RESEARCH REPORT

SWINE HEALTH

Title: Use of an uncapsulated *Haemophilus* Parasuis Vaccine in
NPB #00-030

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I. Abstract

Haemophilus parasuis is the causative agent of the porcine polyserositis, arthritis and meningitis, also known as Glasser's disease. Control of *H. parasuis* infections by current available commercial vaccines is variable and unsatisfactory. An uncapsulated *H. parasuis* vaccine was tested in colostrum-deprived pigs and its effectiveness in protecting susceptible animals against homologous challenge was evaluated. A *H. parasuis* strain highly prevalent in swine herds was submitted to a heat treatment for capsule extraction and uncapsulated bacteria were used to produce a killed vaccine. Colostrum-deprived pigs were vaccinated twice, with a two-week interval, by the intra-muscular route. Homologous challenge was performed two weeks after the second vaccination. The nonimmunized control pig developed swollen joints and high fever (105°) 24h post-challenge. At 72h post-challenge, central nervous system signs were observed and the pig died before euthanasia could be performed. Vaccinated animals showed no clinical signs after challenge. The nonimmunized control pig exhibited macro and microscopic lesions characteristic of *H. parasuis* infection. Swabs taken from lesions were PCR and culture positive for *H. parasuis*. Vaccinated animals showed no evident lesions and were PCR and culture negative for *H. parasuis*. The uncapsulated *H. parasuis* vaccine was successful in protecting colostrum-deprived pigs against homologous challenge.

II. Introduction

Haemophilus parasuis is the causative agent of the porcine polyserositis, arthritis and meningitis, a severe and often fatal syndrome that affects swine at all stages of production, especially in high-health herds. Control of *H. parasuis* infections relies on two major factors: early diagnosis of infection and development of immunity against the agent. To date there is no universally effective vaccine against *H. parasuis*. This is in part due to the fact that there are 15 antigenically distinct capsular serotypes as well as antigenic subtypes of some serotypes. Vaccination with a killed whole cell vaccine prepared from one serovar does not always confer protection against other serotypes,

or even against subtypes of the same serovar. This scenario is further complicated by the high number of nontypable *H. parasuis* isolates, which indicates that more that 15 different serovars could be circulating within a swine population at a given time[1].

Studies have shown that the presence of anti-capsule antibodies in vaccinated animals is not necessarily related to protection. Conversely, protective immunity was found to be consistent with the presence of anti-outer membrane proteins (OMP) antibodies [2]. Virulent *H. parasuis* strains tend to have similar OMP profiles[3], and also to induce effective protection against both homologous and heterologous *H. parasuis* challenge[4].

The capsular material present in Gram-negative bacteria is a potential inhibitor of phagocytosis[5]. Since bacterial antigen processing and presentation by macrophages is a critical event during the development of an effective humoral and cellular immune response[6], expression of a thick capsule by virulent *H. parasuis* strains could be one of the major factors involved in lack of cross-protection between heterologous strains.

Considering that the majority of *H. parasuis* virulent strains have similar OMP patterns and that protective immunity is mainly related to the recognition of these subcapsular antigenic determinants, we hypothesize that vaccination of naive animals using a uncapsulated virulent *H. parasuis* strain will protect against homologous challenge.

III. Objectives

1. To develop a protocol for capsule extraction without altering substantially outer-membrane proteins profiles.
2. To evaluate the effectiveness of an uncapsulated *H. parasuis* killed vaccine in protecting colostrum-deprived pigs against homologous challenge.

IV. Procedures

Bacterial strain: *Haemophilus parasuis* strain 29775 was selected for vaccine production and challenge. The characteristics of this strains have been described previously[7]. Routine genotyping in our laboratory has shown that this is one of the most prevalent *H. parasuis* strains involved in outbreaks in swine herds.

***In vivo* passages**: To enhance capsule expression *H. parasuis* strain 29775 was submitted to an *in vivo* passage using chicken eggs. Bacteria was grown in chocolate agar for 18 hours at 37°C in a 5% CO_2 atmosphere and harvested from plate using sterile PBS. Two hundred microliters of the bacterial suspension were inoculated in the corium-allantoid membrane (CAM) of a 10-day-old embryonated chicken egg. A noninoculated egg was used as control. Inoculated and control eggs were incubated at 37°C for 72h. After incubation, *H. parasuis* was re-isolated from CAM using sheep blood agar to check purity. Non-passed and egg-passed *H. parasuis* were compared by Indian ink staining, acryflavine agglutination and Transmission Electron Microscopy (TEM) for capsule expression. To enhance the virulence potential of the challenge strain, *H. parasuis* strain 29775 was inoculated into a colostrum-deprived pig, by the intra-peritoneal route, using a 3 ml dose of a 1 x 10^4 CFU/ml bacterial culture. This pig was euthanized at 24h post-inoculation and swabs were collected from joints, abdominal and thoracic cavities and from the brain. Swabs were then plated in blood-agar for *H.*

parasuis isolation. A sample of the peritoneal fluid was collected for direct observation of *H. parasuis* by TEM.

***Haemophilus parasuis* capsule extraction:** Egg-passed *H. parasuis* was used for capsule extraction trials. Eighteen-hour bacterial growth was harvested from agar and suspended in sterile PBS. Bacterial suspension was incubated in water-bath at 65°C for 5 minutes. After heat treatment, bacterial suspension was washed in sterile PBS 5 times. The final pellet was suspended in PBS and used for vaccine production. Samples were taken from the bacterial suspensions before and after heat treatment and were compared for presence of capsule by Indian ink staining, acryflavine agglutination and TEM.

Vaccine production: Egg-passed *H. parasuis* was grown in chocolate agar for 18 hours at 37°C at a 5% CO_2 atmosphere. Bacterial growth was harvested from plates using sterile PBS. A sample was taken for CFU counting. Formalin was added to the bacterial suspension to a final concentration of 2% and a oil-in-water adjuvant was added to a final concentration of 10%.

Colostrum-deprived animals: In order to obtain "pathogen-free-like" newborn pigs, sows were attendant-farrowed so that pigs were caught as they were born. The umbilical cord was gently detached from the sow and disinfected with a 2% iodine solution. Each pig was freed from adhering membranes, dried with disposable paper towels, placed into a clean plastic container and transferred to an isolation room where they were kept warm with a heat lamp. To overcome the lack of passive immunity, newborn pigs were fed bovine colostrum obtained from pools of the first-day's secretions from dairy cows during the first 5 days after birth. After the fifth day of life, pigs were fed regular dry food. A total of five colostrum-deprived pigs were used in the vaccine trail.

Vaccination and evaluation of vaccine efficacy: Four colostrum-deprived piglets were vaccinated twice, with a two-week interval, by the intra-muscular route using a 3ml dose (1×10^9 CFU/ml). The first vaccination was performed at 10 days of age and the second vaccination at 24 days of age. One control piglet was not vaccinated. Blood samples were collected before the first vaccination and weekly after that. Sera were submitted to ELISA test (Biovet) for detection of anti-*H. parasuis* antibodies. Animals were challenged two weeks after the second vaccination by the intra-tracheal route, using a 3 ml dose of a 1×10^9 CFU/ml bacterial culture (pig-passed *H. parasuis* strain). Tonsillar swabs and rectal temperature were taken before challenge and daily after that. Animals were observed daily and clinical signs were scored according to severity (0-3). Three animals, including two vaccinated and the nonvaccinated control were necropsied on day 3 post-challenge. The two remaining pigs were euthanized on day 7 post-challenge. At necropsy, animals were observed for macroscopic lesions and samples from brain, lung, heart, joint, liver and spleen were taken for histological evaluation bacterial isolation and PCR to detect *H. parasuis*[8]. All *H. parasuis* isolates recovered from pigs were further genotyped using the rep-PCR technique[3] in order to confirm the re-isolation of the challenge strain.

V. Results

***In vivo* passages:** Comparison of the capsule expressed by non-passed and egg-passed *H. parasuis* isolates by TEM, Indian ink staining and acryflavine agglutination demonstrated that the *in vivo* passage considerably enhanced *H. parasuis* capsule expression. Egg-passed *H. parasuis* isolate showed a thicker capsule when compared with the non-passed isolate (Figure 1 A and B). Based on these results, the egg-passed isolate was further used for capsule extraction trials. Direct observation by TEM of peritoneal fluid obtained from the colostrum-deprived pig inoculated by the intra-peritoneal route also revealed further enhancement of capsule expression by the pig-passed isolate compared with the non-passed and the egg-passed isolates (Figure 1 C and D). *H. parasuis* was isolated in pure culture from the peritoneal fluid.

***Haemophilus parasuis* capsule extraction:** Transmission electron microscopy revealed that *H. parasuis* capsule was successfully extracted by the heat treatment (Figure 2A). Capsule extraction was further confirmed by Indian ink staining and positive acryflavine agglutination of the heat-treated isolate. Outer membrane proteins profiles showed that the major OMP (36 Kd) was not affected by the heat treatment. Conversely, OMPs weighting around 31 kd and 45 kd became weaker after the treatment (Figure 1B).

Vaccination and evaluation of vaccine efficacy: ELISA results are shown in Figure 3. Despite the fact that all pigs were negative by the test cut-off (0,6), all vaccinated pigs had increasing antibody titers against *H. parasuis* compared with the nonvaccinated pig. On day one after challenge, the non-vaccinated control pig developed a fever of 105°F (Figure 4). No high fever was observed for the vaccinated group. After challenge the control pig developed swollen joints, which was aggravated on day two post-challenge. On day three post-challenge, the control pig developed severe central nervous system signs characterized by trembling, prostration and pedaling. The vaccinated animals did not showed any clinical signs after challenge. The control pig died on day three post-challenge. Two vaccinated pigs (pigs 30 and 32) were also euthanized on day three post-challenge to allow comparison of lesions with the nonvaccinated pig (pig 64). Pigs 31 and 34 were maintained until day 7 post-challenge and were euthanized by then. Vaccinated animals showed no macroscopic or microscopic lesions, and swabs taken during the necropsy were PCR and isolation negative for *H. parasuis*. The nonvaccinated animal developed severe fibrinous polyserositis, polyarthritis and meningitis, which were evident in both macroscopic and histological evaluation. *Haemophilus parasuis* was isolated in pure culture from all tissues and swabs taken during the necropsy were all positive by PCR (Figure 5A). The re-isolation of the *H. parasuis* strain used for challenge was confirmed by rep-PCR (Figure 5B).

Conclusions

We have developed a suitable technique to extract the capsule of highly capsulated *H. parasuis* strains without substantially changing the OMP profiles. The uncapsulated vaccine successfully protected vaccinated colostrum-deprived pigs against homologous challenge. This study supports the idea that development of protective immunity against *H. parasuis* may not involve the capsule. Considering that virulent *H. parasuis* strains have similar OMP profiles and that capsular type do not participate in development of

protective immunity, this vaccine is a suitable candidate for cross-protection among different *H. parasuis* serovars. Further studies will be conducted in order to better access the effectiveness of this uncapsulated *H. parasuis* vaccine in protecting susceptible animals against heterologous challenge.

References

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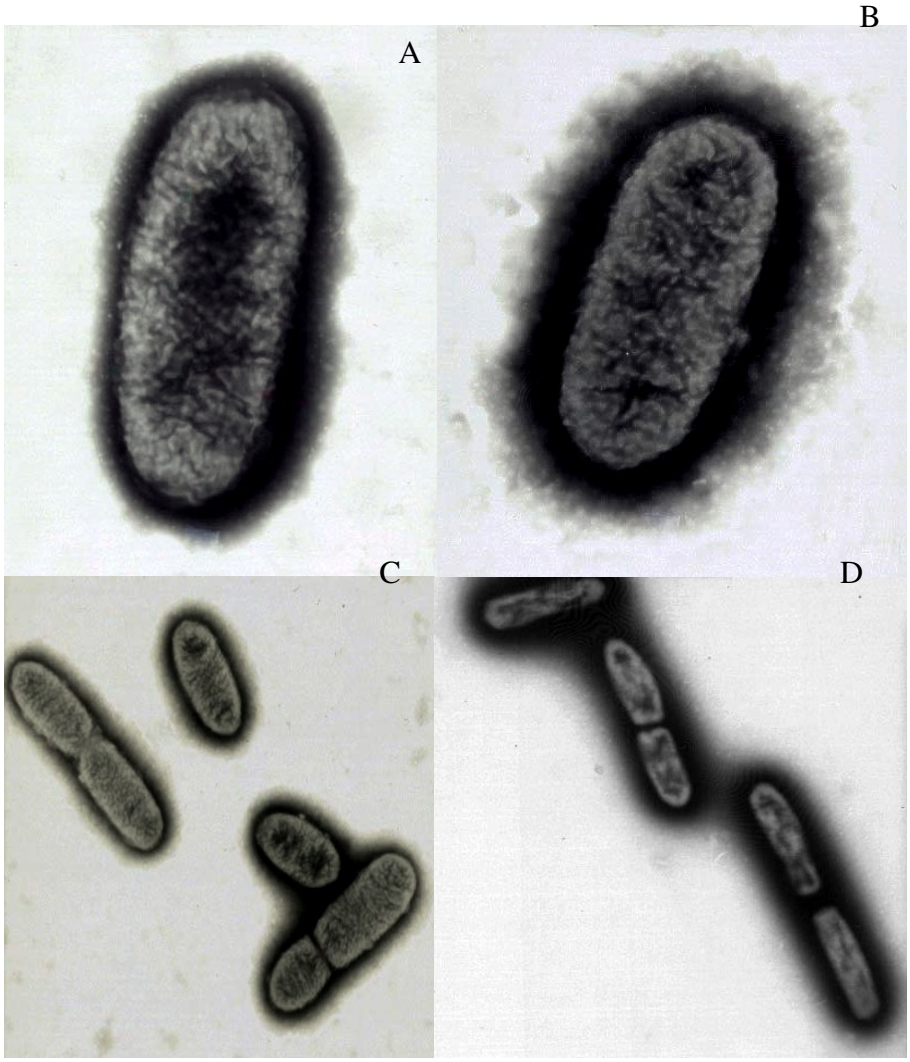

Figure 1. Transmission Electron Microscopy of *Haemophilus parasuis* isolates. A. *In vitro* passed *H. parasuis* isolate (45,000x). B. Egg-passed *H. parasuis* isolate (45,000x). C. Egg-passed *H. parasuis* isolate (13,000x). D. Direct observation of peritoneal fluid (pig-passed *H. parasuis* isolate) (13,000x).

1 2 3 4

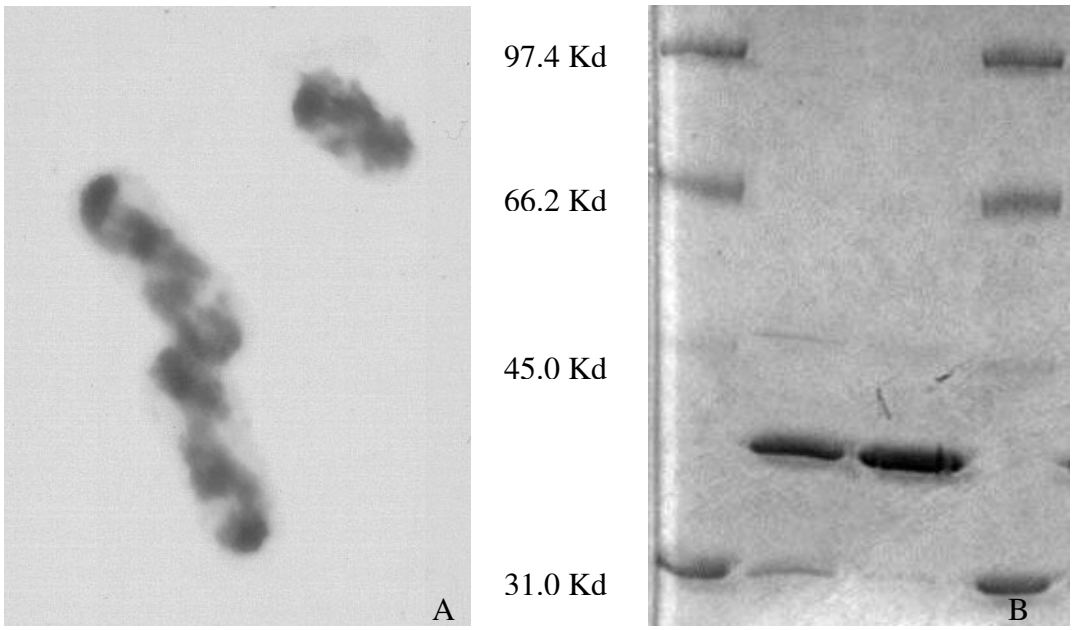

Figure 2. A. Transmission Electron Microscopy of heat-treated *H. parasuis*. B. Outer Membrane Protein profiles of non-treated (lane 2) and heat-treated (lane 3) *H. parasuis.* Lanes 1 and 4 – Molecular weight marker.

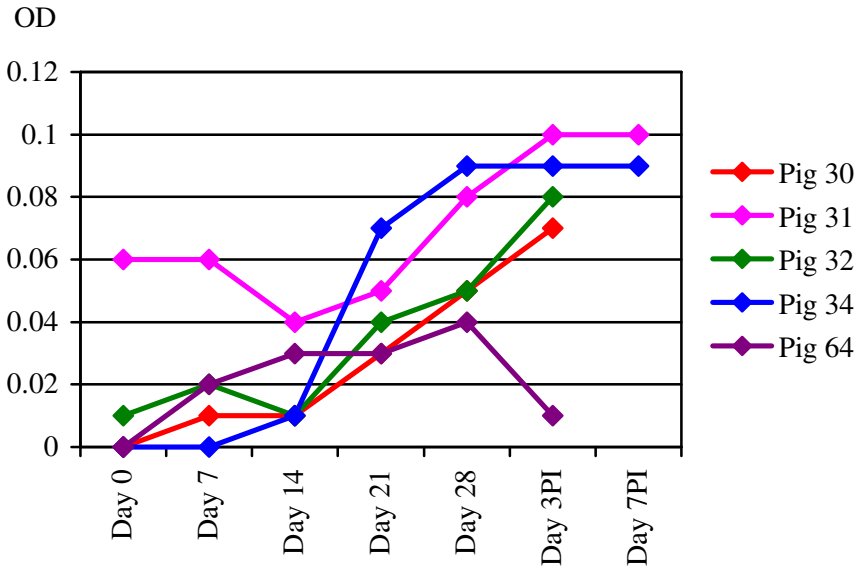

Figure 3. ELISA titers for anti-*Haemophilus parasuis* antibodies. Vaccinated group - Pigs 30, 31, 32 and 34. Nonvaccinated control - Pig 64. Day 0 – First vaccination. Day 14 – Second vaccination. Day 28 – Challenge. PI – Post-infection. Test cut-off – 0.6 OD.

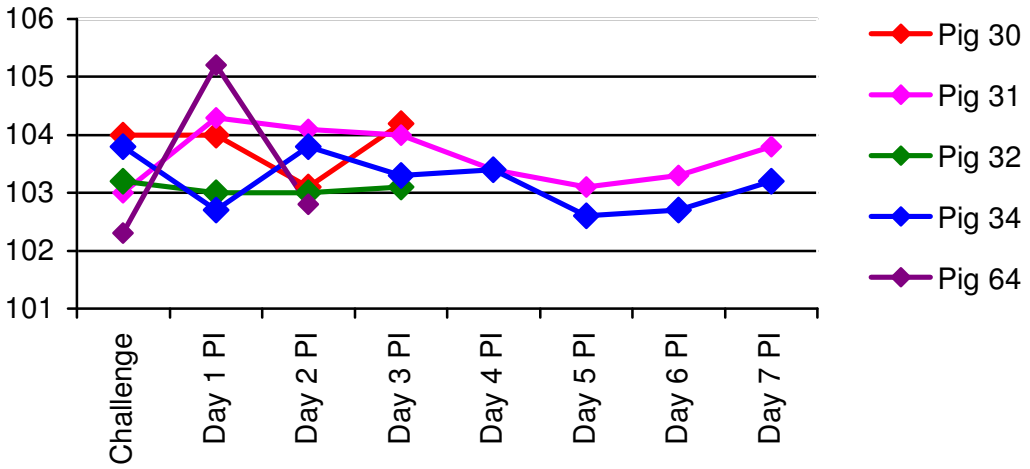

Figure 4. Temperature values before and after challenge. Vaccinated group - Pigs 30, 31, 32 and 34. Nonvaccinated control - Pig 64.

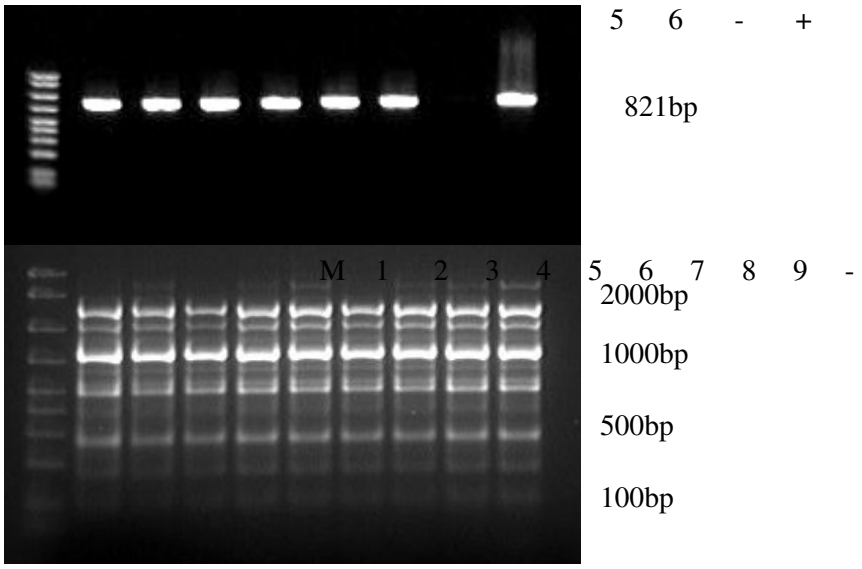

Figure 5. A. PCR results for brain, spinal-fluid, pleura, pericardium, peritoneum, joint swabs (lanes 1 to 6) collected from the nonvaccinated control pig. B. Genotyping of *Haemophilus parasui*s isolates recovered from the nonvaccinated pig. 1 - Challenge strain. 2 - Pleura isolate. 3 -Spleen isolate. 4 – Liver isolate. 5 – Joint isolate. 6 – Brain isolate. M – Base pairs marker.