Immunization of Pigs Against *Streptococcus suis* Serotype 2 Infection Using a Live Avirulent Strain

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ABSTRACT

***Sreptococcus suis* capsular type 2 is still an important cause of economic losses in the swine industry. At the present time, vaccination of pigs against this infection is generally carried out with autogenous bacterins and results are equivocal. In this study, the protective effect of a live avirulent *S. suis* type 2 strain (#1330) which had induced a good protection in mice, was evaluated in swine. The experiment was performed in triplicate using 4 week-old piglets. A total of 15 piglets were vaccinated 3 times, 15 others were vaccinated 2 times, and 15 piglets were injected 3 times with sterile Todd-Hewitt broth. Using an indirect ELISA, an increase in the IgG response to *S. suis* antigens was noted in 27 of the 30 vaccinated piglets. On day 21 post-vaccination, all animals were challenged intravenously with a virulent *S. suis* type 2 strain (#999). In the 2 vaccinated groups, 26 animals were fully protected. Only 1 out of the 15 piglets vaccinated 3 times developed mild clinical signs. In the group vaccinated twice, 3 piglets showed clinical signs and 1 of them died after the challenge. In the control group, 7 animals died out of the 11 with clinical signs of infection. In conclusion, a protective immunity was observed in swine when using strain 1330. However, more studies are needed to assess the use of a live *S. suis* strain in a vaccine for pigs.**

RÉSUMÉ

Les infections dues à *Streptococcus suis* sérotype 2 sont toujours une cause importante de pertes économiques pour l'industrie porcine. Jusqu'à maintenant, la majorité des essais de vaccination ont été effectués avec des bactérines autogènes et les résultats sont très mitigés. Cette étude avait pour objectif d'évaluer la capacité d'une souche vivante et avirulente de *S. suis* type 2 (#1330) d'induire une protection chez le porc. Cette souche souche avait auparavant induit une protection chez la souris. Dans la présente étude, comportant trois expériences répétées avec des porcelets âgés de quatre semaines, un nombre total de 15 porcelets ont été vaccinés trois fois, 15 autres ont été vaccinés deux fois et 15 porcelets ont reçu trois injections du bouillon de culture stérile Todd-Hewitt. À l'aide d'un test ELISA indirect, une augmentation du titre d'anticorps contre les antigènes de *S. suis* a été notée chez 27 des 30 porcelets vaccinés. Au jour 21 post-vaccination, tous les animaux ont reçu, par voie intra-veineuse, une injection de défi avec une souche virulente de *S. suis* sérotype 2 (#999). Dans les deux groupes d'animaux vaccinés, 26 porcelets sur 30 ont été protégés complètement. Parmi ceux ayant reçu trois doses du vaccin, un seul porcelet a manifesté des signes cliniques. Dans le groupe d'animaux vaccinés deux fois, trois porcelets ont présenté des signes cliniques et l'un d'eux est mort après l'injection de défi. Dans le groupe des témoins, 11 animaux ont présenté des signes cliniques et sept d'entre eux sont morts. En conclusion, une immunité protectrice a été observée chez l'espèce porcine lors de l'utilisation de la souche 1330 comme vaccin. Toutefois, d'autres études sont nécessaires avant de permettre l'utilisation d'une souche vivante de *S. suis* comme vaccin.

INTRODUCTION

Streptococcus suis is an important pathogen of swine causing mainly septicemia, meningitis and endocarditis. Serotype 2 is the most common capsular type recovered from cases of meningitis in weaned pigs in the United Kingdom, North America and the Netherlands (1–3). It is also associated with various types of infections in different animal species, as well as in humans (4,5). Economic losses due to *S. suis* are important and conventional control measures, such as vaccination, have so far given unsatisfactory results (6). Holt et al (7) found that numerous repeated vaccinations with inactivated cells were needed to induce a good protection. Injections of purified capsular polysaccharides in pigs failed to induce adequate protection (8) and poorly encapsulated strains appeared to be as immunogenic as fully encapsulated ones (9). However, passive and active immunization using different cell wall proteins succeeded in protecting mice against the infection (10–12). A few *S. suis* capsular type 2 strains were recently shown to be avirulent in pigs as well as in the mouse model of infection (13). One of them, strain 1330, harbored a highly immunogenic 135 kDa protein which is also present in virulent isolates. It succeeded in inducing a complete protection in mice against the experimental infection with virulent strains (14). The purpose of this study was to monitor the protective effect of vaccination with *S. suis* avirulent strain 1330 and

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TABLE I. Evaluation of antibody response and protection induced by vaccination of piglets with the live avirulent *Streptococcus suis* serotype 2 strain 1330

Experiment	Vaccination protocol	Mean of titer increase[a]	No. of sick pigs[b]/ No. of challenged pigs	No. of dead pigs[c]/ No. of challenged pigs	No. of pigs with *S. suis* in tissues[d]/ No. of challenged pigs
1	3 doses	5.4	0/5	0/5	0/5
	2 doses	3.5	0/5	0/5	0/5
	control	0	4/5	4/5	4/5
2	3 doses	8.5	0/5	0/5	0/5
	2 doses	2.5	2/5	1/5	1/5
	control	0	4/5	2/5	3/5
3	3 doses	8.8	1/5	0/5	0/5
	2 doses	1.8	1/5	0/5	0/5
	control	0	3/5	2/5	2/5

[a] Value represents mean antibody titer increase of animals from 1 group
[b] Nervous signs, lameness, and decubitus for more than 12 h were considered
[c] Number of euthanized animals showing decubitus or nervous signs for more than 12 h
[d] Bacteriological analyses following post-mortem examination; presence of *S. suis* in at least 1 organ or in blood

to study the antibody response in the natural host.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Two *S. suis* capsular type 2 strains were used in this study. Strains 999 and 1330 were isolated from pig tissues in the laboratory of clinical bacteriology of the Faculty of Veterinary Medicine, University of Montreal. They were identified as *S. suis* capsular type 2 using a procedure already described (2). These strains had previously been tested with an experimental mouse model of infection and in pigs (12,15) and their virulence estimated as follows: 999 highly virulent and 1330 avirulent. For each strain, 3–4 colonies from a 24 h culture on blood agar plates (5% bovine blood) were inoculated in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Michigan, USA) and grown overnight. From this broth, 500 µL were added to 50 mL of fresh medium and grown without agitation at 37°C with 5% CO_2 until the desired absorbance (540 nm), or number of bacteria, was reached.

IMMUNIZATION OF PIGS

Upon their arrival, all pigs were tested serologically for the presence of antibodies against *S. suis* serotype 2 antigens. Using an ELISA assay, piglets with low levels of antibodies were kept for the study. Fifteen 4 week-old crossbred piglets were alloted to each of 3 separate, but identically designed experiments (Table I). In each experiment, pigs were divided into 3 groups of 5 animals and immunizations were carried out via the intra-muscular route. In group 1, 5 piglets were injected 3 times with 10^9 colony forming unit (CFU) of strain 1330 (days 0, 7 and 14). In group 2, 5 other piglets were injected 2 times with the same bacterial concentration (days 0 and 14). In group 3, 5 control animals were injected 3 times with sterile THB (days 0, 7, 14). Between days 0 and 21, pigs were examined twice daily to detect clinical signs of infection. Seven days before the first immunization and 7 d after the last immunization, 2 mL of blood were collected in order to evaluate the antibody response to *S. suis* capsular type 2 cellular proteins by ELISA and to some specific *S. suis* proteins by Western blot. Guidelines from the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care were followed during the experiment.

EXPERIMENTAL INFECTION AND ASSESSMENT OF CLINICAL SIGNS

Pigs were challenged on day 21 by intravenous injection of 10^8 CFU of strain 999 as described by Quessy et al (13). Experimental infection was carried out for a period of 10 d and clinical signs were monitored twice daily for 1 h. Recorded clinical signs included lameness, persistent lateral or ventral decubitus, fever (> 40.5°C), as well as nervous signs such as incoordination, paddling, and opisthotonos. Pigs in decubitus or manifesting nervous signs for more than 12 h, were euthanized for ethical reasons. A necropsy was performed on these animals and tissues such as blood, lung, liver, spleen and brain were cultured for the presence of *S. suis* according to a procedure already described (2). Remaining pigs were euthanized after the 10 d period and submitted for necropsy, where special attention was paid to the immunization site in order to detect any lesions caused by intramuscular injection of the vaccinal strain.

SDS-PAGE

Twenty-five milliliters (mL) of an overnight culture of the strain 999 were centrifuged (12 500 x *g*) for 20 min and resuspended in 1 mL of K_2HPO_4 (0.1 M, pH 7.0), processed in a French Press (16) (SLM, Amico, Urbana, Illinois, USA) (Mini-cell, 20 000 PSI, 3 times), treated with lysozyme (5 mg mL^{-1}) for 4 h at 37°C and centrifuged again (12 500 x *g*) for 20 min at 4°C. Cellular proteins present in the supernatant were harvested, mixed with equal volume of solubilization buffer, boiled 4 min and processed in 10% polyacrylamide vertical slab gels (with 4.5% stacking gels) (17). Gels were stained with Coomassie blue or transferred to nitrocellulose.

WESTERN BLOT ANALYSES

Following SDS-PAGE, material was transferred to the nitrocellulose membrane by electroblotting in a transblot apparatus (Bio-Rad, San Francisco, C) with methanol-Tris-glycine buffer for 1 h at 100 volts (18). Non-reacting sites on nitrocellulose membrane were blocked for 1 h with casein 2% (w/v). The membrane was incubated for 1 h with 1:200 (v/v) dilutions of each pig serum before and after vaccination. After washing, the

membrane was incubated for 1 h with a peroxidase conjugated goat anti-pig IgG (Jackson Immuno Research,West Grove, Pennsylvania, USA). After washing, the presence of bound antigens was visualized by reacting the nitrocellulose membrane with 0.06% 4-*chloro*-1-naphtol (Sigma) in cold methanol mixed to 0.02% H_2O_2 in Tris-NaCl. Apparent molecular weights were calculated by comparison with standards of known molecular weight (Bio-Rad).

SEROLOGICAL RESPONSE OF PIGS FOLLOWING VACCINATION

Porcine sera were tested using an ELISA procedure. Flat-bottom polystyrene microtiter plates (NUNC Immunoplates, Copenhagen, Denmark) were coated at 4°C for 18 h with 0.4 μg of protein extract (strain 1330) in 100 mL of 10 mM phosphate buffer, pH 7.4 (PBS) per well. Then, 100 mL of PBS containing 0.3% (w/v) of casein and 0.005% (v/v) of Tween 20 (Sigma) were added and left 1 h at room temperature in order to block free sites; plates were then washed 3 times. Pig sera were diluted serially in PBS, added in 100 μL amounts to appropriate wells and incubated for 1 h at room temperature. Serum from an axenic pig was used as negative control. The positive control was an anti-strain 999 hyperimmune pig serum obtained from a pig after 6 consecutive immunizations with inactivated bacteria. Well contents were discarded and the plates washed. A volume of 100 μL of goat anti-pig IgG conjugated to horseradish peroxidase (Jackson Immuno Research) diluted 1:2000 in PBS was added to each well and left for 1 h at room temperature. The plates were washed and 100 μL of 0.4 mM 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) dissolved in 0.05 M citrate buffer (pH 4.0) with 0.5 M H_2O_2 were added to each well. The absorbance was measured after 30 min of incubation at 22–23°C. Optical density was corrected by substraction of background binding in control wells (coated with PBS). ELISA titers were estimated as the highest dilution that gave an increase in light absorbance at 414 nm (A_{414}) more than twice the mean of the corresponding blank values (without antibody but with conjugate and substrate). Increase in antibody titer was calculated using this formula: titer obtained on day 21 divided by titer obtained on day 0. The mean of titer increase was calculated using this formula: total titer increase for each animal in the same group divided by the total number of pigs in this group.

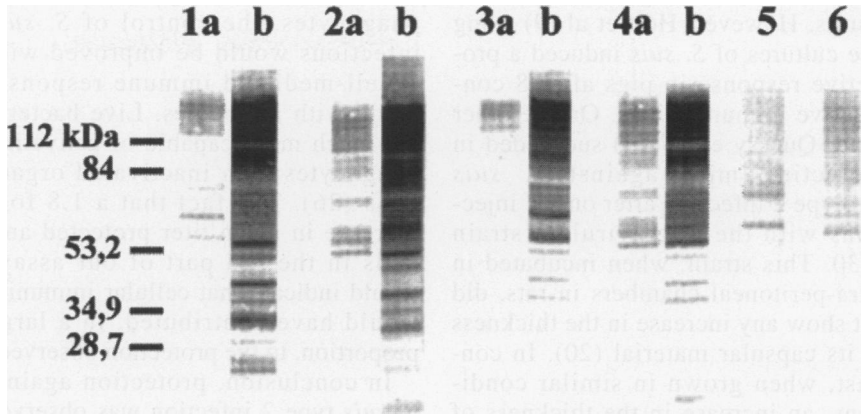

Figure 1. Comparison of the IgG response of pigs to *Streptococcus suis* proteins before (a) and after (b) immunization with the live avirulent strain 1330 by Western blot analysis. Piglets were immunized 3 times (lanes 1 and 2) and 2 times (lanes 3 and 4). Sera from control animals after they received 3 immunizations with sterile Todd-Hewitt broth (lanes 5 and 6). Results using antisera from animals selected from experiment 1 are shown; data were similar in experiments 2 and 3. Western blots were performed after transfer from a 12.5% polyacrylamide gel loaded with virulent strain 999 proteins.

RESULTS

IMMUNIZATION AND SEROLOGICAL RESPONSE OF PIGS

None of the pigs showed lameness, decubitus or nervous signs following immunization with the vaccinal strain of *S. suis*. Necropsy did not reveal any detectable lesions at the immunization site. In the first experiment, a 5.4 fold mean elevation in antibody titer against *S. suis* antigens was observed in pigs that received 3 doses of vaccine (Table I). Antibody titers between 40 000 and 80 000 were observed in these animals, while in animals vaccinated twice, antibody titers varied between 10 000 and 40 000, which corresponds to a 3.5 fold increase. In the control group, titers ranged from 5 000 to 10 000. A similar increase in the mean titer was observed in the 2 other experiments.

WESTERN BLOT ANALYSES

Western blot analyses carried out on sera from immunized pigs, before and after vaccination, showed an apparent increase in the IgG response against the virulent strain 999 cellular proteins (Figure 1 for experiment 1). An IgG response was observed against high molecular weight proteins and against proteins of approximately 40 and 70 kDa (Figure 1). Western blot analyses performed on sera in experiments 2 and 3 gave similar results (data not shown).

EXPERIMENTAL INFECTION

In the 3 experiments, when challenged by an intravenous injection of virulent strain 999, 1 out of the 15 pigs vaccinated 3 times and 3 out of the 15 pigs vaccinated twice showed clinical signs compatible with *S. suis* infection. In contrast, 11 out of the 15 control pigs manifested lameness, decubitus or nervous signs. Finally, 1 out of the 30 immunized pigs and 9 out of the 15 control pigs died (Table I). Lesions attributed to *S. suis* infection were observed and the microorganism was recovered from at least one organ or in blood from all animals having shown decubitus and/or nervous signs. Pathological lesions, and isolation of *S. suis* were recorded in only one of the other pigs (Table I).

DISCUSSION

Previous attempts to protect pigs against *S. suis* type 2 infection, either by injection of formalin killed bacteria (6,7,19) or purified capsular

material (8), have given equivocal results. However, Holt et al (9) using live cultures of *S. suis* induced a protective response in pigs after 8 consecutive immunizations. On the other hand, Quessy et al (14) succeeded in protecting mice against *S. suis* serotype 2 infection after only 2 injections with the live avirulent strain 1330. This strain, when incubated in intra-peritoneal chambers in rats, did not show any increase in the thickness of its capsular material (20). In contrast, when grown in similar conditions, an increase in the thickness of the capsular material was noted for virulent strains and was accompanied by a better resistance to killing by porcine polymorphonuclear leukocytes (12). In mice, it was previously demonstrated that immunization with the avirulent strain 1330 led to the production of IgG recognizing many proteins of different virulent strains (14).

A persistent high-level bacteremia usually precedes the onset of bacterial meningitis (21). Thus the ability of a bacteria to induce and maintain a bacteremia is a major determinant of pathogenicity (22). In this kind of infection, humoral immunity plays an important role. Moreover, the importance of humoral immunity in the pathogenesis of *S. suis* infection was confirmed by Holt et al who found that the protective response was serum-mediated and associated with both IgM and IgG (23). Presence of antibodies and particularly IgG at the bacterial surface could increase recognition and then stimulate uptake by phagocytes (24). However, the outcome of interactions between bacteria and phagocytes is important in determining the level of bacteremia and the incidence of meningitis (25). Since replication of virulent strains of *S. suis* within murine macrophages was shown by Williams (22), cellular immunity could also be determinant. Thus, in a live vaccine, organisms act as endogenous antigens and tend to trigger a response dominated by cytotoxic T-cells (26). In contrast, inactivated organisms, act as exogenous antigens and stimulate a response dominated by helper T-cells (26). If *S. suis* can survive inside phagocytes, both types of immunity would be required to eliminate all bacteria. Since activation is important in the control of organisms inside phagocytes, the control of *S. suis* infections would be improved with T-cell-mediated immune response, along with antibodies. Live bacteria are much more capable of activating phagocytes than inactivated organisms (26). The fact that a 1.8 fold increase in mean titer protected animals in the 3rd part of our assays would indicate that cellular immunity would have contributed, in a large proportion, to the protection observed.

In conclusion, protection against *S. suis* type 2 infection was observed in pigs after 2 or 3 vaccinations with the live avirulent strain 1330. This protection seems to be related to the presence of antibodies against some of *S. suis* cellular proteins and to cellular immunity. However, more studies are needed to assess the use of a live strain of *S. suis* in a vaccine for pigs.

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