Comp Med 1978; 42: 428-437.
8. **MacINNES J, ROSENDAL S.** Analysis of major antigens of *Haemophilus (Actinobacillus) pleuropneumoniae* and related organisms. Infect Immun 1987; 55: 1626-1634.
9. **BARENKANP SJ, MUNSON RS, GRANOFF DM.** Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profiles. J Infect Dis 1981; 143: 668-676.
10. **DARVEAU RP, HANCOCK REW.** Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J Bacteriol 1983; 155: 831-838.
11. **BOSSÉ JT, JOHNSON RP, ROSENDAL S.** Capsular polysaccharide antigens for detection of serotype-specific antibodies to *Actinobacillus pleuropneumoniae*. Can J Vet Res 1990; 54: 320-325.
12. **BLOCH I, NICOLET J.** Epidemiological studies on the distribution of *H. parasuis* in pigs. Proc 9th Int Pig Vet Soc Congr, Barcelona, Spain, 1986: 278.
13. **NIELSEN R, DANIELSEN V.** An outbreak of Glasser's disease. Studies of etiology, serology, and effect of vaccination. Nord Vet Med 1975; 27: 20-25.