Production of Capsular Material by *Streptococcus suis* Serotype 2 under Different Growth Conditions

M. Gottschalk, R. Higgins and M. Jacques

ABSTRACT

The procedure currently used for the production of *Streptococcus suis* antigen is very long and includes several subcultures. The aim of the present work was to study the *in vitro* production of capsular material by *S. suis* serotype 2 after each of these subcultures. The amount of capsular material produced was evaluated by electron microscopy using bacterial cells grown on blood-agar plates and in Todd-Hewitt broth (THB) or THB supplemented with serum. In addition, the production of antibodies in rabbits with antigens produced using different growth conditions was compared. Antigens produced after only three subcultures possessed as much capsular material as cells obtained after the complete procedure and induced a similar antibody response. The use of serum as a supplement to the broth did not assure a higher production of capsule; in addition, antibody titers obtained with antigens produced in THB were as high as those obtained with antigens produced in THB supplemented with serum. We recommend the use of three subcultures in nonsupplemented broth for the production of immunogens. This revised protocol offers two main advantages: it is less time-consuming because of the limited number of subcultures and is also less expensive since nonsupplemented broths are used.

RÉSUMÉ

Le protocole présentement utilisé afin de produire des antigènes de *Streptococcus suis* nécessite plusieurs sous-cultures. Le but de la présente étude était d'évaluer la production du matériel capsulaire de *S. suis* sérotype 2 après chacune de ces sous-cultures. La quantité de matériel capsulaire a été évaluée par microscopie électronique en utilisant des cellules bactériennes cultivées sur géloses au sang ou en bouillon Todd-Hewitt (THB) supplémenté ou non avec du sérum. De plus, nous avons comparé la production d'anticorps chez des lapins immunisés à l'aide d'antigènes obtenus après différentes conditions de croissance. Les résultats obtenus indiquent que les cellules obtenues après seulement trois sous-cultures semblent avoir une capsule aussi abondante que celle des cellules obtenues après le protocole complet et induisent une aussi bonne réponse d'anticorps chez le lapin. L'addition de sérum au THB n'a pas augmenté la production de matériel capsulaire; les titres des antisérums préparés à partir des cellules bactériennes cultivées dans un bouillon non-supplémenté étaient équivalents à ceux des antisérums préparés avec des cellules bactériennes cultivées dans un milieu supplémenté. Nous recommandons l'utilisation de trois sous-cultures en bouillon THB non-supplémenté pour la production des antigènes. Ce protocole révisé offre deux principaux avantages : il est plus rapide à cause du nombre limité de sous-cultures et il est aussi moins dispendieux à cause de l'utilisation de milieux non-supplémentés.

Streptococcus suis is a worldwide cause of a variety of infections in swine (1,2). So far, 29 capsular types have been described (3,4,5). The serotyping system is based on antigenically different capsular material in each serotype; this capsular material is mainly composed of carbohydrates. Previous work has shown that the capsular polysaccharide of *S. suis* capsular type 2 has a molecular weight of at least 100,000 Da and contains five different sugars, including sialic acid (6). The morphology of the capsule of serotypes 1 to 8, 1/2 and 23 through 28 has extensively been studied (4,7). As the number of capsular types increases, serotyping becomes more complex and could soon be limited to reference laboratories. Some tests routinely used in North America, such as the slide agglutination and the coagglutination tests, detect nonspecific cross-reactions (8). The capsular reaction test, which is considered the reference test, requires an antiserum with a high titer to be easily interpreted. As a consequence, the preparation of reagents becomes critical. This might explain some interlaboratory variations in the identification of capsular types of *S. suis* (9).

Encapsulation of bacteria by polysaccharides has been suggested to be one of the factors responsible for the virulence of a microorganism, and the capsular thickness seems to play an important role (10). Isolates of *S. suis* capsular type 2 from diseased animals were shown to possess a thicker capsule than those from clinically healthy animals (11). Different amounts of capsular material produced after *in vitro* culture might have been responsible for the difficulty found by some researchers in reproducing clinical disease in pigs after

Groupe de Recherche sur les Maladies Infectieuses du Porc. Faculté de Médecine vétérinaire, Université de Montréal, C.P. 5000, Saint-Hyacinthe, Québec J2S 7C6.

Reprint requests to Dr. R. Higgins.

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TABLE I. Capsule thickness of *Streptococcus suis* capsular type 2 reference strain #735 after subcultures in Todd-Hewitt broth (THB) in the presence or in the absence of 5% bovine serum

No. of subcultures	Incubation time (hours)	Capsule thickness (range in nm)[a]	
		THB with serum	THB without serum
1	18	130–150	150–200
2	6	110–150	150–170
3	6	175–200	200–250
4	6	150–175	200–250
5	6	150–200	220–260
6	2	150–200	200–220

[a]Capsule was stabilized using anticapsular type 2 antiserum. A total of 20 to 25 measurements per preparation were made

experimental infection with *S. suis* serotype 2 (12,13).

Equivocal results have so far been obtained following vaccination of animals against *S.suis* infection using autogenous bacterins. As antibodies against the capsule have been demonstrated to be responsible, at least in part, for the protection against infection, the expression of the capsular material *in vitro* may have some influence on the quality of the vaccine.

Capsule expression may vary with different growth conditions. It has been demonstrated for *Actinobacillus pleuropneumoniae* that the amount of capsular material varied according to the age of cultures when isolates were grown in liquid media (14). To enhance the production of capsular material in *S. suis* strains, most workers have used young cultures in a broth medium during the exponential phase of growth and, in some instances, they have incorporated normal serum or blood into the media. For example, media have been supplemented with 6% horse blood (15), 10% horse serum (12,16), 5% and 10% bovine serum (8, J. Henrichsen, personal communication, 1988). However, the actual effect of these supplements has never been evaluated. Other authors did not use any supplement in their broths (13,17) or simply used a blood agar medium (18).

Several subcultures in serum supplemented broths have been recommended for the production of *S. suis* antigens (8, J. Henrichsen, personal communication, 1988), based on experience obtained with *Streptococcus pneumoniae* (19). However, this is a very long and cumbersome procedure, which consists of six subcultures. Production of antigens to be used as immunogens should be as easy and inexpensive as possible. The aim of the present work was to study the *in vitro* production of capsular material by *S. suis* serotype 2 after each subculture of the long procedure currently used for the production of antigens. The amount of capsular material produced was evaluated by electron microscopy using bacterial cells grown on blood agar plates, or in Todd-Hewitt broth supplemented or not with serum. In addition, the production of antibodies in rabbits with antigens produced using different growth conditions was compared.

Streptococcus suis capsular type 2 reference strain #735 (5) was used. The strain was stored in 1 mL aliquots at −70°C and thawed before being used. The liquid medium used in the experiments was Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Michigan) with or without serum. Bovine serum used as a 5% supplement for the THB originated from a single animal, did not contain antibodies agains *S. suis*, and was inactivated at 56°C for 30 min. The protocol of antigen production currently used in our laboratory was followed (8). After 18 h of incubation on blood agar plates at 37°C in aerobic atmosphere as a purity control, a first 18 h subculture in THB was performed (subculture #1). Then, four passages of 6 h of incubation using 10 mL of broth were done (subcultures #2 to 5). After each one of the 6 h passages, cultures were kept at 4°C overnight, and then six drops were inoculated into 10 mL of fresh, prewarmed broth. Five mL of the last 6 h subculture were inoculated into 50 mL of a prewarmed broth, which was incubated for only two hours (subculture #6). A culture obtained after 18 h of incubation on tryptic soy agar (Difco) with 5% bovine blood was also studied. The procedure was repeated twice.

The thickness of the capsule of bacterial cells from each of the cultures and subcultures was measured after immunostabilization for electron microscopy (7). The same whole-cell antiserum raised against the capsular type 2 reference strain was used throughout the study. This antiserum possessed a titer of 1/16 as revealed by the capsular reaction test (8). Agar- and broth-grown bacteria were harvested, washed with phosphate-buffered saline (0.01 M, pH 7.2), adjusted to an optical density of 1.8 at 540 nm and exposed to undiluted, capsular type 2 antiserum for 1 h at 4°C. Bacterial cells were then suspended in cacodylate buffer (0.1 M, pH 7.0) containing glutaraldehyde and ruthenium red. Fixation was of 2 h at 20°C. Controls with normal rabbit serum or without stabilization were processed simultaneously. Thin sections were examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV.

After 18 h of incubation, the capsule of cells cultured in either supplemented or nonsupplemented broth was considerably thicker (130–150 and 150–200 nm, respectively) than that of cells grown on blood agar plates (90–110 nm). As a consequence, the study demonstrated that culture in broth is more suitable than culture on solid media, as it was previously demonstrated for *A. pleuropneumoniae* (14). Capsule integrity was not maintained by a high portion of cells after 18 h of incubation in broth; the degradation could be attributed to the presence of high amounts of enzymes (unpublished observations). It has been previously described for some serotypes of *S. pneumoniae* that isolates tend to show autolysis in the stationary phase of growth and cultures should sometimes be interrupted after only 4–7 h of growth to preserve capsule integrity (19,20).

When the amount of capsular material was studied after each subculture during the complete protocol using THB and THB supplemented with

TABLE II. Antibody response in rabbits against *Streptococcus suis* capsular type 2 after the inoculation of different antigenic preparations

No. of subcultures [b]	Culture conditions	Anticapsular type 2 titer[a]	
		Capsular reaction test	Slide agglutination test
6	THB	1/32 (1/8–1/64)	1/16 (1/8–1/32)
6	Supplemented THB	1/32 (1/8–1/64)	1/8 (1/8–1/8)
3	THB	1/64 (1/16–1/128)	1/32 (1/8–1/64)
3	Supplemented THB	1/16 (1/4–1/32)	1/16 (1/8–1/32)
1	THB	<1/2	<1/2
1	Blood agar plate	<1/2	<1/2

[a]Mean titer (range)
[b]See Table I for details
THB: Todd-Hewitt broth

serum, a slightly thicker capsule was always obtained in each subculture with a broth without serum (Table I). However, the total number of cells obtained using the THB supplemented with serum was always tenfold higher than that obtained with the nonsupplemented broth after the same period of time (data not shown). The age of the cultures also seemed to influence the production of capsular material, since 6 h subculture showed a thicker and more stable capsule than an 18 h culture. However, the amount of capsule was not increased when six subcultures were performed (Table I, Fig. 1a); a thick capsular layer was obtained with only three subcultures (Fig. 1b). These results showed that neither the use of serum nor the use of many subcultures increased the production of capsule of *S. suis* serotype 2.

The second objective of the present work was to investigate if the production of rabbit antibodies against the capsule could vary using cells grown under the different growth conditions above mentioned. Six and four rabbits were immunized with antigens produced in THB and THB supplemented with serum respectively using the complete protocol (six subcultures). In addition, six and four rabbit antisera were also produced with antigens obtained after three subcultures in THB and THB supplemented with serum respectively; these antigens were shown, by electron microscopy, to possess as much capsular material as the six-subculture antigens in each category. Finally, four rabbits were incubated with cells grown in an 18 h THB culture and six animals were inoculated with antigens produced on blood agar plates after 18 h of incubation. The inoculation schedule was the same for all antigens and has been described previously (8). Titers of antibodies against *S. suis* capsular type 2 were measured in triplicate by the slide-agglutination and the capsular reaction tests; sera were diluted in a twofold base in phosphate buffer solution.

In general, titers of antibodies obtained in this study were similar to those previously obtained for *S. suis* (21) and for *S. pneumoniae* (5) using the same techniques. The results indicated that similar titers were obtained using the six- and the three-subculture antigens produced in both serum supplemented and nonsupplemented broths (Table II). Despite the fact that cells produced in THB possessed a thicker capsule, differences in antibody titers were not significant. Even though antigens produced in broth media after 18 h of incubation possessed more capsular material than cells grown for a similar time on blood agar plates (see above), differences in antibody titers obtained with those antigens were not evident; only a weak response was obtained in both cases. This could probably be explained by the capsule degradation observed after the overnight incubation. These results showed that neither the use of serum nor the use of the long protocol increased the production of rabbit antibodies against the capsule of *S. suis* serotype 2.

In conclusion, the long procedure currently used for the production of antigens (8) is no longer justified as antigens produced after only three subcultures seemed to possess as much capsular material as cells obtained after the complete procedure and induced good antibody titers. The use of serum as a supplement to the broth did not assure a higher production of capsular material; in addition, antibody titers obtained with antigens produced in nonsupplemented media were as high as those obtained with serum supplemented media. Nevertheless, a higher number of cells can be obtained in the same period of time using serum as a supplement and, in addition, it has recently been demonstrated that the use of serum as a supplement can increase the virulence in mice (22). We recommend the use of three subcultures in THB for the production of immunogens. This revised protocol offers two main advantages: it is less time-consuming because of the limited number of subcultures and

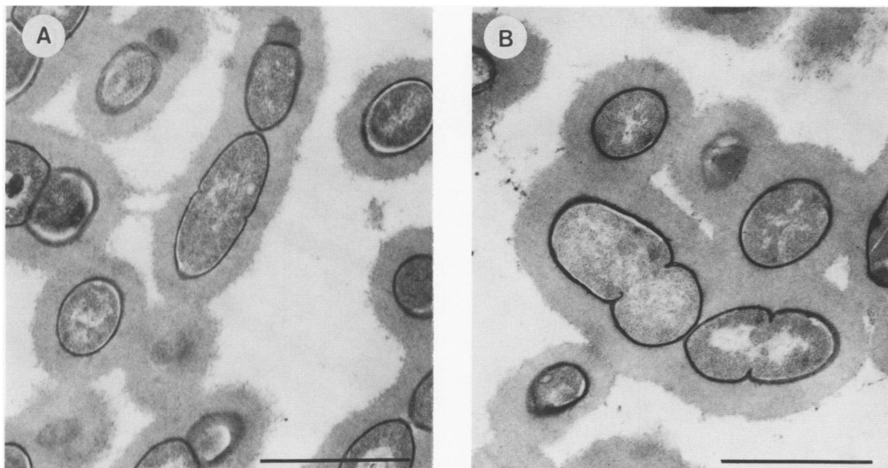

Fig. 1. Transmission electron micrographs of cells of *Strepcoccus suis* strain #735 stabilized with whole-cell serotype-specific antiserum and stained with ruthenium red. (A) Cells were grown in Todd-Hewitt broth (THB) supplemented with 5% bovine serum and subcultured a total of six times (once incubated for 18 h, four times incubated for six hours and the last incubation for two hours). (B) Cells were grown in THB without serum and subcultured only three times (once incubated for 18 hours and twice incubated for six hours). Bars : 1 μm.

it is also less expensive since nonsupplemented broths are used.

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