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A novel intranasal mouse model for mucosal colonisation of *Streptococcus suis* serotype 2

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Maren Seitz[1], Andreas Beineke[2], Jana Seele[1], Marcus Fulde[3], Peter Valentin-Weigand[1] and Christoph Georg Baums[1]*

[1] Institute for Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany

[2] Institute for Pathology, University of Veterinary Medicine Hannover, Hannover, Germany

[3] Medical Microbiology Research Group, Helmholtz Centre for Infection Research, Braunschweig, Germany

*Corresponding author. Mailing address:

Stiftung Tierärztliche Hochschule Hannover

Zentrum fuer Infektionsmedizin

Institut fuer Mikrobiologie

Bischofsholer Damm 15

D-30173 Hannover, GERMANY

Phone: ++49-511 856-7563

Fax: ++49-511 856-7697

Email:christoph.baums@gmx.de

ABBREVIATION LIST

CDC	cholesterol-dependent cytolysin
d.p.i.	days post infection
Ex.	Experiment
S.	*Streptococcus*
SLY	suilysin
TNL	tracheonasal lavarge
S. aureus	*Staphylococcus aureus*

ABSTRACT

Streptococcus (*S.*) *suis* causes meningitis and various other diseases in pigs and humans. Healthy piglets carrying virulent *S. suis* strains on their mucosal surfaces are epidemiologically very important. The objective of this study was to establish an intranasal *S. suis* mouse model for invasion and colonisation of the respiratory tract. CD1 mice were intranasally infected with a highly virulent *S. 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 *S. suis* was a common manifestation. Furthermore, a novel model to study nasopharyngeal colonisation was established by reducing the volume of 1 % acetic acid per nostril to 5 µl prior *S. suis* application. This model mimics asymptomatic carriage in swine as all mice carried *S. suis* on their respiratory mucosa 7 days post infection in moderate to high numbers without development of pneumonia or any other invasive *S. suis* disease. This intranasal *S. suis* model was applied to investigate the function of suilysin in colonisation. Though an isogenic suilysin mutant was isolated from the upper respiratory tract at a lower recovery rate than its wild type parental strain 14 days post infection, differences were not significant and did not indicate severe attenuation in colonisation. In conclusion, this work describes the first intranasal mice model to study colonisation of the respiratory tract by a highly virulent *S. suis* pathotype.

INTRODUCTION

Streptococcus (*S.) 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 *S. suis* infections in humans (Tang *et al.*, 2006; Gottschalk *et al.*, 2007). Noteworthy, in 2005 a *S. suis* outbreak in China affected 204 humans, of which 38 died mainly because of streptococcal toxic shock-like syndrome (Tang *et al*., 2006). In Vietnam, *S. 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 intestine (Nghia *et al.*, 2011). Occupational exposure to pigs and pork is worldwide the most important risk factor for *S. suis* infections (Arends & Zanen, 2005).

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

S. suis shows a high diversity as reflected by the presence of at least 33 serotypes. Serotype 2 is worldwide the most prevalent among invasive isolates of pigs and humans (Wisselink *et al.*, 2000; Wei *et al.*, 2009). The serotype is determined by the

polysaccharide capsule. The capsule 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 *S. suis* diseases (Baums & Valentin-Weigand, 2009; Fittipaldi *et al.* 2012). The majority but not all invasive *S. suis* isolates secrete a pore-forming cholesterol-dependent cytolysin (CDC) called suilysin (SLY) (King *et al.*, 2001). SLY might exhibit different functions in the pathogenesis of *S. suis* diseases as it has been shown to cause cytotoxicity of various cells *in vitro* (Allen *et al.*, 2001; Jacobs *et al.*, 1994; Segura & Gottschalk, 2002). It may also contribute to bacterial escape of opsonphagocytosis at sublytic concentrations (Chabot-Roy *et al.*, 2006; Lecours *et al.*, 2011; Benga *et al.*, 2008). Though the pathogenesis of *S. suis* meningitis is still not well understood, even less is known about the mechanisms employed by *S. suis* to colonise mucosal surfaces. As a matter of fact, not a single factor of *S. suis* has been demonstrated to be crucial for colonisation.

A *S. suis* mouse meningitis model was first described by Williams *et al.* (1988) using intravenous application. Histopathological lesions in the brain and inflammatory responses were more recently characterized in detail in an intraperitoneal *S. 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 proinflammatory cytokines. Animals surviving septicaemia frequently developed meningitis. As the upper respiratory tract is considered to be the port of entry for *S. suis* (Williams & Blakemore, 1990), early steps in the pathogenesis of *S. suis* diseases cannot be studied in the described mouse models. Furthermore, colonisation of the respiratory mucosa can only be investigated in mice using intranasal infection. Here, we report for the first time that *S. suis* colonises the murine

respiratory tract efficiently after intranasal application following predisposition. As CDC, in particular pneumolysin, have been demonstrated to contribute to mucosal colonisation (Richards *et al.*, 2010; Kadioglu *et al.*, 2002) the novel *S. suis* mouse model was applied to investigate the contribution of suilysin to colonisation of the murine respiratory tract.

METHODS

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

Experimental intranasal infection of mice. Streptococci grown to late exponential growth phase (OD_{600} 0.8) were harvested by centrifugation and resuspended in PBS (pH 7.4) for intranasal infection. Inoculum concentrations were verified by plating 10-fold serial dilutions. Four week old specific pathogen free female mice of the outbred

strain Crl:CD1 (ICR) obtained from Charles River Laboratories (Sulzfeld, Germany) were used for all experiments (ex.) except ex. 2, in which mice of the *S. aureus* free outbred strain Hsd:ICR (CD1®) were used that had been purchased from Harlan Laboratories (AN Venray, The Netherlands). Animals were randomly divided into groups consisting of 5 to 6 animals each. Mice were allowed to acclimate for one week and cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animal 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 anesthetised via inhalation of isofluran (IsoFlo®, Albrecht). In ex. 2 mice were pretreated with 12.5 µl, and in ex. 3 and 4 with 5 µl 1 % acetic acid (pH 4.0) placed in each nostril 1 h prior intranasal infection. After a controlled recovery phase and further anaesthesia, mice were infected with 5×10^9 c.f.u. of either *S. suis* wild type strain 10 or strain 10Δsly. The inoculum was applied in two drops of 12.5 µl placed in front of the nostrils.

Clinical score. Animals were examined every 8 hours. The health status was rated by using a clinical score sheet (Table 1), including 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 to 4 indicated mild clinical signs, 5 to 6 moderate clinical signs and a score greater 6 severe clinical signs with specific regards to neural failure, respectively. Mice with a cumulative score equal or greater than 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 CO_2 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, lumbar) encased within vertebral bodies and sagittal sections of the nasal cavity were formalin fixed, decalcified in 10 % ethylenediaminetetraacetic acid solution for 48 hours and subsequently embedded in paraffin wax. The histological screenings were carried out as blinded experiments. Fibrinosuppurative and purulent necrotizing lesions were scored as described for piglets (Baums *et al.*, 2006). The group score ω was calculated by dividing the sum of the highest scores of each animal for any of the investigated organs through the number of animals. Rhinitis was not included.

Reisolation of *S. suis* strains from tissue and tracheonasal lavage (TNL). One half of each organ was suspended in 5 ml cold PBS (pH 7.4). After weighing suspensions were homogenized with an Ultra Turrax (IKA). Ten-fold serial dilutions of the tissue-PBS-suspensions were plated on blood agar plates. Colony forming units were counted after incubation at 37 °C for 24 h and bacterial load per mg organ was calculated.

As a read out parameter for colonisation 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 c.f.u. per ml TNL of α-haemolytic streptococci with colony morphology typical for *S. suis* strain 10 were determined. Noteworthy, mice were also colonized in the respiratory tract by other α-haemolytic streptococci showing different colony morphology, namely

a much larger zone of α-haemolysis and formation of smaller colonies. Isolated typical α-haemolytic streptococci were profiled in a *S. suis* multiplex PCR for the detection of *mrp*, *epf*, *sly*, *arcA*, *gdh*, *cps1*, *cps2*, *cps7*, and *cps9* (Silva *et al.*, 2006). Isolates from mice challenged with 10Δsly were additionally investigated in a *sly*-specific PCR using the primer pair slyAgefor (TGTACCGGTGATTCCAAACAAGATATTAA) and slyAge3new (TTAACCGGTTACTCTATCACCTCATCCG) with a final concentration of 0.5 µM as described previously (Silva *et al.*, 2006). Based on c.f.u. per mg organ or per ml TNL, bacterial loads were classified as low (+; < 100), moderate (++; ≥ 100 but ≤ 1000) or high (+++; > 1000).

Statistical analysis. The 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

A main objective of this study was to establish an intranasal model for colonisation and/or invasion for *S. suis* in CD1 mice. In the first experiment, a high dose of 5×10^9 c.f.u. *S. suis* serotype 2 was applied intranasally without previous predisposition. Nine of 10 mice did not show any clinical signs of infection during the observation period of 5 or 12 days, respectively (Table 2). Apathy, continuing anorexia and weight loss of more than 20 % was registered in one mouse starting on the 4th d.p.i. (day post infection, Table 2). Histopathological screening revealed a severe purulent meningitis and encephalitis associated with recovery of 4,000 c.f.u. of the challenge

strain per mg brain (Tables 3 and 4). In accordance with these results, Williams *et al*. (1988) recorded also only disease in one of five mice intranasally infected with *S. suis* serotype 2.

Colonisation of the respiratory tract was monitored in this study through quantification of the specific bacterial content in TNL and the lungs. The *S. suis* challenge strain was detected in 4 of 5 mice in the TNL and in 2 of 5 mice in the lungs on the 5th d.p.i.. However, the mean bacterial load of *S. suis* in the TNL was only 439 c.f.u. ml^{-1} (SD: $\pm$ 820). Twelve d.p.i. *S. suis* was recovered from TNL of only 2 mice (Table 4). These results indicated that *S. suis* serotype 2 colonised the respiratory tract of CD1 mice transiently in low numbers after intranasal application without any predispositon.

Intranasal infection model for invasion after predisposition with acetic acid

In piglets, *S. suis* mucosal infection models have been described which include experimental predisposition, such as infection with *Bordetella bronchiseptica* (Smith *et al.*, 1996; Smith *et al*., 1999) or local application of 1 % acetic acid (Baums *et al.*, 2006; Pallares *et al.*, 2003). Therefore we treated CD1 mice intranasally with 12.5 µl 1 % acetic acid per nostril 1 h prior infection with *S. suis*. Morbidity and mortality were 67 % and 25 %, respectively (results not shown). Severe fibrinosuppurative or purulent necrotizing pneumonia associated with a high load of *S. suis* (up to 23,000 c.f.u. mg^{-1} lung tissue) was a common finding in these mice. However, in 8 of 24 mice *S. aureus* was also detected in inner organs. A likely explanation for this finding was that local application of acetic acid allowed *S. aureus* colonising the respiratory tract to invade deeper tissues. Nevertheless, infection with *S. aureus* was not a prerequisite for *S. suis* infection as in more than 50 % of mice positive for *S. suis* in the brain, lung, heart or any other organ *S. aureus* was not detected in these tissues.

Noteworthy, we have never observed coinfections in piglets predisposed through intranasal application of 1 % acetic acid.

To exclude coinfection with commensal *S. aureus* a further experiment with 12.5 µl acetic acid predisposition was conducted with 10 *S. aureus* free CD1 mice (ex. 2 in Tables 2 to 4). Morbidity and mortality was 40 % and 20 %, respectively (Table 2). Moderate to severe purulent necrotizing or fibrinosuppurative rhinitis was detectable in all infected animals and severe pneumonia in 4 of 10 mice. Accordingly, the group pathoscore ω was higher in comparison to the non-predisposed group (Table 3). *S. suis* colonised 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 52 x 10^4 c.f.u. ml^{-1} (SD: ± 94 x 10^4). As expected *S. aureus* coinfection was not recorded in this experiment.

The results indicated that application of 12.5 µl 1 % acetic acid per nostril prior to experimental *S. suis* infection predisposed mice to rhinitis and pneumonia rather than to meningitis, the most important pathology of *S. suis* infection in swine and humans. This is in contrast to the effect of intranasal acetic acid predisposition in swine, leading mainly to *S. suis*-associated pleuritis, peritonitis and meningitis (Baums *et al.*, 2006; Baums *et al.*, 2009; Pallares *et al.*, 2003) and the intraperitoneal CD1 model reported by Dominguez-Punaro *et al*. (2007) with a 40 % prevalence of meningitis. However, as demonstrated by bacteriology, *S. suis* invaded different inner organs in 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 *S. suis* in mice, using bacteriological screening of different inner organs as an important read out parameter. However, availability of *S. aureus* free CD1 mice is at present very limited. Alternatively, animals might be treated with antibiotics prior to *S. suis* challenge as conducted with piglets by Allen *et al.* (2001).

Intranasal infection model for mucosal colonisation

The high rate of severe fibrinosuppurative or purulent necrotizing rhinitis and pneumonia observed in ex. 2 was inappropriate for a model designed to study colonisation 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 ex. 3 in order to establish a mucosal colonisation model for *S. suis* in CD1 mice. None of the 10 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 7 d.p.i. in the TNL and lung, respectively (Table 4). Ninety percent of the mice had a specific bacterial load above 1000 c.f.u. ml^{-1} TNL (mean of the group ± SD: $34 \times 10^4 \pm 63 \times 10^4$ 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 an exception of one mouse for one day) or a developing chronic infection. Based on these results, we propose a mouse model for mucosal colonisation of the reference *S. suis* serotype 2 strain 10 including predisposition with 5 µl 1 % acetic acid per nostril.

Comparison of colonisation of an isogenic *sly*-mutant with its wildtype strain

The intranasal mouse model for colonisation including predisposition with 5 µl 1 % acetic acid was used to investigate the possible contribution of SLY to mucosal

colonisation of *S. suis*. None the mice either infected with the wild type strain 10 or 10Δsly developed severe clinical signs (Table 2). In accordance, none of the infected animals died and only two cases of general sickness including rough coat, moderate depression and persistent weight loss $\geq$ 5 % were observed in each group. Accordingly, severe inflammations were not recorded in any of these mice. However, one mouse infected with strain 10 showed moderate purulent pleuritis and one moderate purulent nephritis (Table 3).

The challenge strain was detected in TNL of all mice infected either with the wild type strain or the isogenic suilysin mutant (10Δsly) 7 d.p.i. in comparable concentrations (mean in 10^3 c.f.u. ml^{-1} TNL ± SD of the two groups: 45 ± 60 and 62 ± 86, respectively), indicating no attenuation in early colonisation of the *sly* mutant (Table 4). Fourteen d.p.i. the wt strain was detected in TNL of 4 out of 6 mice (mean c.f.u. ml^{-1} TNL ± SD: 511 ± 947). In contrast, the *sly* mutant (10Δsly) was isolated in only 1 out of 6 mice 14 d.p.i. (mean c.f.u. ml^{-1} TNL ± SD: 78 ± 191). However, this difference was not significant (P = 0.13). It has been reported, that pneumolysin has an effect on long-term nasopharyngeal carriage since a pneumolysin-deficient mutant was faster cleared than the wildtype in an intranasal *S. pneumoniae* carriage model (Richards *et al.*, 2010; Kadioglu *et al.*, 2002). As the differences of the *sly* mutant and the wt strain were not significant further studies are warranted to elucidate a putative contribution of SLY to sustained colonisation of the respiratory epithelium. Many wt *S. suis* strains do not express SLY, including virulent serotype 2 strains in North America (King *et al.*, 2001). However, as different *S. suis* strains might exhibit also differences in colonisation mechanisms, SLY might well be a factor contributing to sustained colonisation of virulent European serotype 2 strains.

In conclusion, different putative intranasal murine models for *S. suis* invasion and colonisation were evaluated in this work. Predisposition with 12.5 µl 1 % acetic acid

per nostril promoted invasion of different inner organs by the *S. suis* serotype 2 challenge strain. Severe purulent necrotizing pneumonia 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 *S. suis* meningitis. However, a new intranasal mouse model established in this work including predisposition with 5 µl 1 % acetic acid per nostril should allow investigation of mucosal colonisation mechanisms employed by *S. suis* in mice in the future.

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340 **Table 1.** Scoring of parameters for calculation of the cumulative clinical score* of a *S.*
341 *suis* infected mouse
342

Para-meter	Score		
	0	**1**	**2**
Body weight	Constant or gain	> 5 % weight los	> 20 % weight loss
Coat	Flat, glossy	Rough, reduced grooming	Scrubby, failure of grooming
Breathing	Adequate, rhythmic	Rapid, shallow	Rapid, abdominal
Dehydration	Normal skin elasticity	Moderately reduced skin elasticity	Persisting skin fold, sunken eyes
Bearing	Normal	Moderately curved back	Cowering, highly curved back
Eyes	Normal	Moderately protruding	Highly swollen
Activity	Normal active	Depression	Apathy, social isolation
Locomotion	No anomaly	Moderate incoordination	Apraxia, stagger

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344 * The sum of the scores of the 8 parameters of a mouse for a defined time point of control

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Table 2. Clinical course of CD1 mice intranasally infected with 5 x 10^9 c.f.u. of the *S. suis* wildtype (wt) strain 10 or its isogenic suilysin mutant (10Δsly)

Ex.	No. of mice	Acetic acid pre-treat-ment*	*S. suis* strain	d.p.i.†	No. of mice/total no. of mice				
					Morbidity‡	Mortality	Severe clinical signs§	Maximum weight loss (%)	
								≥ 5	≥ 20
1	10	no	wt	5‖, 12‖	1/10	0/10	0/10	2/10	1/10
2	10	12.5 µl	wt	2¶, 3	4/10	2/10	2/10	7/10	1/10
3	10	5 µl	wt	7	1/10	0/10	0/10	2/10	0/10
4	12	5 µl	wt	7‖, 14‖	2/12	0/12	0/12	10/12	0/12
4	12	5 µl	10Δsly	7‖, 14‖	2/12	0/12	0/12	6/12	0/12

* Volume of 1 % acetic acid applied to each nostril 1 h prior to infection
† Days post infection, on which mice were killed
‡ Mice with a cumulative clinical score greater or equal 3 were regarded as diseased
§ In particular persistent anorexia, apathy, and/or neural disorder leading to a cumulative clinical score > 6
‖ Half of the mice were sacrificed on each of these d.p.i.
¶ Two mice were killed on the 2nd day post infection for reasons of animal welfare

Table 3. Scoring of fibrinosuppurative and purulent necrotizing lesions of CD1 mice intranasally infected with 5 x 10^9 c.f.u. of the *S. suis* wildtype (wt) strain 10 or its isogenic suilysin mutant (10Δsly)

Ex.	No. of mice	Acetic acid pre-treat-ment*	*S. suis* strain	d.p.i.	No. of mice/total no. of mice												ω¶
					Nose			Spleen, liver, kidney			Lung			Brain and spinal cord			
					Rhinitis			Splenitis‖, hepatitis, nephritis, peritonitis			Pneumonia, pleuritis			Meningitis encephalitis,			
					4†	2‡	1§	4†	2‡	1§	4†	2‡	1§	5†	3‡	1§	
1	10	no	wt	5, 12	0/10	1/10	0/10	1‖/10	0/10	0/10	1/10	0/10	2/10	1/10	0/10	0/10	0.7
2	10	12.5 µl	wt	2, 3	7/10	3/10	0/10	0/10	1‖/10	5‖/10	4/10	0/10	0/10	0/10	0/10	0/10	1.9
3	10	5 µl	wt	7	1/10	1/10	1/10	0/10	0/10	2‖/10	0/10	0/10	0/10	0/10	0/10	0/10	0.2
4	6	5 µl	wt	7	0/6	2/6	3/6	0/6	1/6	1‖/6	0/6	0/6	0/6	0/6	0/6	0/6	0.5
4	6	5 µl	wt	14	0/6	0/6	0/6	1/6	0/6	5‖/6	1#/6	0/6	0/6	0/6	0/6	0/6	1.8
4	6	5 µl	10Δsly	7	0/6	2/6	0/6	0/6	0/6	3‖/6	0/6	0/6	2/6	0/6	0/6	0/6	0.5
4	6	5 µl	10Δsly	14	0/6	1/6	0/6	0/6	0/6	3‖/6	0/6	0/6	0/6	0/6	0/6	0/6	0.5

* Volume of 1 % acetic acid applied to each nostril 1 h prior infection
† Scoring of 4 and 5 indicates moderate to severe diffuse or multifocal fibrinosuppurative or purulent necrotizing inflammations
‡ Scoring of 2 and 3 indicates mild focal fibrinosuppurative or purulent necrotizing inflammation
§ Individual single perivascular immune cells received a score of 1
‖ Infiltration of splenic red pulp with neutrophilic granulocytes
¶ $\omega = \Sigma\ score_{max}/n_{animals}$ (Baums *et al*, 2006); rhinitis is not included in the score ω
# Moderate focal purulent pleuritis was registered in one mouse

Table 4. Reisolation of the wildtype (wt) *S. suis* challenge strain 10 or its isogenic suiylsin mutant (10Δsly) in intranasally infected mice

Ex.	Acetic acid pre-treat-ment*	*S. suis* strain	d.p.i.†	No. of mice in which the *S. suis* challenge strain‡ was isolated/total no. of mice											
				TNL§‖			Spleen, liver, kidney, heart‖			Lung‖			Brain‖		
				+++	++	+	+++	++	+	+++	++	+	+++	++	+
1	no	wt	5	1/5	1/5	2/5	0/5	0/5	0/5	0/5	0/5	2/5	1/5	0/5	0/5
1	no	wt	12	1/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
2	12.5 µl	wt	2	2/2	0/2	0/2	0/2	1/2	1/2	0/2	0/2	2/2	0/2	0/2	2/2
2	12.5 µl	wt	3	7/8	1/8	0/8	0/8	1/8	4/8	0/8	1/8	3/8	0/8	0/8	2/8
3	5 µl	wt	7	9/10	1/10	0/10	0/10	0/10	0/10	0/10	1/10	5/10	0/10	0/10	0/10
4	5 µl	wt	7	3/6	3/6	0/6	0/6	0/6	0/6	0/6	0/6	1/6	0/6	0/6	1/6
4	5 µl	wt	14	1/6	1/6	2/6	0/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6	1/6
4	5 µl	10Δsly	7	5/6	1/6	0/6	1/6	0/6	3/6	0/6	1/6	0/6	0/6	0/6	1/6
4	5 µl	10Δsly	14	0/6	0/6	1/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6

* Volume of 1 % acetic acid applied to each nostril 1 h prior to infection

† Days post infection, on which mice were sacrificed

‡ Isolation of the challenge strain was confirmed in a multiplex PCR for detection of *mrp*, *sly*, *epf*, *arcA*, *cps1*,*cps2*, *cps7*, and *cps 9* (Silva *et al*., 2006). All typical α-hemolytic colonies recovered in this work were positive for the profile of virulence-associated genes of the challenge strains.

§ Tracheo-nasal lavage

‖ Bacterial loads in c.f.u. per mg organ or per ml TNL were classified as low (+ ; < 100), moderate (++; ≥ 100 but ≤ 1000) or high (+++; > 1000)

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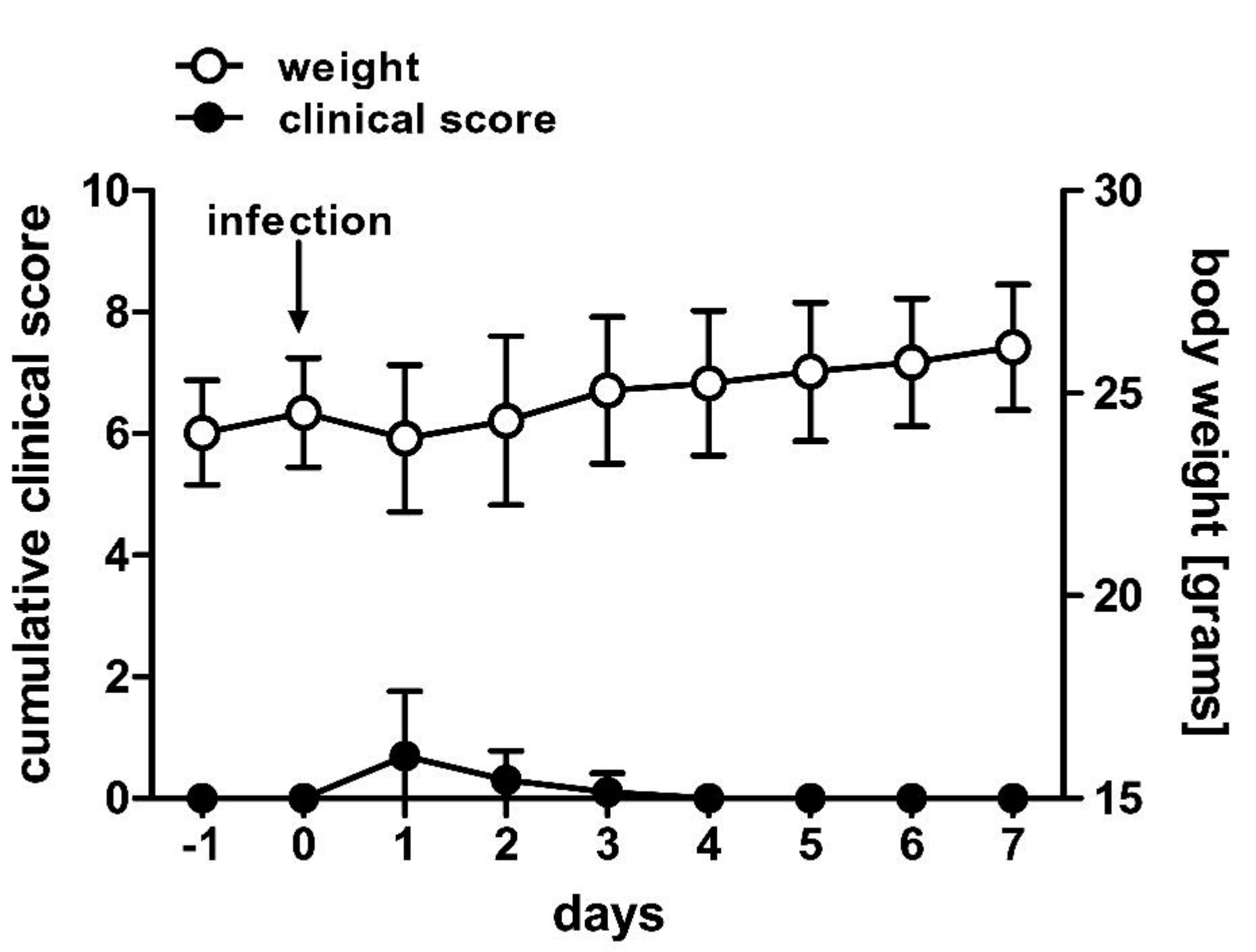
Figure legend

Fig. 1. Body weight and cumulative clinical score (see Table 1 for definition) of CD1 mice intranasally infected with 5 x 10^9 c.f.u. of *S. suis* strain 10 after predisposition through application of 5 µl 1 % acetic acid per nostril (ex. 3). The *S. suis* challenge strain was detected in all 10 mice with a specific bacterial load greater than 100 c.f.u. per ml in the tracheo-nasal lavage taken 7 days post infection (Table 4). Mice with a cumulative clinical score greater or equal 3 were regarded as diseased. Data points represent mean values (n = 10) ± SD.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