A novel intranasal mouse model for mucosal colonization by *Streptococcus suis* serotype 2

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**INTRODUCTION**

*Streptococcus suis* is one of the most important swine pathogens worldwide, causing severe diseases such as meningitis, septicaemia and bronchopneumonia (Higgins & Gottschalk, 2005). Furthermore, it is an emerging zoonotic agent. Meningitis and septicaemia are also important manifestations of *Streptococcus suis* infections in humans (Gottschalk et al., 2007; Tang et al., 2006). It is noteworthy that, in 2005, a *Streptococcus suis* outbreak in China affected 204 humans of whom 38 died, mainly because of streptococcal toxic shock-like syndrome (Tang et al., 2006). In Vietnam, *Streptococcus suis* is the most important cause of meningitis in adults (Mai et al., 2008). This is related to eating ‘high-risk’ dishes, such as undercooked blood and intestines (Ho et al., 2011). Occupational exposure to pigs and pork is the most important risk factor for *Streptococcus suis* infections worldwide (Arends & Zanen, 1988).

Pigs and wild boars are considered the natural reservoir of *Streptococcus suis* (Baums et al., 2007; Clifton-Hadley & Alexander, 1980; Higgins & Gottschalk, 2005). Different mucosal surfaces might be colonized by *Streptococcus suis*. In weaning piglets, *Streptococcus suis* is among the most abundant colonizers of the upper respiratory and alimentary tracts (Baaks et al., 2001; Su et al., 2008). Healthy carriers of virulent *Streptococcus suis* strains play an important role in the epidemiology of *Streptococcus suis* diseases in pigs and humans (Hoa et al., 2011). Their movement to uninfected herds leads to spreading of disease. As *Streptococcus suis* is a facultative pathogen, different biotic and abiotic factors such as virus infections, corrosive gases and crowding are thought to promote *Streptococcus suis* diseases in modern swine production.

*Streptococcus suis* shows a high diversity, as reflected by the presence of at least 33 serotypes. Serotype 2 is the most virulent one and has been responsible for the majority of *Streptococcus suis* infections worldwide (Hoa et al., 2011). In this study, we describe the establishment of an intranasal mouse model for colonization of the respiratory tract with a highly virulent *Streptococcus suis* serotype 2 strain.

**Streptococcus suis** causes meningitis and various other diseases in pigs and humans. Healthy piglets carrying virulent *Streptococcus suis* strains on their mucosal surfaces are epidemiologically very important. The objective of this study was to establish an intranasal *Streptococcus suis* mouse model for invasion and colonization of the respiratory tract. CD1 mice were infected intranasally with a highly virulent *Streptococcus suis* serotype 2 strain under different conditions. Clinical, histological and bacteriological screenings revealed that invasion of host tissue occurred in the majority of mice only after predisposition with 12.5 μl 1% acetic acid per nostril. Severe fibrinosuppurative or purulent necrotizing pneumonia associated with *Streptococcus suis* was a common manifestation. Furthermore, a novel model to study nasopharyngeal colonization was established by reducing the volume of 1% acetic acid per nostril to 5 μl prior to *Streptococcus suis* application. This model mimics asymptomatic carriage in swine, as all mice carried *Streptococcus suis* on their respiratory mucosa at 7 days post-infection (p.i.) in moderate to high numbers without the development of pneumonia or any other invasive *Streptococcus suis* disease. This intranasal *Streptococcus suis* model was applied to investigate the function of suilysin (SLY) in colonization. Although an isogenic SLY mutant was isolated from the upper respiratory tract at a lower recovery rate than its wild-type parental strain at 14 days p.i., the differences were not significant and did not indicate severe attenuation in colonization. In conclusion, this work describes to the best of our knowledge the first intranasal mouse model to study colonization of the respiratory tract by a highly virulent *Streptococcus suis* pathotype.
prevalent serotype worldwide among invasive isolates from pigs and humans (Wei et al., 2009; Wisselink et al., 2000). The serotype is determined by the polysaccharide capsule, which protects the bacteria against opsonophagocytosis and functions as an important virulence factor (Charland et al., 1998; Smith et al., 1999). A number of other surface-associated factors have also been demonstrated to contribute to the pathogenesis of *Streptococcus suis* diseases (Baums & Valent-Wei gand, 2009; Fittipaldi et al., 2012). The majority of, but not all, invasive *Streptococcus suis* isolates secrete a pore-forming cholesterol-dependent cytolsin called suilysin (SLY) (King et al., 2001). SLY might exhibit different functions in the pathogenesis of *Streptococcus suis* diseases, as it has been shown to cause cytotoxicity for various cells *in vitro* (Allen et al., 2001; Jacobs et al., 1994; Segura & Gottschalk, 2002). It may also contribute to bacterial escape of opsonophagocytosis at sublytic concentrations (Benga et al., 2008; Chabot-Roy et al., 2006; Lecours et al., 2011). The pathogenesis of *Streptococcus suis* meningitis is still not well understood and even less is known about the mechanisms employed by *Streptococcus suis* to colonize mucosal surfaces. In fact, not a single factor of *Streptococcus suis* has been demonstrated to be crucial for colonization.

A *Streptococcus suis* mouse meningitis model was first described by Williams et al. (1988) using intravenous application. Histopathological lesions in the brain and inflammatory responses were characterized more recently in detail in an intraperitoneal *Streptococcus suis* model in CD1 mice (Dominguez-Punaro et al., 2007). In the latter model, 20 % of infected mice died shortly after infection in association with high levels of systemic pro-inflammatory cytokines. Animals surviving septicemia frequently developed meningitis. As the upper respiratory tract is considered to be the port of entry for *Streptococcus suis* (Williams & Blakemore, 1990), early steps in the pathogenesis of *Streptococcus suis* diseases cannot be studied in the described mouse models. Furthermore, colonization of the respiratory mucosa can only be investigated in mice using intranasal infection. Here, we report that *Streptococcus suis* colonizes the murine respiratory tract efficiently after intranasal application following predisposition. As cholesterol-dependent cytolsins, in particular pneumolysin, have been demonstrated to contribute to mucosal colonization (Kadioglu et al., 2002; Richards et al., 2010), our novel *Streptococcus suis* mouse model was applied to investigate the contribution of SLY to colonization of the murine respiratory tract.

**METHODS**

**Bacterial strains and culture conditions.** *Streptococcus suis* serotype 2 wild-type (wt) strain 10 was kindly provided by H. Smith (Leystad, The Netherlands). This strain expresses extracellular factor, muramidase-released protein, SLY, fibrinectin and fibrinogen-binding protein, and opacity factor of *Streptococcus suis*. It has been used by different groups successfully for mutagenesis and experimental intranasal infections of pigs (Baums et al., 2006; de Greeff et al., 2002; Smith et al., 1999; Vecht et al., 1997). The isogenic SLY-deficient mutant of strain 10 (designated 10 Δsly) was constructed during a previous study (Benga et al., 2008). *Staphylococcus aureus* was identified by typical colony morphology and a positive coagulase and hyaluronidase reaction (note that all putative isolates showed typical golden-coloured colonies). Bacteria were grown on Columbia agar supplemented with 6 % sheep blood (Oxoid) or in Todd–Hewitt broth (Difco) under aerobic conditions at 37 °C.

**Experimental intranasal infection of mice.** *Streptococci* grown to late exponential growth phase (OD600 of 0.8) were harvested by centrifugation and resuspended in PBS (pH 7.4) for intranasal infection. Inoculum concentrations were verified by plating tenfold serial dilutions. Four-week-old specific-pathogen-free female mice of the outbred strain Crl:CD1 (ICR) obtained from Charles River Laboratories were used for all experiments except for Experiment 2, in which mice of the *Staphylococcus aureus*-free outbred strain Hsd : ICR (CD1) were used, purchased from Harlan Laboratories. Animals were divided randomly into groups consisting of five to six animals each. Mice were allowed to acclimate for 1 week and were cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series, no. 123: http://conventions.coe.int/treaty/en/treaties/html/123.htm; permit no. 33.9-42502-04-08/1589). Before infection, mice were anaesthetized via inhalation of isofluran (IsoFlo; Albrecht). In Experiment 2, mice were pre-treated with 12.5 μl and in Experiments 3 and 4 with 5 μl 1 % acetic acid (pH 4.0) placed in each nostril 1 h prior to intranasal infection. After a controlled recovery phase and further anaesthesia, the mice were infected with 5 × 10^6 cfu *Streptococcus suis* wt strain 10 or strain 10 Δsly. The inoculum was applied in two drops of 12.5 μl placed in front of the nostrils.

**Clinical score.** Animals were examined every 8 h. Health status was rated using a clinical score sheet (Table 1), and included weight development, clinical signs of general sickness (rough coat, rapid breathing, dehydration) and clinical signs indicating meningitis (apathy, apraxia) or septicaemia (swollen eyes, depression). A cumulative score of 3–4 indicated mild clinical signs, 5–6 indicated moderate clinical signs and >6 indicated severe clinical signs with specific regard to neural failure. Mice with a cumulative score of >3 were classified as diseased (calculation of morbidity). In the case of severe weight loss (>20 %) and/or enduring severe clinical signs, mice were euthanized for reasons of animal welfare by inhalation of CO2 and cervical dislocation.

**Histological screening.** Immediately after euthanasia, necropsy was conducted and the following organs were aseptically removed and split for histological and bacteriological screenings: spleen, liver, kidney, heart, lung and brain. For histology, organs were fixed in 10 % formalin and embedded in paraffin wax. In addition, spinal cord segments (cervical, thoracic and lumbar) encased within vertebral bodies and sagittal sections of the nasal cavity were formalin-fixed, decalcified in 10 % EDTA solution for 48 h and subsequently embedded in paraffin wax. Histological screenings were carried out as blinded experiments. Fibrinogenic and purulent necrotizing lesions were scored as described previously for piglets (Baums et al., 2006). The group score (Gs) was calculated by dividing the sum of the highest scores of each animal for any of the investigated organs by the number of animals. Rhinitis was not included in the score.

**Reisolation of Streptococcus suis strains from tissue and tracheosaline lavage (TLL).** One half of each organ was suspended in 5 ml cold PBS (pH 7.4). After weighing, the suspensions were homogenized with an Ultra Turrax (IKA). Tenfold serial dilutions of the tissue suspensions were plated on blood agar plates. The number...
Concentration of 0.5 mM TGTACCGGTGATTCCAAACAAGATATTAA-3 associated with the recovery of 4000 c.f.u. of the challenge revealed a severe purulent meningitis and encephalitis infection (p.i.) (Table 2). Histopathological screening during the observation periods of 5 or 12 days, respectively.

Based on c.f.u. (mg organ)^-1 was calculated. As a read-out parameter for colonization of the upper respiratory tract, TNL was obtained and investigated as follows. The trachea was opened and a retrograde irrigation of the nasal cavity with 300 µl PBS was collected. After serial platings, the number of c.f.u. (ml TNL)^-1 of z-haemolytic streptococci with a colony morphology typical for Streptococcus suis strain 10 was determined. It should be noted that mice were also colonized in the respiratory tract by other z-haemolytic streptococci showing different colony morphology, namely a much larger zone of z-haemolysis and the formation of smaller colonies. Isolated typical z-haemolytic streptococci were profiled in a Streptococcus suis multiplex PCR for detection of the mep, efp, sly, arcA, gdh, cps1, cps2, cps7 and cps9 genes (Silva et al., 2006). Isolates from mice challenged with 10^4 sly were investigated additionally in a sly-specific PCR using the primer pair slyAgefor (5’-TGTAACGGTGATTCCAAAAGATATTAA-3’) and slyAge3new (5’-TTAACCGGTACTCTATCACCCTCATCCG-3’) with a final concentration of 0.5 µM, as described previously (Silva et al., 2006). Based on c.f.u. (mg organ)^-1 or (ml TNL)^-1, bacterial loads were classified as low (+; <100), moderate (+ +; ≥ 100 but ≤1000) or high (+ + +; >1000).

Statistical analysis. A Mann–Whitney test was performed to analyse differences between two groups of mice. Statistical significance was defined at P<0.05.

RESULTS AND DISCUSSION

Intranasal infection without predisposition

The main objective of this study was to establish an intranasal model for colonization and/or invasion for Streptococcus suis in CD1 mice. In the first experiment, a high dose of 5 x 10^9 c.f.u. Streptococcus suis serotype 2 was applied intranasally without previous predisposition. Nine of the ten mice did not show any clinical signs of infection during the observation periods of 5 or 12 days, respectively (Table 2). Apathy, continuing anorexia and weight loss of >20% was registered in one mouse starting on day 4 post-infection (p.i.) (Table 2). Histopathological screening revealed a severe purulent meningitis and encephalitis associated with the recovery of 4000 c.f.u. of the challenge strain (mg brain)^-1 (Tables 3 and 4). In agreement with these results, Williams et al. (1988) also recorded disease in only one of five mice infected intranasally with Streptococcus suis serotype 2.

Colonization of the respiratory tract was monitored in this study by quantification of the specific bacterial content in the TNL and lungs. The Streptococcus suis challenge strain was detected in four of five mice in the TNL and in two of five mice in the lungs on day 5 p.i. However, the mean bacterial load of Streptococcus suis in the TNL was only 439±820 c.f.u. ml^-1 (mean±st). At 12 days p.i., Streptococcus suis was recovered from the TNL of only two mice (Table 4). These results indicated that Streptococcus suis serotype 2 colonized the respiratory tract of CD1 mice transiently in low numbers after intranasal application without any predisposition.

Intranasal infection model for invasion after predisposition with acetic acid

In piglets, Streptococcus suis mucosal infection models have been described that include experimental predisposition, such as infection with Bordetella bronchiseptica (Smith et al., 1996, 1999) or local application of 1% acetic acid (Baums et al., 2006; Pallarès et al., 2003). Therefore, we treated CD1 mice intranasally with 12.5 µ l 1% acetic acid per nostril 1 h prior to infection with Streptococcus suis. Morbidity and mortality were 67 and 25%, respectively (results not shown). Severe fibrinosuppurative or purulent necrotizing pneumonia associated with a high load of Streptococcus suis [up to 2.3 x 10^4 c.f.u. (mg lung tissue)^-1] was a common finding in these mice. However, in eight of 24 mice, Staphylococcus aureus was also detected in inner organs. A probable explanation for this finding was that local application of acetic acid allowed Staphylococcus aureus colonizing the respiratory tract to invade deeper tissues. Nevertheless, infection with Staphylococcus aureus was not a prerequisite for Streptococcus suis infection – in >50% of mice positive for Streptococcus suis in the brain, lung, heart or any other organ, Staphylococcus aureus was not detected in these

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Table 1. Scoring of parameters for calculation of the cumulative clinical score of a Streptococcus suis-infected mouse

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td></td>
<td>Constant or gain</td>
<td>&gt;5% weight loss</td>
<td>&gt;20% weight loss</td>
</tr>
<tr>
<td>Coat</td>
<td></td>
<td>Flat, glossy</td>
<td>Rough, reduced grooming</td>
<td>Scrubby, failure of grooming</td>
</tr>
<tr>
<td>Breathing</td>
<td></td>
<td>Adequate, rhythmic</td>
<td>Rapid, shallow</td>
<td>Rapid, abdominal</td>
</tr>
<tr>
<td>Dehydration</td>
<td></td>
<td>Normal skin elasticity</td>
<td>Moderately reduced skin elasticity</td>
<td>Persisting skin fold, sunken eyes</td>
</tr>
<tr>
<td>Bearing</td>
<td></td>
<td>Normal</td>
<td>Moderately curved back</td>
<td>Cowering, highly curved back</td>
</tr>
<tr>
<td>Eyes</td>
<td></td>
<td>Normal</td>
<td>Moderately protruding</td>
<td>Highly swollen</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td>Normal active</td>
<td>Depression</td>
<td>Apathy, social isolation</td>
</tr>
<tr>
<td>Locomotion</td>
<td></td>
<td>No anomaly</td>
<td>Moderate incoordination</td>
<td>Apraxia, stagger</td>
</tr>
</tbody>
</table>

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tissues. It should be noted that we have never observed co-infections in piglets predisposed through intranasal application of 1% acetic acid.

To exclude co-infection with commensal *Staphylococcus aureus*, a further experiment with 12.5 µl acetic acid predisposition was conducted with ten *Staphylococcus aureus*-free CD1 mice (Experiment 2 in Tables 2–4). Morbidity and mortality were 40 and 20%, respectively (Table 2). Moderate to severe purulent necrotizing or fibrinosuppurative rhinitis was detectable in all infected animals and severe pneumonia in four of the ten mice. Accordingly, the group pathoscore was higher in comparison with the non-predisposed group (Table 3). *Streptococcus suis* colonized the respiratory tract very efficiently in these mice, as indicated by reisolation of the challenge strain from TNL in all animals, with a mean specific bacterial load of $5.2 \times 10^5 \pm 9.4 \times 10^5$ c.f.u. ml$^{-1}$ (mean $\pm$ SD). As expected, *Staphylococcus aureus* co-infection was not recorded in this experiment.

The results indicated that application of 12.5 µl 1% acetic acid per nostril prior to experimental *Streptococcus suis* infection predisposed the mice to rhinitis and pneumonia rather than to meningitis, the most important pathology of *Streptococcus suis* infection in swine and humans. This is in contrast to the effect of intranasal acetic acid predisposition in swine, which leads mainly to *Streptococcus suis*-associated pleuritis, peritonitis and meningitis (Baums et al., 2006; Pallarés et al., 2003) and the intraperitoneal CD1 model reported by Domínguez-Punaro et al. (2007) with a 40% prevalence of meningitis. However, as demonstrated by bacteriology, *Streptococcus suis* invaded different inner organs in the mice after intranasal application following 1% acetic acid predisposition (12.5 µl per nostril). Therefore, this model might be used to study invasion and spreading of *Streptococcus suis* in mice, using bacteriological screening of different inner organs as an important read-out parameter. However, the availability of *Staphylococcus aureus*-free CD1 mice is at present very limited. Alternatively, animals might be treated with antibiotics prior to *Streptococcus suis* challenge, as has been done with piglets (Allen et al., 2001).

### Intranasal infection model for mucosal colonization

The high rate of severe fibrinosuppurative or purulent necrotizing rhinitis and pneumonia observed in Experiment 2 was inappropriate for a model designed to study colonization of mucosal surfaces in asymptomatic carriers. Therefore, the volume of 1% acetic acid applied to each nostril prior to infection was reduced to 5 µl in Experiment 3 in order to establish a mucosal colonization model for *Streptococcus suis* in CD1 mice. None of the ten mice included in this experiment developed pneumonia or severe clinical signs (Tables 2 and 3). Only one mouse received a cumulative clinical score above 3 and was thus classified as diseased. Furthermore, only one case of severe rhinitis was recorded in the histological screening (Table 3). Importantly, the challenge strain was detected in 100 and 60% of these mice at 7 days p.i. in the TNL and lung, respectively (Table 4). Ninety per cent of the mice had a specific bacterial load above 1000 c.f.u. (ml TNL)$^{-1}$ (mean $\pm$ SD: $3.4 \times 10^5 \pm 6.3 \times 10^5$ c.f.u. ml$^{-1}$). The daily weight increase and the course of the cumulative clinical score after challenge (Fig. 1), as well as the histological findings (Table 3), indicated that these mice were asymptomatic carriers and not affected by an acute disease (with the exception of one mouse for 1 day) or a developing chronic infection. Based on these results, we propose a mouse model for mucosal colonization of the reference *Streptococcus suis* serotype 2 strain 10 including predisposition with 5 µl 1% acetic acid per nostril.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. mice</th>
<th>Acetic acid pre-treatment*</th>
<th><em>Streptococcus suis</em> strain</th>
<th>Days p.i.+</th>
<th>No. mice/total no. mice</th>
<th>Morbidity‡</th>
<th>Mortality</th>
<th>Severe clinical signs§</th>
<th>Maximum weight loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>None</td>
<td>wt</td>
<td>5</td>
<td></td>
<td>, 12</td>
<td></td>
<td></td>
<td>1/10</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>12.5 µl</td>
<td>wt</td>
<td>2</td>
<td></td>
<td>, 3</td>
<td>4/10</td>
<td>2/10</td>
<td>0/10</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>5 µl</td>
<td>wt</td>
<td>7</td>
<td>1/10</td>
<td>0/10</td>
<td>0/10</td>
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<td>0/10</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>5 µl</td>
<td>wt</td>
<td>7</td>
<td></td>
<td>, 14</td>
<td></td>
<td></td>
<td>2/12</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>5 µl 10Δsly</td>
<td>7</td>
<td></td>
<td>, 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Volume of 1% acetic acid applied to each nostril 1 h prior to infection.
†Days p.i. on which mice were killed.
‡Mice with a cumulative clinical score of $\geq 3$ were regarded as diseased.
§In particular, persistent anorexia, apathy and/or neural disorder leading to a cumulative clinical score of $\geq 6$.
||Half of the mice were sacrificed on each of these days p.i.
||Two mice were killed on day 2 p.i. for reasons of animal welfare.
Table 3. Scoring of fibrinosuppurative and purulent necrotizing lesions of CD1 mice infected intranasally with $5 \times 10^9$ c.f.u. *Streptococcus suis* wt strain 10 or its isogenic SLY mutant ($10^{DSly}$)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. mice</th>
<th>Acetic acid pre-treatment*</th>
<th><em>Streptococcus suis</em> strain</th>
<th>Days p.i.</th>
<th>Nose: rhinitis</th>
<th>Spleen, liver, kidneys: splenitis, hepatitis, nephritis, peritonitis</th>
<th>Lung: pneumonia, pleuritis</th>
<th>Brain and spinal cord: meningitis encephalitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4§</td>
<td>2‖</td>
<td>1‖</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>None</td>
<td>wt</td>
<td>5, 12</td>
<td>0/10</td>
<td>1/10</td>
<td>0/10</td>
<td>1‖/10</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>12.5 µl</td>
<td>wt</td>
<td>2, 3</td>
<td>7/10</td>
<td>3/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>5 µl</td>
<td>wt</td>
<td>7</td>
<td>1/10</td>
<td>1/10</td>
<td>1/10</td>
<td>0/10</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>5 µl</td>
<td>wt</td>
<td>7</td>
<td>0/6</td>
<td>2/6</td>
<td>3/6</td>
<td>0/6</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>5 µl</td>
<td><em>10^{DSly}</em></td>
<td>14</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>1/6</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>5 µl</td>
<td><em>10^{DSly}</em></td>
<td>7</td>
<td>0/6</td>
<td>2/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>4</td>
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<td>5 µl</td>
<td><em>10^{DSly}</em></td>
<td>14</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
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</table>

*Volume of 1 % acetic acid applied to each nostril 1 h prior to infection.
†$\omega=\Sigma \text{score}_{\text{max}}/n_{\text{animals}}$ (Baums *et al.*, 2006); rhinitis was not included in the $\omega$ score.
‡Infiltration of splenic red pulp with neutrophilic granulocytes.
§A score of 4 and 5 indicated moderate to severe diffuse or multifocal fibrinosuppurative or purulent necrotizing inflammations.
‖A score of 2 and 3 indicated mild focal fibrinosuppurative or purulent necrotizing inflammation.
¶Individual single perivascular immune cells received a score of 1.
#Moderate focal purulent pleuritis was registered in one mouse.
Table 4. Reisolation of the wild-type (wt) *Streptococcus suis* challenge strain 10 or its isogenic SLY mutant (10Δsly) in intranasally infected mice

Isolation of the challenge strain was confirmed in a multiplex PCR for detection of *mrp*, *sly*, *epf*, *gdh*, *arcA*, *cps1*, *cps2*, *cps7* and *cps 9* (Silva et al., 2006). All typical β-haemolytic colonies recovered in this work were positive for the profile of virulence-associated genes of the challenge strains. Bacterial loads in c.f.u. (mg organ)^{−1} or (ml TNL)^{−1} were classified as low (+ ; <100), moderate (+ + ; ≥100 but ≤1000) or high (+ + + ; >1000).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Acetic acid pre-treatment*</th>
<th>Streptococcus suis strain</th>
<th>Days p.i.†</th>
<th>No. mice in which <em>S. suis</em> challenge strain was isolated/total no. mice‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TNL</td>
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<tr>
<td>1</td>
<td>None</td>
<td>wt</td>
<td>5</td>
<td>1/5 1/5 2/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>wt</td>
<td>12</td>
<td>1/5 0/5 1/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>2</td>
<td>12.5 μl</td>
<td>wt</td>
<td>2</td>
<td>2/2 0/2 0/2 0/2 1/2 1/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2</td>
</tr>
<tr>
<td>2</td>
<td>12.5 μl</td>
<td>wt</td>
<td>3</td>
<td>7/8 1/8 0/8 0/8 1/8 4/8 0/8 1/8 3/8 0/8 0/8 2/8 0/8 0/8 2/8</td>
</tr>
<tr>
<td>3</td>
<td>5 μl</td>
<td>wt</td>
<td>7</td>
<td>9/10 1/10 0/10 0/10 10/10 0/10 0/10 0/10 0/10 10/10 0/10 0/10 0/10 0/10 0/10</td>
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<td>4</td>
<td>5 μl</td>
<td>wt</td>
<td>7</td>
<td>3/6 3/6 0/6 0/6 0/6 0/6 0/6 0/6 1/6 0/6 0/6 1/6 0/6 0/6 1/6</td>
</tr>
<tr>
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<td>wt</td>
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<tr>
<td>4</td>
<td>5 μl</td>
<td>10Δsly</td>
<td>7</td>
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<tr>
<td>4</td>
<td>5 μl</td>
<td>10Δsly</td>
<td>14</td>
<td>0/6 0/6 1/6 1/6 0/6 0/6 0/6 0/6 0/6 0/6 0/6 0/6 0/6 0/6 0/6</td>
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</tbody>
</table>

*Volume of 1% acetic acid applied to each nostril 1 h prior to infection.
†Days p.i. on which mice were sacrificed.
‡(ml TNL)^{−1}.

Comparison of colonization of an isogenic sly mutant and its wt strain

The intranasal mouse model for colonization including predisposition with 5 μl 1% acetic acid was used to investigate the possible contribution of SLY to mucosal colonization of *Streptococcus suis*. None of the mice infected with the wt strain 10 or the isogenic SLY mutant (10Δsly) developed severe clinical signs (Table 2). None of the infected animals died and only two cases of general sickness including rough coat, moderate depression and persistent weight loss ≥5% were observed in each group. Severe inflammations were not recorded in any of these mice. However, one mouse infected with strain 10 showed moderate purulent pleuritis and one showed moderate purulent nephritis (Table 3).

The challenge strain was detected in the TNL of all mice infected with either the wt strain or 10Δsly at 7 days p.i. at comparable concentrations [mean ± SD of the two groups: 4.5 ± 6.0 × 10^4 and 6.2 ± 3.8 × 10^4 c.f.u. (ml TNL)^{−1}, respectively], indicating no attenuation in early colonization of the sly mutant (Table 4). At 14 days p.i., the wt strain was detected in the TNL of four of six mice [mean ± SD: 511 ± 947 c.f.u. (ml TNL)^{−1}]. In contrast, the sly mutant (10Δsly) was isolated in only one of six mice at 14 days p.i. [mean ± SD: 78 ± 191 c.f.u. (ml TNL)^{−1}]. However, this difference was not significant (P=0.13). It has been reported that pneumolysin has an effect on long-term nasopharyngeal carriage, as a pneumolysin-deficient mutant was cleared faster than the wt in an intranasal *Streptococcus pneumoniae* carriage model (Kadioglu et al., 2002; Richards et al., 2010). As the differences between the sly mutant and wt strain were not significant, further
studies are warranted to elucidate a putative contribution of SLY to sustained colonization of the respiratory epithelium. Many wt Streptococcus suis strains do not express SLY, including virulent serotype 2 strains in North America (King et al., 2001). However, as different Streptococcus suis strains might exhibit differences in colonization mechanisms, SLY may well be a factor contributing to sustained colonization of virulent European serotype 2 strains.

In conclusion, different putative intranasal murine models for Streptococcus suis invasion and colonization were evaluated in this work. Predisposition with 12.5 µl 1% acetic acid per nostril promoted invasion of different inner organs by the Streptococcus suis serotype 2 challenge strain. Severe purulent necrotizing meningitis was a common finding among infected mice in this model. As the rate of meningitis was rather low, intravenous or intraperitoneal mouse models are more appropriate to study the later stages in the pathogenesis of Streptococcus suis meningitis. However, the new intranasal mouse model established in this work including predisposition with 5 µl 1% acetic acid per nostril should allow future investigation of mucosal colonization mechanisms employed by Streptococcus suis in mice.

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