Studies on a murine model for evaluation of virulence of Streptococcus suis capsular type 2 isolates

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1. SUMMARY

Five different parameters, time of incubation of the culture, type of culture medium, inoculum, strain of inbred mice, and age of mice, were tested using the LD₅₀ technique to standardize a murine model for the evaluation of the virulence of Streptococcus suis capsular type 2 isolates. A model using 28 day-old mice belonging to CF1 strain appeared to give the best results. The inoculum size was the parameter most influencing the 50% lethal dose obtained with mice. Inoculation with 1-ml volume of a bacterial suspension instead of 0.1 or 0.5 ml decreased the LD₅₀. The standardized model was used to evaluate the virulence of some isolates of known pathogenicity for pigs. The minimum lethal dose was used in the model and it appeared that the virulence of Streptococcus suis capsular type 2 isolates can be measured from highly virulent to totally avirulent.

2. INTRODUCTION

Streptococcus suis is an important pathogen which has been associated with a wide variety of infections in swine such as septicemia, meningitis, and pneumonia [1]. It has also been isolated from other animal species [2,3] as well as from humans [4,5]. Until now, 29 different capsular types of Streptococcus suis have been described [6-8]. In many countries, S. suis capsular type 2 is considered to be the most virulent as well as the most prevalent capsular type in diseased pigs [2,3], but isolates belonging to this capsular type are also found in nasal cavities of clinically healthy pigs [9,10].

Although experimental infections of pigs with S. suis have already been realized [11,12], little is known about S. suis capsular type 2 pathogenesis, due in part to the lack of a suitable experimental model. Inoculation of mice with S. suis capsular type 2 was originally carried out by Williams and co-workers [13] who reported an indication that the behaviour of this microorganism in mice resembled that reported in pigs. Later, Kebede and co-workers [14] also used a model of experimen-
tal infection with *S. suis* capsular type 2 in young mice for immunization studies. Also, Robertson and Blackmore [15,16] reported the use of laboratory animals such as mice, rats and rabbits to determine the pathogenicity of different isolates of *S. suis* capsular type 2; they concluded that mice are the best indicator species to assess the pathogenicity of isolates for pigs. In 1991, Kataoka and co-workers [17] tested the susceptibility of five inbred strains of mice. Most of these studies also demonstrated that there were virulent and avirulent strains against mice and pigs among the strains of *S. suis* serotype 2. The findings are in accordance with those of Drolet and co-workers [18], and Clifton-Hadley [19].

Previous authors have used different methodologies in their mouse model for *S. suis* making difficult the comparison of results from one study to another. Moreover, previous models did not allow a comparative measurement or titration of the virulence of a group of *S. suis* strains of different origin. The main objective of this study was to evaluate different parameters necessary for the standardization of an experimental murine model of infection with *S. suis* capsular type 2. This standardized model was then used to titrate the virulence of various isolates of *S. suis* capsular type 2 in comparison with a virulence control strain, and a reference strain. Such a standardized model would be an invaluable tool to study virulence factors and carry out protection trials.

3. MATERIALS AND METHODS

3.1. Bacterial strains

*S. suis* capsular type 2 strain 89-1591 was chosen as the virulence control strain and all evaluations of the different parameters in the experimental model were solely based on this strain. It originated from a case of septicemia and meningitis in a pig [20] and its virulence had been maintained at its maximum by serial passages in mice. In addition, 10 different capsular type 2 isolates, the reference strain of the same capsular type (S735, J. Henrichsen, Statens Seruminstitute, Copenhagen, Denmark), and two avirulent mutants of the strain 89-1591 were tested with the murine model. Eight of the field isolates were of swine origin. Of them, three were recovered from cases of pneumonia, two from cases of septicemia, and three were isolated from the nasal cavities of clinically healthy animals. The two other field isolates corresponded to a case of abortion in a cow [2] and to a case of meningitis in a human (AR 770297, J.P. Arends, Groningen, The Netherlands). These 10 field isolates were selected according to data obtained from studies based on restriction endonuclease fingerprinting and ribotyping [21].

All the strains or isolates used in this experiment had their identity confirmed by 11 different biochemical tests: growth in 6.5% NaCl, arginine dihydrolase, production of acetoin, production of acid from lactose, salicin, glycerol, inulin, trehalose, sorbitol, sucrose and mannitol [1,3]. All of them had the characteristic biochemical profile of *S. suis* [22], except isolate 4/3 H1 which was mannitol-positive and originated from a clinically healthy pig. With the exception of the two mutants, all strains were confirmed as capsular type 2 members using both the coagglutination and the capsular reaction tests [23,24].

3.2. Evaluation of parameters

A total of five different parameters were evaluated for the experimental model of infection: time of incubation (8 h vs. 18 h) at 37°C in aerobiosis; presence (THS) or absence (TH) of 10% inactivated bovine serum in the culture medium (Todd-Hewitt broth; Difco), volume of the inoculum to mice (0.1 ml, 0.5 ml, or 1.0 ml); strain of inbred mice (CD1 vs. CF1); and age of mice (28 days, 50 days, and 80 days). Each parameter was individually evaluated using the LD₅₀ technique with the optimal virulence control strain 89-1591. The standardization was carried out in several assays. For each of these assays, only one parameter was tested and the best one was incorporated into the next step in which another parameter was evaluated (Table 1).

3.3. Fifty percent lethal dose

The 50% lethal dose (LD₅₀) was determined according to the method of Reed and Muench [25]. Each LD₅₀ was estimated with groups of five
mice. As in previous experiments the cell-free supernatants of cultures were demonstrated to be innocuous for mice; cells were not washed (data not shown). Bacterial suspensions were adjusted to an optical density of 0.4 (540 nm) which corresponded approximately to a concentration of 10⁹ cfu/ml and were injected intraperitoneally to mice. The exact number of viable cells present was counted by a pour-plate method, using blood-agar plates incubated for 18 h at 37°C. Death or presence of meningitis was monitored over the next 9 days. The test was repeated in duplicate for each strain. Mice were also inoculated with sterile TH and THS as controls.

3.4. Evaluation of the virulence of S. suis isolates

Once the most adequate parameter for each category was chosen, the field isolates, the reference strain as well as the two mutant strains of S. suis serotype 2 were tested using the murine model. Since the use of the LD₅₀ for routine testing of virulence in mice was not a very practical means, it was decided to use the minimal lethal dose (MLD) as measured with the 89-1591 control strain. This dose was established by adjusting OD₅₄₀ to 0.09 with Todd-Hewitt broth, which corresponded to a concentration of 10⁷ cfu/ml. This eliminated the possibility of classifying low-virulent isolates as high-virulent isolates by inoculating too many bacteria. In order to establish a virulence titer, the isolates were classified according to the number of dead mice. A total of 10 mice were used per isolate or strain, and the experiment was repeated twice always using the strain 89-1591 as a positive control. Isolates and strains were classified as highly virulent (seven or more dead mice); weakly virulent (between three and six dead mice); and avirulent (two or less dead mice).

4. RESULTS

4.1. Evaluation of parameters

Each of the five different parameters involved in the standardization of the murine experimental model of infection with S. suis capsular type 2 was chosen on a sequential basis as described in Table 1.

The LD₅₀ method was used as the performance indicator to compare the different alternatives in each assay (Table 2). In assay 1, an 8-h culture of the virulence control strain (89-1591) had an LD₅₀ fifteen times lower than that of an 18-h culture of the same strain. In assay 2, the presence of inactivated bovine serum in the medium did not decrease the LD₅₀. However, the number of bacterial cells was higher in the serum-supplemented medium after 8 h of incubation, due to more rapid growth. Inoculation of sterile TH and THS did not affect control mice.

In assay 3, the LD₅₀ was lowered when giving the inoculum in a 1-ml volume rather than in a 0.1- or 0.5-ml volume. The comparison of the two inbred of mice (CD1 vs. CF1) in assay 4 did not demonstrate any tangible difference in the LD₅₀ value; for the rest of the assay, the CF1 inbred strain of mice was chosen. Finally, when three different age groups of mice, 28, 50 and 80 days, were compared (assay 5), the LD₅₀ values were similar. Then, for practical reasons, 28-day-old

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steps involved in the evaluation of five different parameters for the experimental model of infection with Streptococcus suis capsular type 2</td>
</tr>
<tr>
<td>Assay No.</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>1.</td>
</tr>
<tr>
<td>2.</td>
</tr>
<tr>
<td>3.</td>
</tr>
<tr>
<td>4.</td>
</tr>
<tr>
<td>5.</td>
</tr>
</tbody>
</table>

a Parameter chosen in the previous assay.

TH = Todd-Hewitt broth.

THS = Todd-Hewitt broth with 10% inactivated bovine serum.
Table 2
Sequential analysis of the different parameters of the murine model using the LD_{50} method

<table>
<thead>
<tr>
<th>Assay No.</th>
<th>Time of incubation (h)</th>
<th>Medium</th>
<th>Inoculum (ml)</th>
<th>Inbred strain</th>
<th>Age of mice (days)</th>
<th>LD_{50} (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>TH</td>
<td>0.5</td>
<td>CD1</td>
<td>28</td>
<td>(4.4 \times 10^6)</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>TH</td>
<td>0.5</td>
<td>CD1</td>
<td>28</td>
<td>(2.9 \times 10^6)</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>THS</td>
<td>0.5</td>
<td>CD1</td>
<td>28</td>
<td>(6.1 \times 10^6)</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>THS</td>
<td>0.1</td>
<td>CD1</td>
<td>28</td>
<td>(1.6 \times 10^6)</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>THS</td>
<td>1.0</td>
<td>CD1</td>
<td>28</td>
<td>(2.5 \times 10^6)</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>THS</td>
<td>1.0</td>
<td>CF1</td>
<td>28</td>
<td>(1.5 \times 10^6)</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>THS</td>
<td>1.0</td>
<td>CF1</td>
<td>50</td>
<td>(1.6 \times 10^6)</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>THS</td>
<td>1.0</td>
<td>CF1</td>
<td>80</td>
<td>(1.8 \times 10^6)</td>
</tr>
</tbody>
</table>

* As indicated in Table 1.
* Chosen parameter in the standardized model.

mice were chosen. Thus, the final choice of the different parameters was the following: 28-day-old mice belonging to CF1 strain, inoculated with a 1-ml volume of a bacterial suspension obtained from an 8-h culture in THS.

Table 3
Evaluation of the virulence of different strains of *Streptococcus suis* type 2 with an experimental murine model of infection

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin/No. of dead mice</th>
<th>Degree of virulence</th>
<th>Ribotyping profile (BglII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>89-1591</td>
<td>P/sep. 9</td>
<td>HV</td>
<td>B</td>
</tr>
<tr>
<td>LM90-559</td>
<td>P/sep. 8.5</td>
<td>HV</td>
<td>B</td>
</tr>
<tr>
<td>90-999</td>
<td>P/sep. 9</td>
<td>HV</td>
<td>B</td>
</tr>
<tr>
<td>4/40 H2</td>
<td>P/norm. 9</td>
<td>HV</td>
<td>B</td>
</tr>
<tr>
<td>4/3 H1</td>
<td>P/norm. 8.5</td>
<td>HV</td>
<td>D</td>
</tr>
<tr>
<td>4/39 H1</td>
<td>P/norm. 8.5</td>
<td>HV</td>
<td>B</td>
</tr>
<tr>
<td>90-741</td>
<td>B/abor. 7.5</td>
<td>HV</td>
<td>B</td>
</tr>
<tr>
<td>S735</td>
<td>P/men. 7</td>
<td>HV</td>
<td>C</td>
</tr>
<tr>
<td>AR770297</td>
<td>H/men. 3.5</td>
<td>WV</td>
<td>C</td>
</tr>
<tr>
<td>89-4223</td>
<td>P/pneu. 3.5</td>
<td>WV</td>
<td>C</td>
</tr>
<tr>
<td>89-6891</td>
<td>P/pneu. 4</td>
<td>WV</td>
<td>A</td>
</tr>
<tr>
<td>90-1330</td>
<td>P/pneu. 0</td>
<td>AV</td>
<td>A</td>
</tr>
<tr>
<td>M2, M42</td>
<td>89-1591 0</td>
<td>AV</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

P = pig; H = human; B = bovine; men = meningitis; pneu = pneumonia; sep = septicemia; norm = normal; abor = abortion; HV = highly virulent; WV = weakly virulent; AV = avirulent; N.D. = not determined

* Optimal virulence control strain.
* Mannitol-positive isolate;

4.2. Evaluation of the virulence of *S. suis* isolates

With the standardized model, the virulence control strain (89-1591) always killed nine mice out of 10, whereas the mutant strains (M2, M42) failed to kill mice. Five swine isolates, the reference strain (S735) and the bovine isolate were classified as highly virulent (Table 3). Their virulence was comparable to that of the virulence control strain 89-1591. Of those five swine isolates, three had been recovered from nasal swabblings of clinically healthy pigs, and the two others originated from cases of septicemia. It is interesting to note that four of the virulent strains showed an identical fingerprinting profile while hybridizing with a riboprobe (Table 3).

Two out of three pig isolates associated with cases of pneumonia were classified as weakly virulent, along with the human isolate responsible for meningitis. Finally, only one swine-field isolate failed to kill mice. Its ribotyping profile was similar to that of a weakly virulent isolate. As expected, none of the avirulent mutants could kill any of the mice, nor cause meningitis.

5. DISCUSSION

5.1. Experimental murine model of infection

The choice of an 8-h culture for the preparation of the inoculum was based on the previous demonstration that the capsule was thicker on cells after a 6-h culture than after an 18-h culture [20]. In this experiment, the LD_{50} value was lower.
with the 8-h culture than with the 18-h culture, suggesting that the amount of capsular material could influence the virulence of *S. suis* capsular type 2. The use of serum to supplement the Todd-Hewitt broth was also shown not to affect the production of capsular material by *S. suis* serotype 2, but it did increase the number of bacterial cells in a same period of time [20].

In the present study, the bacterial suspension was made with Todd-Hewitt broth. Interestingly, according to Baltimore and coworkers [26], the bacterial suspension for the preparation of the inoculum has to be done in Todd-Hewitt broth or in blood broth and the use of saline resulted in the absence of mortality in mice when injected with group B streptococci. However, Katoaka and co-workers [17] succeeded in killing mice when using saline as dilutant.

The volume of the inoculum, for a same number of bacterial cells, seemed to affect the outcome of the experimental infection. In this study, the LD$_{50}$ value was lowered when a 1-ml inoculum was used rather than a smaller (0.1 or 0.5 ml) inoculum was injected. This is in accordance with Baltimore and coworkers [26] who demonstrated a decrease of the LD$_{50}$ when increasing the volume of the inoculum to 1 ml. However, they did not succeed in a further decrease of the LD$_{50}$ when the inoculum volume was increased to 2 ml.

Although no difference in susceptibility was observed between mice belonging to three different groups of age, the choice of four week-old mice was based on practical reasons. Williams and co-workers [13] had concluded that four-week-old mice were more susceptible to *S. suis* capsular type 2 than older mice, whereas Robertson and Blackmore had succeeded in obtaining a good response with 6–12-week-old mice [15]. In the present study, both inbred strains of mice, CD1 and CF1, appeared to be susceptible to the *S. suis* inoculum. Kataoka and co-workers [17] had failed to reproduce the disease in CF1 mice, but other parameters in their experiment differed from those evaluated in this study.

In mice, there appeared to be a dose response for the development of clinical signs. According to Robertson and Blackmore [15], only mice receiving more than $10^6$ organisms show signs of disease. Williams and co-workers [13] had reported that at least $10^8$ organisms had to be inoculated intravenously for meningitis to develop in mice. In our experience, an inoculum of $10^7$ can reproduce the disease in mice. These differences in the number of bacterial cells in the inoculum could be attributed to differences in one or more of the parameters tested in this study. It should be possible with a standardized experimental murine model of infection, to make more uniform the different requirements for the reproduction of the disease in mice.

5.2. Evaluation of the virulence of *S. suis* isolates

To the best of our knowledge, this is the first report about titration of virulence carried out on *S. suis* isolates with a standardized experimental murine model of infection. As mentioned by other authors [13,27,28], it appears that the virulence of *S. suis* capsular type 2 isolates may vary from highly virulent to totally avirulent. Highly virulent isolates could probably be identified as such, by the presence of specific DNA bands as shown by ribotyping techniques [21]. It is interesting to note that only highly virulent strains had a double band in the 10-kDa area. Vecht and co-workers [27] demonstrated that two proteins could be found in *S. suis* type 2 isolates and that one or two of these proteins were virulent factors that play a role in the pathogenesis of this microorganism. Moreover, Gottschalk and co-workers [17] demonstrated that isolate 89-1591 possessed a 44-kDa protein which was absent in avirulent mutants strains M2 and M42. This model could then be used to make a correlation, among these isolates, between the presence of a particular protein and their virulence. It could also be very useful for further studies on protection.

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REFERENCES