Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2

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Abstract

*Streptococcus suis* capsular type 2 has a capsule rich in sialic acid (NANA). Sialic acid, known to be an antiphagocytic factor for many bacterial species, inhibits the activation of the alternative complement pathway. The role of capsular NANA in virulence, resistance to phagocytosis and intracellular survival of *S. suis* capsular type 2 was evaluated. In general, a low concentration of NANA was observed for all the *S. suis* strains tested. In addition, no difference could be found in NANA concentrations between strains of different virulence degrees. Sialic acid concentration increased in the virulent strain 89-1591 and the avirulent strain 90-1330 after *in vivo* growth with an increased capsular material thickness compared to growth in vitro. No significant difference could be found in the phagocytosis rate by porcine blood monocytes of either strain and strain 89-1591 treated with sialidase or the sialic acid-binding lectin from *Sambucus nigra* (SNA I). Intracellular survival of strain 89-1591 decreased after treatments with sialidase or lectin, becoming comparable to that of strain 90-1330. Finally, no difference could be seen in virulence using a murine model, even if strain 89-1591 was treated with the enzyme or the lectin. Thus, NANA does not seem to be a critical virulence factor for *S. suis* capsular type 2.

Keywords: *Streptococcus suis*; Sialic acid; Capsule; Virulence; Phagocytosis

1. Introduction

*Streptococcus suis* is a worldwide causative agent of many swine infections such as meningitis, arthritis, septicemia and endocarditis [1] as well as being a zoonotic agent for meningitis and endocarditis [2,3]. However, virulence factors of *S. suis* have not been well identified. Indeed, within capsular type 2 isolates, the capsular type mostly isolated from diseased animals, some are highly virulent whereas others are completely avirulent [4–6]. Different bacterial structures or products, such as the capsule, fimbriae, extracellular and cell-wall-associated proteins, and hemolysin, have been considered to be virulence factors [7–9]. Among them, the polysaccharidic capsule may play an important role since it has been
recognized as an important feature for successful invasion and disease production by many bacterial pathogens [10,11]. *S. suis* type 2 possesses a capsule composed of five sugars, the third most important being sialic acid (*N*-acetyl neuraminic acid, Neu5Ac or NANA) [12]. Little is known about the role of NANA in *S. suis* type 2. Recently, the type of linkage involving NANA in the capsule of *S. suis* type 2 has been identified as a possible Neu5Ac(α2,6)Gal/GalNAc sequence [13]. Sialic acid is known to modulate the activation of the alternative complement pathway by increasing the affinity constant of C3b binding to factor H relative to factor B, thereby preventing bacterial phagocytosis by blocking the formation of C3 convertase C3bBb, and inhibiting further C3b deposition [14]. Sialic acid is involved in the virulence of many bacteria such as group B streptococci [15,16] and gonococci [17]. Arends and Zanen [2] have suggested that the presence of NANA in the capsule of *S. suis* type 2 could be involved in the virulence of the bacteria.

Although capsular NANA plays a well recognized role in the resistance to phagocytosis in other bacteria, similar functions in *S. suis* infections have not been studied. In fact, the pathogenesis of *S. suis* infections seems to be different from that reported for other streptococci also responsible for meningitis, such as group B streptococci and pneumococci [15,18]. For these pathogens, the capsule seems to play an important antiphagocytic role. In the case of *S. suis*, there are some reports which indicated that both well encapsulated virulent and avirulent isolates were easily phagocytosed by murine macrophages in absence of antibodies and/or complement but only virulent isolates survived within phagosomes [6,19].

The aim of this study was to evaluate the role of capsular sialic acid in virulence, resistance to phagocytosis and intracellular survival of *S. suis* capsular type 2.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Six different capsular type 2 isolates and the reference strain (R735, J. Henrichsen, Statens Seruminstitute, Copenhagen, Denmark) used in this study came from our collection. In addition, an unencapsulated avirulent strain, M2, derived from strain 89-1591, was also used [20]. The strains were chosen according to their different degree of virulence in both mice and pigs (R735, 89-1591, 89-999, JL 590, LM-90-559, highly virulent; 90-1330, 0891, TD 10, avirulent) [4]. Strain TD 10 was kindly provided by Dr. T. Alexander (University of Cambridge, UK). Cells were grown overnight on bovine blood agar plates at 37°C and single colonies were used as inocula for Todd-Hewitt broths (THB; Difco Laboratories, Detroit, MI).

2.2. Sialic acid concentration determination

Sialic acid concentration was determined using the thiobarbituric acid method of Warren-Aminoff [21]. Sialic acid extraction was done as follows. Cells were grown in THB for 6 h at 37°C and centrifuged at 1300 X g for 10 min. Absorbances of the suspensions were measured at 540 nm and suspensions concentrated to an A540-equivalent of 5 in phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 1.5 mM KH2PO4, 10 mM Na2HPO4, pH 7.4). Cells were lyophilized and hydrolysis in hot acid (0.4 M H2SO4, 80°C, 1 h) was performed on lyophilized cells (20 mg ml-1) to release NANA. *N*-acetylneuraminic acid was used as a standard (Sigma Chemicals, St. Louis, MO). Sialic acid concentrations were measured in at least three independent experiments.

2.3. Sialidase treatment

Sialidase from *Clostridium perfringens* (Boehringer-Mannheim, Laval, Québec, Canada) was used at a concentration of 0.6 U ml-1 sodium acetate buffer pH 5.5, where one unit liberates 1.0 μmol of NANA per min at pH 5.0 at 37°C. Treatment was performed as previously described [22]. Briefly, strain 89-1591 grown for 6 h (4 x 108 colony forming units (cfu) ml-1) was centrifuged and suspended in the enzyme solution prewarmed to 37°C. Cells were incubated for 3 h at 37°C and washed twice in PBS to remove free NANA.
2.4. Treatment with lectin I from *Sambucus nigra*

Lectin SNA I from the elderberry *Sambucus nigra* recognizes *S. suis* type 2 NANA [13] and was kindly provided by Dr. Jan T.C. Kellens (University of Maastricht, the Netherlands). Strain 89-1591 from a 6-h broth was centrifuged, suspended in a 1 mg ml⁻¹ solution of SNA I diluted 1/50 in PBS (final concentration of 20 μg ml⁻¹) such as to obtain minimal visual agglutination reactions, and left to incubate for 1 h at room temperature. Cells were then washed to remove unbound lectin and suspended in PBS.

2.5. In vivo growth conditions

Diffusion chambers were prepared using a modified methodology already described [23]. Ten-ml polypropylene syringe barrels were cut to have internal volumes of 6 ml. Filters of 0.22 μm, cut to the diameter of the syringes, were fixed to each end by melting the barrel on a hot knife. Syringes were punctured using a heated 20-G needle. Chambers were then autoclaved. A virulent (89-1591) and an avirulent (90-1330) strain was chosen for these experiments. Cells grown for 6 h in THB were centrifuged and suspended in sterile PBS to an A₅₄₀ of 0.4, corresponding to 2–4 × 10⁸ cfu ml⁻¹. Four milliliters of the bacterial suspension were injected in each chamber and the holes closed with a hot glass rod. A total of eight chambers per pig were prepared. Specific pathogen free piglets (born from hysterectomy-derived sows) aged between 5 and 10 weeks were used. These animals were kept in high-security barns until the sacrifices and were free of *S. suis*. Two pigs per strain were used each time. Pigs were anesthetized with Stresnil (2 mg kg⁻¹) by intramuscular route and with Hypnodil (10 mg kg⁻¹) (Janssen Laboratories, Belgium) by intraperitoneal route. A laparotomy was performed to install the chambers in the peritoneal cavity. The peritoneum and the skin were sewed independently with suture thread PDS II 36 mm⁻¹/2 c. 70 cm (Ethicon, France). Chambers were left to incubate for 16–18 h. The pigs were then sacrificed by exsanguination and the peritoneal cavity opened to retrieve the chambers. Chambers were washed thoroughly with sterile saline and the suspension retrieved with a sterile needle and syringe. Cells were centrifuged and suspended in PBS until used.

2.6. Phagocytic assay

Monocytes from pig blood were used in this assay and isolated using modified methods previously described [24,25]. Fresh heparinized pig blood was diluted 1:1 with sterile Hank's Balanced Saline Solution (HBSS, Gibco, Burlington, Canada), mixed with 6% (w/v) dextran (in HBSS) in a 2:1 ratio and left to incubate for 1 h at 4°C. The supernatant was then removed, centrifuged at 400 × g, 15 min, 4°C and cells washed with HBSS. Monocytes were then isolated in a Percoll (Pharmacia, Uppsala, Sweden) gradient, prepared from isotonic and Percoll stock solutions as already described [25]. Cell purity was determined by a non-specific esterase staining and was more than 80%. Cell viability was more than 99% as determined by Trypan blue exclusion. The cell concentration was then adjusted to 1 × 10⁶ monocytes ml⁻¹ in Iscove medium (Gibco, Burlington, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were distributed in 1-ml volumes in wells of a 24-well plate containing sterile microscope cover slips. Monocytes were left to incubate for 1 h at 37°C, 5% CO₂ to permit adhesion, washed three times with HBSS and reincubated in Iscove medium plus 10% FBS until use. Cells from the chambers or cells grown overnight in THB were used in this assay. To observe the effect on complement activation, cells grown in vivo were opsonized naturally in the peritoneal cavity of the pigs whereas cells grown in THB were opsonized with 5% fresh pig serum before the phagocytic assay. The assay was performed as already described [19] with some modifications. Opsonized bacteria were diluted in Iscove medium plus 10% FBS to a concentration of 5 × 10⁶ cells ml⁻¹ and added to the monocytes for a final ratio of five bacteria for one monocyte. Phagocytosis was left to proceed for 3 h at 37°C, 5% CO₂. After incubation, the medium was removed and phagocytic cells with engulfed bacteria were stained with acridine orange (Sigma Chemicals, St. Louis, MO; 0.0144%, w/v, in HBSS) for 45 s and counterstained with crystal violet (Sigma Chemicals, St. Louis, MO; 0.12 mM in 0.15 M NaCl) for
45 s to quench fluorescence of bacteria external to phagocytes. Viable bacteria fluoresced green whereas dead ones were red under ultraviolet light [26]. Calculations were made as follows:

\[
\text{Percentage of phagocytosis} = \frac{\text{number of monocytes that phagocyted bacteria}}{200 \text{ monocytes}} \times 100
\]

\[
\text{Percentage of survival} = \frac{\text{number of viable bacteria in 30 monocytes}}{\text{total number of bacteria in these 30 monocytes}} \times 100
\]

2.7. Electron microscopy of S. suis grown in vivo and in vitro

Bacteria retrieved from diffusion chambers or from THB were prepared in order to stabilize the capsule [8]. Briefly, cells were fixed in 5% (w/v) glutaraldehyde in cacodylate buffer for 2 h at room temperature. All the following solutions until acetone washing contained 0.05% (w/v) ruthenium red without antibodies. Fixed bacteria were then suspended in cacodylate buffer containing 1 mg ml\(^{-1}\) of polycationic ferritin (Sigma Chemical) and incubated for 30 min at room temperature. The reaction was slowed by ten-fold dilution with buffer, cells centrifuged and washed three times in the same buffer. Bacteria were immobilized in 4% (w/v) agar, washed five times in buffer and postfixed with 2% (v/v) osmium tetroxide for 2 h. Cells were washed again as above and dehydrated in a graded series of acetone washes. Samples were washed twice in propylene oxide and embedded in Spurr low-viscosity resin. Thin sections were finally post-stained with uranyl acetate and lead citrate and examined with an electron microscope (Phillips 201) at an accelerating voltage of 60 kV. A total of 20 to 25 capsular material thickness measurements were made on different cells of each strain grown under different conditions.

2.8. Evaluation of the virulence

Virulence of strain 89-1591 untreated, sialidase-treated and SNA I-treated was assessed using a murine model already described with some modifications [27]. Briefly, three sets of cells were grown in THB + 10% FBS to an \(A_{540}\) of 0.4. The dose of bacteria injected was increased from \(10^7\) cfu ml\(^{-1}\) to \(10^8\) cfu ml\(^{-1}\) to prevent false negative reactions i.e. too few bacteria. One set was left untreated, another set was treated with SNA I lectin and suspended in PBS supplemented with 10% FBS and the last set was treated with sialidase, concentrated ten times to compensate for the loss in viability and also suspended in PBS + 10% FBS. One milliliter of the suspensions was injected intraperitoneally to three groups of five 28-day-old BALB/c mice and mortality was monitored twice a day for 48 h. Mice were handled according to the ‘Guide to the Care and Use of Experimental Animals’ from the Canadian Council on Animal Care. Viable counts were done on each suspension.

2.9. Statistics

Data were analyzed by Student’s unpaired \(t\)-test (two-tailed) [28].

3. Results

3.1. Sialic acid concentrations

Of the nine strains tested after in vitro growth, only the unencapsulated strain M2 possessed a remarkable difference in its NANA concentration (< 1 \(\mu\)g mg\(^{-1}\) of cells; \(P < 0.05\)) (Table 1). All other strains had similar NANA concentrations without significant differences between strains of different virulence degrees \((P > 0.05)\). Strains 89-1591 and 90-1330 had the highest NANA concentration, even though their virulence differed. After in vivo growth in diffusion chambers, an increase in NANA concentration could be observed in both strains 89-1591 and 90-1330 \((P < 0.05)\). No significant difference in NANA concentration between both strains grown in vivo could be seen \((P > 0.05)\). On the other hand, strain 89-1591 treated with sialidase had little NANA left but NANA could be found in the supernatant after the treatment (results not shown).
Table 1
Sialic acid concentrations of *Streptococcus suis* capsular type 2 strains grown under in vitro and in vivo conditions.

<table>
<thead>
<tr>
<th><strong>Strain</strong></th>
<th><strong>Sialic acid concentration (μg mg⁻¹ of cells)</strong></th>
<th><strong>Treatment No. of dead mice (n = 15)</strong></th>
<th><strong>Viable counts (cfu × 10⁸ ml⁻¹)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro, mean ± S.D.</td>
<td>In vivo, mean + S.D.</td>
<td></td>
</tr>
<tr>
<td>R735(V)</td>
<td>2.58 ± 0.34</td>
<td>ND</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>89-1591(V)</td>
<td>3.36 ± 0.22</td>
<td>6.39 ± 0.45 a</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>89-999(V)</td>
<td>2.24 ± 0.67</td>
<td>ND</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>LM-90-559(V)</td>
<td>2.47 ± 0.45</td>
<td>ND</td>
<td>6.39 ± 0.45 a</td>
</tr>
<tr>
<td>JL 590(V)</td>
<td>3.03 ± 0.11</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>90-1330(A)</td>
<td>3.14 ± 0.90</td>
<td>6.61 ± 2.47 a</td>
<td>ND</td>
</tr>
<tr>
<td>0891(A)</td>
<td>2.91 ± 0.90</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TD10(A)</td>
<td>2.69 ± 0.45</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M2 (A) b</td>
<td>0.34 ± 0.11 c</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

S.D., standard deviations; ND, not done; V, virulent; A, avirulent.

<table>
<thead>
<tr>
<th><strong>Strain</strong></th>
<th><strong>Phagocytosis (%) after growth</strong></th>
<th><strong>Survival (%) after growth</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D. (n = 3) a</td>
<td>Mean ± S.D. (n = 3) b</td>
</tr>
<tr>
<td>90-1330</td>
<td>23 ± 3</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>89-1591</td>
<td>29 ± 2</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>89-1591Ase</td>
<td>40 ± 2</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>89-1591SNA</td>
<td>37 ± 8</td>
<td>50 ± 19</td>
</tr>
<tr>
<td>89-1591</td>
<td>23 ± 4 b</td>
<td>30 ± 5 b</td>
</tr>
<tr>
<td>89-1591Ase</td>
<td>42 ± 6</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>89-1591SNA</td>
<td>20 ± 9 c</td>
<td>21 ± 6 c</td>
</tr>
<tr>
<td>M2 (A) c</td>
<td>31 ± 8 c</td>
<td>25 ± 9 c</td>
</tr>
</tbody>
</table>

S.D., standard deviations; Ase, sialidase; SNA, SNA 1 lectin.

3.2. Effect of absence or blocking of NANA on phagocytosis and survival

Table 2 presents results of 3 h of phagocytosis after in vitro and in vivo growth, with or without enzyme or lectin treatments. Phagocytosis was similar for both virulent and avirulent strains, regardless of growth conditions (P > 0.05). Enzyme or lectin treatments did not affect phagocytosis, except for lectin-treated 89-1591 after in vivo growth which showed a slight increase in phagocytosis although it was not significant (P > 0.05). Absence of capsule in strain M2 caused an increase in phagocytosis (63 ± 3%, P < 0.05; results not shown). After 3 h, survival was more affected for the avirulent strain 90-1330 in both growth conditions compared to the virulent strain 89-1591 (P < 0.05). On the other hand, after sialidase and lectin treatments, the degree of survival decreased for 89-1591, becoming comparable to the avirulent strain (P > 0.05).

3.3. Effect of in vivo growth on capsular material thickness

After in vitro growth, a similar capsular material thickness was observed for both the virulent 89-1591 and the avirulent 90-1330 strains (Fig. 1). However, after in vivo growth, both strains have a thicker capsule compared to growth in laboratory media. Thickness of capsular material for 89-1591 and 90-1330 after in vitro growth ranged between 70–75 nm, and 60–80 nm, respectively. On the other hand,
Fig. 1. Transmission electron micrographs of thin sections of *Streptococcus suis* capsular type 2 labeled with polycationic ferritin. (A) Virulent strain 89-1591 and (B) avirulent strain 90-1330 grown overnight in THB. (C) Strain 89-1591 and (D) strain 90-1330 grown overnight in pig intraperitoneal chambers. Bars represent 200 nm.

in vivo growth gave capsules of 80–135 nm and 95–150 nm for 89-1591 and 90-1330, respectively.

3.4. Effect of absence or blocking of NANA on virulence

Enzyme treatment decreased the viability by one log when colony counts were compared for treated and non-treated cells. Integrity of capsular material was not affected by the treatment as shown by serotyping and electron microscopy (results not shown). No difference was observed in the mortality of the three groups of mice regardless of the treatments. Untreated, sialidase-treated and lectin-treated bacteria all killed twelve to thirteen mice out of fifteen (Table 3). In addition, clinical signs appeared
simultaneously a few hours after infection in the three groups of animals. No mortality or clinical signs were observed with mice inoculated with the avirulent unencapsulated strain M2 or with the avirulent lectin-treated strain 90-1330 (results not shown).

4. Discussion

Sialic acid does not seem to be a critical virulence factor for \textit{S. suis} capsular type 2 since all strains tested possess a similar concentration of NANA in their capsule, regardless of their virulence. The presence of NANA in the capsule of \textit{S. suis} type 2 is confirmed by the fact that little NANA could be found in the unencapsulated strain M2, the weak reaction being probably due to residual capsular material. Compared to our results, NANA concentration in \textit{S. suis} is one and a half less than that found in the least virulent strains of group B streptococci (GBS) and four times less than that in the most virulent GBS strains [29]. This difference in NANA concentration between the two groups of streptococci, and the difference in the pathogenesis of the infections proposed for both bacterial species [15,16,35], may explain why NANA has a pathogenic role in GBS but not in \textit{S. suis}.

Some virulence factors are not synthesized when bacteria are grown in laboratory media and are only expressed under in vivo conditions [30]. In addition, it was shown that capsular NANA concentration increased after growth of group B streptococci in human serum [31]. To examine such effects on \textit{S. suis} type 2, intraperitoneal chambers were used to grow bacteria in vivo. Sialic acid concentration as well as capsular material thickness increased after in vivo growth for both virulent and avirulent \textit{S. suis} strains. This increase in NANA concentration between the two groups of streptococci, and the difference in the pathogenesis of the infections proposed for both bacterial species [15,16,35], may explain why NANA has a pathogenic role in GBS but not in \textit{S. suis}.

Porcine monocytes instead of neutrophils were chosen since these phagocytes have the most important role in the pathogenesis of \textit{S. suis} infection. Among others, Williams and Blakemore [35] proposed that \textit{S. suis} enters the circulation from tonsils, is taken up by monocytes and transported to the cerebrospinal fluid via the choroid plexus. Our percentages of phagocytosis of virulent and avirulent \textit{S. suis} strains, obtained with porcine monocytes, were similar to those previously obtained with murine macrophages [6,19], and this regardless of the growth conditions. Even though the concentration of NANA doubles after in vivo growth of 89-1591, no difference was found after 3 h in the phagocytosis of this strain after in vivo or in vitro growth, indicating that complement activation does not seem to be affected by the presence of NANA. This confirms data indicating that complement does not seem to play an important role in phagocytosis of \textit{S. suis} [19]. The slight increase in phagocytosis rate for lectin-treated 89-1591 after in vivo growth could be due to microagglutinations produced by the lectin, causing bacteria to clump and facilitating ingestion by monocytes. On the other hand, a difference between the two strains differing in virulence which was greater for the untreated strain 89-1591 than that of the avirulent 90-1330. These results confirm those reporting that both virulent and avirulent strains are phagocytosed with the virulent strains surviving more easily inside macrophages [6,19]. Survival rate decreased for the virulent strain after sialidase or lectin treatments reaching the survival level of the avirulent 90-1330. It is possible that factors other than NANA are modified after enzyme or lectin treatments, which may affect the intracellular fate of \textit{S. suis}. However, other experiments need to be done to confirm this possibility.

It was shown that treatment with sialidase did not
affect the virulence of *S. suis*. It could be thought that sialidase-treated bacteria had time to replicate inside mice and regain their NANA moiety causing bacteria to be more virulent. However, clinical signs appeared within a few hours after infection for all groups of mice. It was shown with group B streptococci that sialidase treatment of virulent strains rendered them less virulent to rats, indicating that NANA-free cells were destroyed more easily than cells with NANA [22].

Despite our results, a possible, indirect role for sialic acid in virulence cannot be ruled out. In fact, NANA could have a role in mimicry to evade host responses. It was demonstrated that no anti-capsular antibodies could be found in convalescent animals experimentally infected and extremely ill with *S. suis* type 2 [36]. These findings could enhance the possibility that the capsular NANA may be involved in the protection of the bacteria against the host’s immune system. Some workers have suggested that NANA may mask epitopes of protective antibodies [22].

In conclusion, sialic acid does not seem to be critical in the virulence of *S. suis* type 2 since all strains tested had the same low sialic acid concentration regardless of their virulence. In addition, blocking or enzymatic removal of sialic acid does not seem to influence the virulence or phagocytosis of the strain used.

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