A Cross-Protection Experiment in Pigs Vaccinated with *Haemophilus parasuis* Serovars 2 and 5 Bacterins, and Evaluation of a Bivalent Vaccine under Laboratory and Field Conditions

Kinya TAKAHASHI\(^1\), Shinya NAGAI\(^1\), Takeshi YAGIHASHI\(^1\), Tsutomu IKEHATA\(^2\), Yoshinori NAKANO\(^2\), Kazuhiro SENNA\(^3\), Takashi MARUYAMA\(^4\) and Junichi MUROFUSHI\(^4\)

\(^1\)Nippon Institute for Biological Science, 9–2221–1 Shinmachi, Ome, Tokyo 198–0024, \(^2\)Abashiri Livestock Hygiene Service Center, 323–5, Taisho, Kitami, Hokkaido 090–0078, \(^3\)Takikawa Animal Husbandry Experimental Station, Higashitakikawa, Takikawa, Hokkaido 073–0026 and \(^4\)Shizuoka Prefectural Swine and Poultry Experiment Station, Nishikata, Kikugawa, Shizuoka 439–0037, Japan

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**ABSTRACT.** Cross-protection between *Haemophilus parasuis* serovars 2 and 5 was examined in pigs using a bacterin based vaccine, and subsequently the safety and efficacy of a bivalent vaccine were evaluated. Upon intratraheal challenge of a serovar 2 or 5 strain, pigs immunized with a monovalent vaccine were protected against challenge with a homologous serovar strain, but not with a heterologous serovar strain. Immunization with a bivalent vaccine containing both serovars 2 and 5 bacterins conferred protection in pigs against lethal challenge with each of the serovar strains. A total of 86 pigs from two SPF herds were injected with the bivalent vaccine intramuscularly twice at a four-week interval. No adverse reactions following the vaccination were observed. On day 7 after the second vaccination, vaccinated and non-vaccinated control pigs from herd A were transferred to herd B, where Glasser’s disease had broken out. Pigs in the control group developed clinical signs of the disease, and 6 of 8 (75%) pigs died until slaughter, in contrast with only 4 of 46 (9%) vaccinated and non-vaccinated control pigs from herd A. Pigs in herd C, where there was no outbreak of Glasser’s disease, complement fixation antibody titer was raised in the vaccinated group. A challenge experiment on days 20 and 79 after the second vaccination showed that only the vaccinated pigs were protected. From these findings, the safety and efficacy of the bivalent vaccine were confirmed under laboratory and field conditions.

**KEY WORDS:** bivalent vaccine, cross-protection, field trial, Glasser’s disease, *Haemophilus parasuis*.

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*Haemophilus parasuis* is the causative agent of Glasser’s disease in swine, which is characterized by acute polyserositis with fibrinous pneumonia, pleuritis and polyarthritis. This bacterium is commonly found in the nasal cavity of apparently healthy conventional pigs [9, 10]. However, it infects systematically when stress factors, such as transport, unfavourable environmental and management conditions, are involved [13]. In recent years, trends in swine production in Japan have resulted in large populations of pigs maintained with high health status, such as specific pathogen free (SPF) pigs. Introduction of *H. parasuis* into such herds may have a devastating effect—infected pigs may spread a contagious disease with high mortality and morbidity, and pigs of all ages may be affected without obvious associated stress factors [15]. Actually, outbreaks of the disease in SPF pigs occur not only in Japan [1] but also in Europe [6], North America [18] and Australia [3], attesting to the increasing importance of *H. parasuis* as a pathogen of economic significance in swine.

Vaccination is an effective way to prevent outbreaks of Glasser’s disease [8, 15–17]. We have developed an inactivated vaccine containing *H. parasuis* serovar 5 bacterin, which is now widely used in Japan for control of the disease. However, a recent study described the gradual increase of Glasser’s disease caused by *H. parasuis* serovar 2 infection in the Japanese field (Sen-na, K. et al.:1995, Abstr. World Vet. Congr. #FC12.1.3).

Fifteen distinct serovars of *H. parasuis* strains, on the basis of immunodiffusion tests using heat-stable antigen extracts, have been reported [5]. However, it has not been established experimentally whether cross-protection among different *H. parasuis* strains actually exists. In this context, the present study was initially conducted to clarify the cross-protection, if any, between serovars 2 and 5, and also to evaluate the safety and efficacy of a bivalent vaccine containing serovars 2 and 5 bacterins.

**MATERIALS AND METHODS**

*Bacterial strains and media:* *H. parasuis* strains Takikawa 188 (serovar 2) and Nagasaki (serovar 5) were used for the preparation of vaccines. Strain Takikawa 188 was isolated from pleural exudate of a pig affected with Glasser’s disease at Hokkaido Prefectural Takikawa Livestock Experimental Station in July, 1992. Strain Nagasaki was isolated from the lung of a pig affected with Glasser’s disease at Nagasaki Chuo Livestock Hygiene Service Center in June, 1972. Both strains were supplied to the Nippon Institute for Biological Science.

For isolation of *H. parasuis*, heart infusion agar supplemented with 5% chicken serum and 100 µg/ml β-NAD
Preparation of the vaccine: *H. parasuis* strains grown to the late log phase in HN-S medium were inactivated with 0.25% formalin. Bacterial cells harvested from the culture were resuspended in phosphate buffered saline (PBS) to a concentration of $1 \times 10^6$ colony forming units (CFU)/ml. Aluminium hydroxide gel was used as the adjuvant.

Antibody assay: Serum antibody titer was measured by a complement fixation (CF) test using heat treated whole cells of strain Takikawa (for serovar 2) or Nagasaki (for serovar 5) [1]. Samples which showed an antibody titer under 4 were judged as negative.

**Experiment 1**: This experiment was carried out to clarify whether the pigs immunized with a monovalent vaccine could be protected against challenge with a heterologous serovar strain. Twenty-eight *H. parasuis* free SPF pigs, which were negative for the isolation of *H. parasuis* from a nasal cavity swab and were also negative for detection of antibody against *H. parasuis*, at the age of 30 to 45 days, were used. These pigs were assigned to the following vaccine/challenge groups: I) serovar 2 bacterin/serovar 2 challenge, II) serovar 2 bacterin/serovar 5 challenge, III) serovar 5 bacterin/serovar 2 challenge, IV) serovar 5 bacterin/serovar 5 challenge, V) saline/serovar 2 challenge and VI) saline/serovar 5 challenge. The pigs in the vaccinated and control groups were injected with 1 ml of the vaccine and saline, respectively, intramuscularly in the neck twice at a two-week interval. On day 14 after the second vaccination, pigs were challenged by intratracheal inoculation with live *H. parasuis* strains as follows: Pigs were anesthetized by intramuscular injection of atropine sulphate (0.05 mg/kg), azaperone (8 mg/kg) and ketamine (10 mg/kg), and intravenous injection of thiopental sodium (10 mg/kg). A cannula was inserted into the trachea and 5 ml ($1 \times 10^5$ CFU) of *H. parasuis* were inoculated through the cannula. Clinical signs of the disease were assessed by monitoring mortality, fever, depression and claudication for ten days after the challenge. Pigs were euthanatized when the clinical disease became severe, and were necropsied immediately. Survivors were euthanatized on day 10, and then necropsied. Gross lesions of the thoracic and abdominal organs were recorded. Specimens for bacteriological examinations were taken from the internal organs, the spinal cord, synovial fluid and bronchial, inguinal and mesenteric lymph nodes. Specimens were cultured to determine the presence of *H. parasuis* and to confirm the serovar by an agar gel precipitation test [12].

**Experiment 2**: This experiment was carried out to clarify whether the pigs immunized with the bivalent vaccine containing serovars 2 and 5 bacterins could be protected against challenge with these serovar strains. Eight *H. parasuis* free SPF pigs at the age of 30 to 45 days were used. These pigs were assigned to the following vaccine/challenge groups: I) bivalent vaccine/serovar 2 challenge, II) bivalent vaccine/serovar 5 challenge, III) saline/serovar 2 challenge and IV) saline/serovar 5 challenge. Challenge procedure, record of clinical signs and postmortem examination were performed as described in Experiment 1.

**Field trial 1**: Fifty-four SPF pigs, originating from SPF grand parent herd A, at the age of 39–59 days were used. Forty-six pigs in a vaccinated group were injected with the bivalent vaccine containing both serovars 2 and 5 bacterins intramuscularly in the neck twice at a four-week interval. The remaining eight pigs served as non-vaccinated controls. The pigs were observed daily to detect adverse reactions following injections of the vaccine. One week after the second vaccination, pigs in both groups were introduced into a commercial fattening herd B, which was suffering from an outbreak of Glasser’s disease. Dead pigs in both groups were necropsied, and gross lesions of internal organs were observed. The effectiveness of the vaccine was judged by comparing mortality rates of Glasser’s disease in the vaccinated and control groups.

**Field trial 2**: Sixty-two pigs at the age of 33–44 days from SPF herd C were used. Forty pigs in the vaccinated group were injected with the bivalent vaccine intramuscularly in the neck twice at a four-week interval. The remaining 22 pigs served as non-vaccinated controls. Since there was no outbreak of Glasser’s disease in this herd, the effectiveness of the vaccine was judged by antibody response and a challenge experiment. For measurement of the CF antibody titer, pigs in both groups were bled prior to each vaccination and on days 19, 54 and 68 after the second vaccination. Four pigs from the vaccinated and control groups, respectively, were transferred to this institute on days 20 and 70 after the second vaccination, and were challenged with serovars 2 and 5 strains as described in Experiment 1.

**RESULTS**

**Experiment 1**: All four pigs immunized with a serovar 2 bacterin survived challenge with a homologous serovar 2 strain without showing any clinical signs of Glasser’s disease (Table 1, Group I). When challenged with a heterologous serovar 5 strain, two of three pigs developed clinical signs and *H. parasuis* was recovered from one of them (Table 1, Group II).

All three pigs immunized with a serovar 5 bacterin survived challenge with a homologous serovar 5 strain (Table 1, Group IV). In contrast, three of four pigs died with severe clinical symptoms after challenge with a heterologous serovar 2 strain (Table 1, Group III). In the non-vaccinated control groups, eight of eight and four of six pigs died after challenge with serovars 2 and 5 strains, respectively (Table 1, Group V and VI). They had severe gross lesions and *H. parasuis* was recovered from them.

**Experiment 2**: Pigs immunized with a bivalent vaccine containing both serovars 2 and 5 bacterins survived challenge with either serovar 2 or 5 strain without showing any clinical signs of Glasser’s disease (Table 2, Group I and II).
In contrast, pigs in the control group developed clinical signs and died after challenge with either serovar 2 or 5 strain (Table 2, Group III and IV). In the protected animals, no gross lesion was observed nor was \textit{H. parasuis} recovered.

\textbf{Field trial 1:} In herd A, no vaccinated pigs showed any adverse reaction to immunization, confirming the safety of the vaccine. When pigs in the vaccinated and control groups were transferred to herd B, which was suffering from an acute outbreak of Glasser’s disease, pigs in the control group developed clinical signs of the disease. Six of eight (75\%) pigs in the control group died until slaughter (Table 3). \textit{H. parasuis} serovars 1, 2 and 4, and untypable strains were isolated from these pigs. In the vaccinated group, four of 46 pigs (9\%) died until slaughter. \textit{H. parasuis} serovar 1 and untypable strains were isolated from these pigs. However, serovars 2 and 5 strains, which were ingredients of the vaccine, were not isolated from pigs in the vaccinated group.

\textbf{Field trial 2:} In herd C, all 40 pigs injected with the bivalent vaccine did not show any adverse reaction to immunization. Since there was no outbreak of Glasser’s disease in this herd, the effectiveness of the vaccine was judged on the basis of an antibody response and a challenge test. In the vaccinated group, 10 of 12 pigs (serovar 2) and 9 of 12 pigs (serovar 5) became seropositive, and the geometric mean antibody titers were 5.3 (serovar 2) and 5.0 (serovar 5) at 19 days after the second vaccination, respectively (Table 4). The rates of the positive antibody response and the geometric mean antibody titers gradually declined until 68 days after the second vaccination. In the control group, a positive antibody response against either a serovar 2 or 5 antigen was not detected throughout the examination period.

After challenge on days 20 and 79 after the second vaccination, all pigs in the vaccinated groups survived without showing any clinical signs of the disease (Table 5, Group I, II, III and IV). At necropsy, they did not show any gross lesions nor was \textit{H. parasuis} recovered. In contrast, typical clinical manifestations were shown in pigs of the control group, two of which (serovar 2 challenge, Table 5, Group V and VI) and one of which (serovar 5 challenge, Table 5, Group VII and IX) died afterwards. The dead pigs showed gross lesions and \textit{H. parasuis} was recovered from them.

\textbf{DISCUSSION}

The present study demonstrated the safety and effectiveness of the \textit{H. parasuis} bivalent vaccine. None of the pigs vaccinated with the bivalent vaccine showed clinical signs of reaction to injection under laboratory or field conditions, confirming the safety of the bivalent vaccine. The efficacy of the vaccine was shown as follows: 1. In Experiment 2, pigs in the vaccinated group were totally protected from lethal challenge with either the \textit{H. parasuis} serovars 2 or 5
strains. 2. In field trial 1, the mortality rate caused by *H. parasuis* infection was reduced in the vaccinated group. 3. In field trial 2, serum CF titers against serovars 2 and 5 rose after the second injection of the vaccine. 4. In field trial 2, pigs in the vaccinated group were protected from lethal challenge for at least 79 days after the second vaccination.

It has not previously been established experimentally whether cross-protection among different *H. parasuis* serovar strains would exist. Kielstein and Raßbach [4] reported that cross-protection was found to occur less often and with lower intensity among different serovars upon challenge experiments using bacterins as immunogens. In contrast, Nielsen [14] reported that pigs given an aerosol with the apathogenic serovars 2, 3, 4 and 7 strains, respectively, resisted challenge with a virulent strain of serovar 5, suggesting cross-protecting immunity. In the present study, the pigs injected with monovalent vaccine containing serovar 2 or 5 bacterin, were not protected from the lethal challenge for at least 79 days after the second vaccination.

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There is controversy about the relationship between serovars and virulence in *H. parasuis*. Kielstein and Rapp-Gabrielson [5] reported that strains of serovars 1, 5, 10, 12, 13 and 14 were most virulent, strains of serovars 2, 4, 8 and 15 were moderately virulent, and strains of serovars 3, 6, 7, 9 and 15 were low or not virulent. The serovar 2 strain, Takikawa, as well as the serovar 5 strain, Nagasaki, used in the present study were confirmed to be highly virulent by intratracheal inoculation in susceptible pigs. Morozumi and Nicolet [11] suggested a correlation between the peptide patterns of *H. parasuis* strains and pathogenicity, and reported that most of the pathogenic strains showed a PAGE type II pattern. The serovar 2 strain, Takikawa, however, had a PAGE type I pattern (data not shown). These findings suggest that the serovar 2 strain, Takikawa, would be a unique strain which does not correspond to a virulent phenotype.

In field trial 1, pigs in the vaccinated group showed a significant reduction in the mortality rate caused by *H. para-
suis infection, although four pigs in the vaccinated group died of Glasser’s disease. H. parasuis serovar 1 and untypable strains were isolated from these pigs. Since this bivalent vaccine contained serovars 2 and 5 bacteria, it might not have been fully effective in the protection against infection with H. parasuis serovar 1 or untypable strains. The serovar 1 infection, however, cannot be overlooked because this serovar 1 strain appears to be highly virulent [2,5]. If outbreaks of serovar 1 infection increase in the Japanese field, the development of a trivalent vaccine containing serovars 1, 2 and 5 would be necessary. However, H. parasuis serovar 4 strain was isolated only from pigs in the control group, suggesting that the bivalent vaccine may be effective at protecting against H. parasuis serovar 4 infection.

In summary, cross-protective immunity between H. parasuis serovars 2 and 5 strains was shown not to be induced by immunization with a monovalent bacterin vaccine. Therefore, we developed a bivalent vaccine and confirmed its safety and efficacy under laboratory and field conditions. The usefulness of this bivalent vaccine to control the disease should be further evaluated by its commercial application on large numbers of animals.

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