Vaccination of Gnotobiotic Primary Specific Pathogen-free Pigs Against *Haemophilus parasuis*

O. Paul Miniats, Nonie L. Smart and Elmer Ewert

ABSTRACT

Three trials were conducted to establish if young primary specific pathogen free (SPF) pigs could be protected from Glasser's disease by vaccination. Three age groups of cesarean-derived isolator-reared gnotobiotic pigs were vaccinated twice at 4 and 6, 3 and 5, and 2 and 4 wk of age respectively with a formalin killed aluminum hydroxide adsorbed bacterin prepared from three strains of *Haemophilus parasuis* isolated from Ontario pigs affected with Glasser's disease. When challenged two weeks later with the homologous strains of virulent bacteria, all the vaccinated pigs remained healthy, while 17/18 nonvaccinated pigs became severely sick or died between three and seven days postchallenge. The one surviving nonimmunized pig was retarded in growth. All of the nonimmunized pigs had visible lesions of polyserositis, the most common being polyarthritis (14/18). Other lesions were fibrinous meningitis, pericarditis, pleurisy and/or peritonitis. Two of the pigs died with a septicemia. *Haemophilus parasuis* was isolated from 15/18 nonimmunized pigs, usually from several of the affected sites. The organisms were not isolated from the immunized pigs, nor from the surviving nonimmunized pig. Attempts to detect the presence of specific antibodies against the *H. parasuis* strains in the sera of the immunized or exposed pigs by the passive hemagglutination test or by enzyme linked immunoassay were unsuccessful. The results of this work indicate that primary SPF pigs can be protected from Glasser's disease by vaccination as early as 2 and 4 wk of age. The nature of this protective mechanism was not established in this study.

RÉSUMÉ

Trois essais ont été réalisés dans le but d'établir si de jeunes porcs exempts d'organismes pathogènes spécifiques (EOPS) pouvaient être protégés par vaccination contre la maladie de Glasser. Trois groupes de porcs gnotobiotiques obtenus par césarienne et élevés en isolateur ont été vaccinés à deux reprises à respectivement 4 et 6, 3 et 5, et 2 et 4 semaines d'âge à l'aide de bactéries tuées à la formaline et adsorbées à l'hydroxyde d'aluminium. Trois souches d'*Haemophilus parasuis* isolées en Ontario de porcs atteints de la maladie de Glasser ont été utilisées. Deux semaines plus tard lors d'une épreuve réalisée avec la souche bactérienne homologue et virulente, aucun des porcs vaccinés n'a développé la maladie alors que 17 des 18 animaux non-vaccinés furent très affectés ou moururent après 3 à 7 jours. D'autre part, le porc non-vacciné qui a survécu à l'infection a de montré un retard de croissance. Tous les porcs non-immunisés ont présenté des lésions de polysérosite, la plus commune étant la polyarthrite (14/18). Les autres lésions observées furent des méningites, péricardites, pleurésies, et/ou péritonites fibrineuses. Deux porcs moururent de septicémie. *Haemophilus parasuis* fut isolé de plusieurs sites infectés et ce chez 15 des 18 porcs non-immunisés. L'organisme ne fut pas isolé des porcs immunisés pas plus que des porcs non-immunisés qui survécurent. Les essais pour détecter la présence d'anticorps spécifiques à *H. parasuis* par hémagglutination passive ou par ELISA dans le sérum des porcs immunisés ou exposés au germe furent négatifs. Les résultats indiquent que des porcs EOPS peuvent être protégés contre la maladie de Glasser grâce à la vaccination faite de 2 et 4 semaines d'âge. La nature de cette protection n'a pas été étudiee. *(Traduit par Dr Daniel Dubreuil)*

INTRODUCTION

Immunization with a formalin-killed bacterin produced from three virulent Ontario isolates of *Haemophilus parasuis* has been found to prevent Glasser's disease in experimentally infected previously nonexposed 7 wk old farm raised specific pathogen free (SPF) pigs (1,2). Subsequent field trials showed that this vaccine had a protective effect also for farm reared SPF pigs of various ages when they were exposed to conventional *H. parasuis* carrier pigs (Smart, unpublished data).

A survey of 19 Ontario SPF herds showed that 16 of these herds had pigs carrying *H. parasuis* in their nasal passages (1,3). Cesarean derived artificially reared SPF pigs are used for introduction of new blood lines into closed SPF herds. This category

Department of Population Medicine, 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, Nepean, Ontario K2H 8P9.

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of pigs should also be considered as being at risk of contracting Glasser's disease after transfer from the rearing laboratory to the recipient farm. The risk may be even greater than that of farm reared SPF pigs because the artificially reared animals are more susceptible to infectious diseases due to their immature immunological defence systems (4) and minimal exposure to microbes, as compared to the former. The isolator reared pigs are placed into new herds at 5 to 6 wk of age which is several weeks earlier than is the case with farm reared weaner SPF pigs. *Haemophilus parasuis* infection being the principal health hazard of the primary SPF pig when moved to new facilities (3), stimulation of immunity against this organism at an early age would be desirable.

This paper describes a series of experiments in which three age groups of primary SPF pigs maintained in a gnotobiotic environment were immunized and subsequently challenged with *H. parasuis* to establish if vaccination of these animals at an early age would protect them from Glasser's disease.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Thirty-six cesarean-derived, gnotobiotic, Yorkshire piglets were used for the immunization-challenge experiments. They were reared in sterile gnotobiotic isolators 1 m^3 in volume, four pigs per isolator, following our method for rearing primary SPF pigs (5). During the second week after delivery, the animals were inoculated orally with lactobacilli, fecal streptococci and a nonvirulent strain of *Escherichia coli*. Until introduction of *H. parasuis* as part of this experiment, the above were the only live microbial associates of the pigs. The experiments followed the guidelines of the Guide to Care and Use of Experimental Animals of the Canadian Council on Animal Care.

EXPERIMENTAL PROTOCOL

As shown in Table I, three age groups (A, B and C) were vaccinated twice at two week intervals with the aluminum hydroxide adsorbed formalin killed bacterin prepared from three virulent Ontario isolates of *H. parasuis* described previously (1,2). Of the four pigs kept in each isolator, two were immunized with the bacterin and two pigs were injected with a placebo vaccine containing no bacterial antigen. The dose was 4 mL. Two mL were injected subcutaneously bilaterally behind each ear. Two weeks after the second vaccination all pigs were challenged with an aerosol containing the three virulent strains of *H. parasuis* homologous to those incorporated in the bacterin. The youngest age group "C" was challenged on two occasions — 14 and 18 days after the second vaccination because no clinical response was observed within the first three days after the initial challenge.

TABLE I. Schedule for immunization and challenge of gnotobiotic pigs with *Haemophilus parasuis*

Group	Number pigs immunized	Number control pigs	Age in weeks when immunized	Age in weeks when challenged
A	8	8	4 and 6	8
B	6	6	3 and 5	7
C	4	4	2 and 4	6 and 6.5

The effectiveness of the vaccine and the challenge were evaluated on the clinical response of the pigs to the challenge, gross necropsy lesions, isolation of *H. parasuis* from selected internal organs and body fluids and on the basis of serology. Necropsies were performed on the pigs that died and samples for bacteriological analysis were collected as early as possible after death. Severely sick pigs were euthanized by intravenous injection of pentobarbital sodium and necropsied immediately. Survivors were killed 7 to 16 days after challenge and necropsied. Blood samples for serology were collected prior to immunization and challenge and from survivors before euthanasia.

PREPARATION OF THE BACTERIN AND INOCULA

The bacteriological techniques followed for the preparation of the vaccine and the challenge material, and the diagnostic methods employed were essentially similar to those already described (1,2). The vaccine and challenge cultures were prepared from three previously freeze-dried virulent strains of *H. parasuis* (1) grown on chocolate agar plates containing 1% nicotinamide adenine dinucleotide (NAD) for 18 h at 37°C.

CHALLENGE PROCEDURES

For challenge, a suspension of live bacteria containing approximately 10^9 colony-forming units/mL was used within 1 h of harvesting. The dose was 8 mL sprayed as a mist into each isolator using 7 L of oxygen at 2000 pounds per square inch as propellant with a DeVilbiss nebulizer (DeVilbiss, Model 65, Summerset, Pennsylvania) as previously described (1,2). To prevent contamination of the gnotobiotic isolators with environmental bacteria, the propelling oxygen was filtered with a 45 μm Millipore filter. Ventilation of the isolator was interrupted for 30 min during the nebulization process.

POSTCHALLENGE PROCEDURES

At necropsy, gross pathological lesions were observed. Samples for bacteriological examination were collected from the cerebrospinal, pericardial, pleural and peritoneal fluids, from the carpal and tarsal joints, and in cases of suspected septicemia, from the blood. They were streaked onto chocolate agar plates previously crossed with a single streak of 1% NAD and incubated at 37°C in a 5% CO_2 atmosphere for 24 h.

Representative gram-negative nonhemolytic satellite colonies were confirmed as *H. parasuis* biochemically by the use of the urease, mannitol, catalase and α-fucosidase tests (3).

SEROLOGY

Serum samples were examined for the presence of specific antibodies against each of the three *H. parasuis* strains by the indirect hemagglutination test (IHA) (6) and by enzyme-linked immunoassay (ELISA) (7). The antigens used for coating sheep red blood cells for the IHA test were the supernatant of sonicated *H. parasuis*

cells or boiled cells. The antigens employed in the ELISA serology were either supernatants from boiled bacteria or dialyzed hot phenol water extracts of the bacteria (lipopolysaccharide antigen). Sera from three rabbits each hyperimmunized with formalin killed antigens of one of the *H. parasuis* strains incorporated in the vaccine were used as controls.

RESULTS

In two of the three trials, the nonimmunized pigs showed clinical signs of Glasser's disease on the second or third day following challenge. In the youngest "C" group, signs of disease were first seen five days after the initial challenge (i.e. two days after the second challenge). This was likely due to reduced virulence of the first inoculum. Notwithstanding this discrepancy, the response to the aerosol challenge with the three strains of *H. parasuis* was similar in all three age groups of the gnotobiotic pigs. None of the immunized pigs had any clinical signs of Glasser's disease, nor did they have any pathological lesions when euthanized and necropsied seven to 14 days postchallenge. *Haemophilus parasuis* was not isolated from them.

All but one of the nonimmunized pigs had clinical manifestations of Glasser's disease. They became anorexic and depressed, most developed swollen joints; recumbency and a moribund state rapidly followed. Two of the pigs had purple discoloration of the ears, the snout, lower parts of the body and extremities. Fourteen of the nonvaccinated pigs either died or were severely sick on days 3 and 5 following challenge. The latter were euthanized for both diagnostic and humane reasons. Another pig died and two were moribund on day 7. One nonvaccinated piglet of the youngest group "C" survived, but it became severely retarded in growth as compared to its vaccinated littermates. When euthanized and necropsied on day 16 postchallenge, it had mild arthritis and an increased volume of synovial fluid in the carpal and tarsal joints. *Haemophilus parasuis* was not isolated from this animal.

The most common lesions in the affected pigs, of all age groups, was polyarthritis which was seen in 14 of the 18 nonvaccinated pigs. It was particularly severe in the younger pigs belonging to group "C" and one pig of group "B". The carpal and tarsal joints of these animals were swollen and contained a purulent exudate. Other lesions, observed in most of the pigs, were one or more of the following: meningitis, fibrinous pleurisy, pericarditis and peritonitis. In two of the pigs, which succumbed to the disease rapidly and apparently died of septicemia, the lesions were not well developed.

Haemophilus parasuis was isolated from 15/18 nonimmunized pigs usually from several of the affected sites. Distribution of the pathological lesions and the sites of isolation of *H. parasuis* are presented in Table II.

The serum passive hemagglutination antibody titers of rabbits injected with the three *H. parasuis* strains ranged from 1280 to 2560, whereas the titers of the vaccinated pigs were either negative or less than 2. Similarly, the ELISA antibody titers of the rabbit sera were high, 1280-10,240. While in the first trial the ELISA on pigs' sera showed some positive results, repeat test results were erratic and frequently negative. They were therefore considered unreliable and are not reported.

DISCUSSION

These experiments showed that primary SPF pigs reared in a gnotobiotic

TABLE II. Response of clinically affected nonvaccinated gnotobiotic pigs to challenge with *H. parasuis*

Group/ Pig	Age when challenged (weeks)	Survival days	Necropsy findings[a]						Bacteriological findings[a]				
			Cya	Br	Peri	Abd	Jt	Pl	Br	Peri	Abd	Jt	Pl
A1	8	3[b]	+	–	–	+	+	+	+	+	+	+	+
A2	8	4	–	–	+	–	–	+	+	+	–	+	+
A3	8	5	–	–	–	–	+	–	–	–	+	–	+
A4	8	5	–	–	–	+	+	–	+	+	+	+	+
A5	8	7 k	–	–	–	+	+	–	–	+	+	+	–
A6	8	3[b]	+	+	–	–	+	+	+	+	–	+	+
A7	8	3 k	+	–	–	–	+	+	–	–	–	–	–
A8	8	3 k	–	–	–	–	+	–	–	–	–	–	–
B1	7	3	–	–	+	+	–	+	–	–	–	+	–
B2	7	3	–	+	–	+	–	+	+	–	+	–	+
B3	7	3 k	–	–	+	–	++	+	+	+	–	+	–
B4	7	3	+	–	+	–	–	–	–	+	+	–	+
B5	7	7	–	–	–	–	+	–	+	–	+	+	–
B6	7	7 k	–	–	–	–	+	–	–	–	–	+	–
C1	6	3 k	–	+	–	+	++	+	+	–	+	+	–
C2	6	4 k	–	–	–	–	++	+	+	–	+	+	–
C3	6	3 k	+	+	–	–	++	+	+	+	+	+	–
C4	6	16 k	–	–	–	–	+	–	–	–	–	–	–

[a]Observations: Cya = cyanosis, Br = brain, Peri = pericardium, Abd = abdomen, Jt = joint, Pl = pleura, – = negative, + = lesion or bacteria present, ++ = severe purulent arthritis and K = killed

[b]*H. parasuis* isolated also from blood

environment can be effectively protected against challenge with virulent *H. parasuis* by vaccination as early as two and four weeks of age. Their non-immunized counterparts, on the other hand, were highly susceptible to a similar challenge.

The concentration of the organisms during the nebulization process was probably considerably greater, but the duration of exposure to the infective dose was shorter than would have been the case under farm conditions. No stress factors other than confinement in isolators, which was a constant factor throughout the life span of the piglets, were involved. It therefore follows that infection via the respiratory tract by *H. parasuis* resulted in the disease in the nonimmunized gnotobiotic pigs without other contributing factors being present. Glasser's disease has also been induced in pigs by intratracheal and intraperitoneal injection of the virulent organisms (8,9). For these reasons, it was assumed that in the case of age group "C" where the first challenge failed to produce clinical signs of Glasser's disease within three days, this must have occurred due to accidental attenuation of the bacterial cultures either during storage or preparation. It is possible that the inoculum was inactivated by the germicidal chemicals used for sterilization of the entry ports of the isolators. *Haemophilus parasuis* is a rather fragile organism and does not survive in the environment for long (10). After the second challenge, the young pigs of group "C" were equally or even more susceptible to the infection than the other groups.

The vaccinated pigs on the other hand had protective immunity against the infection regardless of the age group as determined by their ability to resist challenge. It was, however, impossible to detect the presence of serum antibodies in these pigs against *H. parasuis* by the tests used either after vaccination or after exposure to the live organisms. It, therefore, appears that the protective mechanism against *H. parasuis* infection is not dependent upon serum antibodies detectable by the antigens in these tests. As the hyperimmunized rabbits developed high passive hemagglutination and ELISA antibody titers against all the three strains of *H. parasuis* used in this study, the organisms must be capable of stimulating serum antibody production. The presence of such antibodies in more mature conventional pigs following exposure to *H. parasuis* or vaccination has been demonstrated previously (9,11,12).

The apparent lack of response to the antigens tested may be attributed to the relative immaturity of some of the defence mechanisms of the young pig and particularly of those maintained in a gnotobiotic state (4). The ability of these pigs after immunization to resist a severe challenge with *H. parasuis* indicates that an immunological response did take place. It is possible that the protective immune response was mounted to antigens other than those used in the ELISA passive hemagglutination test. The boiled extract and hot phenol-water extract would predominantly contain polysaccharides and lipopolysaccharides. Further investigation is needed to elucidate the mechanism for the development of immunity against *H. parasuis.*

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