

Comparison of Pig, Rabbit and Mouse IgG Response to *Streptococcus suis* Serotype 2 Proteins and Active Immunization of Mice Against the Infection

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ABSTRACT

The aim of this study was to compare the IgG response of different animal species to *Streptococcus suis* serotype 2 proteins and to evaluate the immunogenic potential of these proteins in the mouse experimental model of infection. The protein profiles of ten different *S. suis* capsular type 2 isolates were compared by Western blotting using antisera produced in mice, rabbits and pigs against the reference strain. Strains were grown overnight in Todd-Hewitt broth, harvested by centrifugation, processed in a French press cell and digested with lysozyme. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was then performed and proteins transferred to nitrocellulose. The rabbit antiserum recognized seventeen common immunoreactive proteins, of which, proteins of 33, 44, 96, 122 kDa were present in all strains. Two, 128 and 136 kDa proteins were recognized by swine serum in many strains. An additional protein of 30 kDa was recognized by the mouse antiserum.

These seven proteins, originating from the reference strain, were excised directly from polyacrylamide gels, mixed with incomplete Freund's adjuvant and given to groups of five mice on days 0 and 10. Immunoglobulin G response to each protein was monitored on day 20 using Western blots. Mice were then experimentally infected on day 21. Results indicated that vaccination with proteins of 33, 44, 128 and 136 kDa resulted in an IgG response and protection against the challenge with the reference strain, but gave

only a partial protection against another virulent *S. suis* serotype 2 strain.

RÉSUMÉ

Cette étude avait pour but de comparer la réponse en immunoglobulines G de différentes espèces animales aux protéines de *Streptococcus suis* sérotype 2, d'identifier les protéines les plus immunogènes et de tester chez la souris leur potentiel protecteur. Les profils protéiques de dix souches de *S. suis* sérotype 2 ont été comparés par la technique de l'immunobuvardage en utilisant des antisérums produits chez la souris, le lapin et le porc contre la souche référence du sérotype 2. Après une croissance de 16 heures en bouillon Todd-Hewitt, les souches ont été récoltées par centrifugation, traitées à la presse de French et digérées avec du lysozyme. Des gels de polyacrylamide ont été réalisés et les protéines transférées sur membrane de nitrocellulose. L'antisérum de lapin reconnaissait dix-sept protéines immunoréactives. De ces protéines, l'antisérum de porc en a reconnu six de respectivement 33, 44, 96, 122, 128, 136 kDa alors que l'antisérum de souris en a reconnu une autre de 30 kDa. En utilisant la souche référence, ces sept protéines ont été prélevées séparément du gel de polyacrylamide, mélangées à de l'adjuvant incomplet de Freund et injectées à des groupes de cinq souris aux jours 0 et 10. La réponse en IgG pour chaque protéine a été évaluée par immunobuvardage au jour 20 et les souris ont été infectées

expérimentalement au jour 21. Il est apparu que la vaccination avec les protéines de 33, 44, 128 et 136 kDa induisait la production d'IgG et donnait une protection contre l'infection avec la souche homologue. Par contre, la protection contre l'infection avec une autre souche virulente n'a été que partielle.

INTRODUCTION

Streptococcus suis capsular type 2 is a swine pathogen, which has an important economical impact, causing mainly septicemia and meningitis (1). Attempts to control the infection by vaccination have given equivocal results; vaccination with inactivated whole cells needs to be repeated many times to give a good protection (2). Injection of purified polysaccharides in pigs has shown that capsular material of *S. suis* capsular type 2, like many other capsular polysaccharides, is poorly immunogenic (3). In addition, immunization using poorly capsulated strains appeared to be as immunogenic in pigs as fully capsulated ones (4). Passive immunization using rabbit antiserum raised against different cell wall proteins succeeded in protecting mice against the infection (5,6).

The serological response of different animal species to various antigens often differs significantly especially if the animal species are unrelated (7). When considering the use of a particular protein in vaccination, the protein must possess good immunogenicity in the natural host. Search for potentially protective proteins can be misleading if one uses antisera from aberrant hosts for passive immunization. Mice

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and rabbits are susceptible to *S. suis* infection (8) and both have been used to study the infection (9,10) and for passive immunization experiments (5,6). The purpose of this study was to compare the immunogenicity of proteins from different *S. suis* capsular type 2 strains in mouse, rabbit and pig; to identify shared most immunogenic proteins and to test their ability to protect against the infection in actively immunized mice (11).

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Ten *Streptococcus suis* capsular type 2 strains were used for this experiment. The reference strain (#735) of *S. suis* capsular type 2 was provided by Dr J. Henriksen, Statens Seruminstitut, Copenhagen, Denmark. The other nine strains were isolated from diseased pigs in the clinical bacteriology laboratory at the Faculty of Veterinary Medicine, University of Montreal, and were identified as *S. suis* capsular type 2 (12). All strains were stored at -20°C .

Three to four colonies from a 24 hour culture on 5% bovine blood agar plates were inoculated in Todd-Hewitt broth (Difco Laboratories, Detroit, Michigan) and incubated overnight in an aerobic atmosphere at 37°C . Cells were harvested by centrifugation, washed with a sterile saline solution and resuspended in 3 mL of 0.1 M K_2HPO_4 (pH 7.0).

PRODUCTION OF ANTISERA

Five six-week-old CF-1 mice, weighing about 20 g, three six-week-old crossbred pigs, weighing about 7 kg, and three adult New-Zealand rabbits were injected once a week for four weeks with 1.0 mL of a formalin-killed (0.5 % v/v, 18 h) suspension of 109 CFU/mL of *S. suis* (#735) grown overnight in Todd-Hewitt broth. Mice were injected intraperitoneally and pigs and rabbits were injected intramuscularly. Animals were euthanized one week later and their blood collected.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Cells of the various *S. suis* strains, suspended in K_2HPO_4 (see above)

were processed in a French press cell (13) (SLM, Amico, Urbana, Illinois) (Mini-cell, 20,000 PSI, three times), treated with lysozyme (5 mg/mL), for four hours at 37°C and centrifuged ($12,500 \times g$, 20 min). Supernatants were harvested and the protein content determined (14) (Bio-rad, San Francisco, California). The fraction was mixed with an equal volume of solubilization buffer, boiled 4 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% and 10% polyacrylamide vertical slab gels (with a 4.5% stacking gel) (15).

WESTERN BLOTTING

Following SDS-PAGE, material was transferred to the nitrocellulose membrane by electroblotting in a transblot apparatus (Hoefer Scientific Instruments, San Francisco, California) with methanol-Tris-glycine buffer (16) for 18 h at 60 volts. Casein (2%, w/v) in Tris-buffered saline was used to block unreacted sites and the nitrocellulose membrane was incubated for two hours with 1:200 (v/v) dilutions of the mouse, rabbit or pig antisera raised against whole cell antigen and 1:100, 1:200, 1:500 (v/v) dilutions of the mouse antisera against specific proteins (see below). After washing in Tris-NaCl, the membrane was incubated with a peroxidase-labelled IgG fraction of goat anti-serum against pig, rabbit or mouse IgGs (heavy + light chain) (Bio-rad) for 60 min at a dilution of 1:1000 in 2% caseine in Tris-NaCl. After repeated washings, the presence of bound antigens was visualized by reacting the nitrocellulose membrane with 0.06% 4-chloro-1-naphthol (Sigma Chemical Company, St. Louis, Missouri) 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).

IMMUNIZATION OF MICE AND EXPERIMENTAL DESIGN

Seven groups of 13 or 14 mice were injected with each protein that appeared to be immunoreactive in five or more of the tested *S. suis* strains. Gels were loaded with reference strain cell proteins, after being processed in the French press cell and separation performed as described for

SDS-PAGE. Bradford (14) (Bio-rad) colorimetric assay was used to evaluate the total amount of loaded protein and a densitometer (Gelscan XL (2.1), Pharmacia, Uppsala, Sweden) was used to evaluate the quantity of protein in each band; different quantities of bands were used to standardize the protein content of preparations. Bands approximately 2 mm wide and containing the proteins were cut from the polyacrylamide gels, homogenized with 1 mL PBS and emulsified with incomplete Freund's adjuvant as described previously (5). Each mouse received, by subcutaneous injection, approximately 30 mg of each protein mixed with incomplete adjuvant (IFA) on day 0 and 20 mg of proteins mixed with IFA on day 10. On day 20, three or four mice were euthanized in each group, their blood collected and their IgG response to each protein was monitored using Western blots. After SDS-PAGE on gels loaded with reference strain proteins, the IgG production by mice against a specific protein was determined by visualization on Western blots of one band corresponding to the molecular weight of the protein injected (see Western blotting, below). The control group received only a mixture of PBS and adjuvant. On day 21, five mice of each group received intraperitoneally 1 mL of strain #735 (O.D. = 0.4 at 450 nm) grown in Todd-Hewitt broth, containing 3×10^8 viable organisms, and the five remaining mice received 1 mL of the virulent strain #1591 (11), containing about the same number of cells. Signs of illness (prostration, nervous signs) and death were recorded twice a day for one week. Every moribund mouse was killed and blood collected by intracardiac puncture for bacterial culture. These experiments were repeated three times and followed the guidelines of the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care.

RESULTS

Western blots using rabbit anti-serum showed seventeen common immunoreactive proteins in the different strains (Fig.1A, lane 3). More *S. suis* serotype 2 proteins appeared to be immunogenic in rabbits than in

mice or pigs. Using mouse antisera, proteins of 30, 33, 44, 96, 122 kDa were detected in all strains examined. A protein of 128 kDa was present in five strains (735, 1591, 6891, 4671, 5382) and a 136 kDa protein was detected in six strains (735, 13A18, 4193, 5303, 1375, 4393) (Fig. 1B). Proteins of 33, 44, 96, 122, 128 and 136 kDa were detected using pig antisera (Fig. 1A, lane 2), the 128 and 136 kDa proteins being present in the same strains. These six proteins and the 30 kDa protein were used as antigens for the active immunization experiments. Western blotting revealed that bands of 33, 44, 128 and 136 kDa gave an IgG response in mice while bands of 30, 96 and 122 kDa failed (Table I). The mice were protected against challenge with the homologous strain 735 if injected with bands of 33, 128 and 136 kDa but these bands failed to protect against infection with strain 1591 (Table II). In some cases, the vaccination did not prevent infection but prevented death as it is shown by a lower mortality rate than morbidity rate (Table II). All three experiments gave similar results. All blood cultures from moribund mice were positive for *S. suis*.

DISCUSSION

Antibodies directed against surface proteins of *S. suis* serotype 2 are thought to be important in protection, since it has been shown that rabbit antibodies directed against such components passively protected mice against the infection (5,6). In addition, it has been demonstrated that protective immunity can be passively transferred from one pig to another by serum (1). The present study confirmed that bacterial protein fractions could generate protection against the infection. However, the differences shown between the IgG response of different animal species to *S. suis* serotype 2 proteins (Fig. 1) confirm that one must be careful in the interpretation of passive immunization assays (5,6) since one protein may be immunogenic in one animal species but not in another. The similarity of pig and mouse IgG response to the *S. suis* proteins tends to confirm the

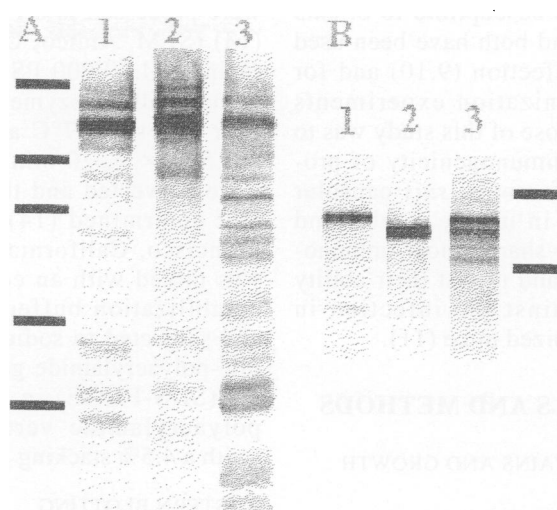


Fig. 1. Comparison by Western blot of the IgG response (panel A) of mouse (lane 1), pig (lane 2) and rabbit (lane 3) to *S. suis* serotype 2 proteins. Western blots were performed after transfer from a 10% polyacrylamide gel loaded with strain #735 proteins. When different isolates were used (panel B), three patterns were seen in high molecular weight proteins, those with a 128 kDa protein (lane 2), those with a 136 kDa protein (lane 1) and those with both (lane 3). Molecular weight markers: Panel A: 140, 106, 80, 49.5 and 32.5 kDa; Panel B: 140 and 106 kDa.

TABLE I. Mouse immunoglobulin G response to active immunization against proteins from *Streptococcus suis* capsular type 2

Protein injected* (kDa)	# of positive mice/ # of tested mice	Serum dilutions		
		1/100	1/200	1/500
30	0/9	— ^b	—	—
33	8/9	100	44	18
44	4/8	32	13	—
96	0/9	—	—	—
122	0/9	—	—	—
128	6/10	23	—	—
136	10/10	75	53	28

* Proteins excised from polyacrylamide gels, mixed with adjuvant and injected twice

^b Evaluation of band intensity (when present) by densitometric scan on a relative scale from 0 to 100

TABLE II. Active immunization of mice using different *Streptococcus suis* capsular type 2 (strain 735) proteins

Protein (kDa)	Challenge strain (3×10^8 CFU)	Sick mice* Tested mice ^b	Dead mice/ Tested mice
30	735 (homologous)	3/5	1/5
	1591 (heterologous)	3/5	2/5
33	735	1/5	0/5
	1591	3.33/5	3/5
44	735	2/5	1/5
	1591	3/5	2/5
96	735	3/5	2/5
	1591	3/5	2/5
110	735	4/5	4/5
	1591	3/5	3/5
128	735	0/5	0/5
	1591	3/5	2.33/5
136	735	0/5	0/5
	1591	4/5	3/5
Adjuvant only	735	4.66/5	4/5
	1591	4.66/5	4.66/5

* Mean number of mice which showed nervous signs and/or prostration during the week following challenge

^b Means of three separate experiments

usefulness of mice for studying *S. suis* infection (9,10). Robertson *et al* (8), studying the experimental infection in different animals species, found that mice were more suitable for predicting the pathogenicity of isolates for pigs while Kataoka *et al* (17) were able to clearly distinguish virulent strains of *S. suis* serotype 2 from avirulent ones using mice. However, despite similarity in the pig and mouse IgG response to proteins tested in this study, any protein or combination of proteins of potential use for vaccination should be tested in the natural host since specific defense mechanisms may vary between animal species (7).

Although vaccination with inactivated bacteria has given equivocal results in the field, better results have been obtained using inactivated autogenous vaccines (1). The finding that proteins from the reference strain gave a much better protection against the challenge with the homologous strain than against the infection with strain 1591 seems to confirm that the use of an autogenous vaccine is more appropriate. However, the two strains used in this study were from different geographical regions and could differ antigenically (18). The lack of a protective effect of antibodies raised against the 136 kDa fraction when mice were experimentally infected with strain 1591 was not unexpected because this protein was not detected in this strain (Table I) using the Western blot technique. Vecht *et al* (19) suggested that a 136 kDa membrane protein and an extracellular factor of 110 kDa were present in virulent *S. suis* serotype 2 strains. The fact that the virulent strain 1591 (11) and other strains isolated from diseased pigs do not possess the 136 kDa protein suggests that important differences could exist between European and Canadian isolates.

Among the proteins studied, the 128 kDa protein was the only one providing partial protection against the heterologous strain 1591 and full protection against the homologous reference strain. This protein was recognized using mouse and pig antisera but was present in only four Canadian isolates and thus might not be useful in a vaccine.

In order to detect most immunogenic proteins, only small quantities of antigen were used (20 to 30 mg) in the present study. Using this protocol, there was no evidence of a protective effect of proteins that failed to induce an IgG response (e.g. 30, 33, 96 kDa). It is thus assumed that these proteins are less immunogenic. On the other hand, it is possible that conformational epitopes could have been altered, since the protocol might denature some proteins.

Pigs infected with *S. suis* have been shown to produce IgM and IgG (1). In this experiment, we have considered the IgG response because IgG production implies previous production of IgM (20). However since it was suggested that virulent strains of *S. suis* serotype 2 can survive in macrophages (21), one cannot exclude cellular immunity in the protection against *S. suis* serotype 2 infections. In order to find a useful vaccine against *S. suis* infections, studies using combination of proteins or combination of proteins and capsular polysaccharides should be considered.

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