



Homologous whole bacterin vaccination is not able to reduce *Streptococcus suis* serotype 9 strain 7997 transmission among pigs or colonization

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ABSTRACT

Streptococcus suis (*S. suis*) is an important porcine pathogen worldwide, and antibiotics are often applied to treat or prevent clinical signs. Vaccination could be an alternative measure to reduce the abundant use of antimicrobials. The aim of this study was to determine the effect of vaccination with homologues whole bacterin vaccine containing *S. suis* serotype 9 strain 7997 on transmission of this serotype among pigs and on mucosal colonization. Caesarean derived, colostrum deprived pigs ($N=50$) were housed pair wise. Thirteen pairs were vaccinated intramuscularly with $2\text{--}3 \times 10^9$ colony forming units (CFU) inactivated *S. suis* serotype 9 per dose and α -tocopherolactetaat as adjuvant at 3 and 5 weeks of age; twelve pairs served as non-vaccinated controls. At 7 weeks of age, one pig of each pair was intranasally inoculated with $1\text{--}2 \times 10^9$ CFU of the homologues strain, whereas the other pig of each pair was contact-exposed. Tonsil brushings and saliva swabs were collected for 4 weeks, and tested for the presence of *S. suis* by bacteriological culture. No differences in number of *S. suis* in the tonsils or saliva samples or in clinical signs were observed between vaccinated and control pigs. In all pairs, transmission between inoculated and contact exposed pigs occurred, and no difference was observed in rate at which this occurred. The estimated transmission rate parameter β between vaccinated pigs was $\beta_v = 5.27/\text{day}$, and for non-vaccinated pigs $\beta_{nv} = 2.77/\text{day}$ ($P=0.18$). It was concluded that vaccination against *S. suis* serotype 9 did not reduce transmission, nor colonization and that there were no indications that protection against clinical signs was induced.

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1. Introduction

Streptococcus suis (*S. suis*) infections are common in commercial swine herds, causing disease, animal welfare problems and economic losses [1–3]. World-wide 33 capsular serotypes of *S. suis* have been described of which serotypes 2 and 9 are most often

associated with clinical signs in commercial pig farms. Infections with *S. suis* in pigs may cause meningitis, arthritis, endocarditis, and sudden death, which mainly occur in pigs between 6 and 10 weeks of age [2–4]. Control measures, implemented by farmers, mainly aim at preventing clinical signs or reduced performance. Some of these measures focus on management and housing conditions, but more often the disease is controlled by (preventive) use of antibiotics [2,3,5]. The abundant use of antibiotics, however, may lead to the induction or selection of antibiotic resistance, which is considered to become a huge problem not only in pig production systems, but mainly for public health [3,6–10]. Therefore, other control measures need to be developed, or improved.

One of the alternative measures is vaccination. Up to now, only few serotype-specific vaccines have been developed, mainly directed against serotype 2, as this serotype 2 has long been considered the most common circulating serotype. The efficacy of these vaccines has not been investigated extensively, but few studies showed that vaccines against this serotype are able to protect pigs against the development of clinical signs induced by an infection with the same serotype [11–13]. In several countries, like Germany, Belgium, Spain, The Netherlands and China, *S. suis* serotype 9 is

Abbreviations: *sly*, sulyisin gene; *mmp*, muramidase-released protein gene; *ef*, extracellular factor gene; COLSH, Columbia agar culture plates with 6% sheep blood; ON, overnight; TH, Todd-Hewitt broth medium; OD₆₀₀, optical density measured at 600 nm; CD/CD, caesarean derived/colostrum deprived; V-i, vaccinated pig that was inoculated; V-c, vaccinated pig that served as contact exposed pig; NV-i, non-vaccinated pig that was inoculated; NV-c, non-vaccinated pig that served as contact exposed pig; DPI, day(s) post inoculation; SI-model, susceptible-infectious model; S, susceptible animal(s); I, infectious animal(s); C, number of new cases per day; β , transmission rate parameter; β_v , transmission rate parameter in vaccinated pigs; β_{nv} , transmission rate parameter in non-vaccinated pigs; CPS, capsular polysaccharide.

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now regarded as a predominant serotype isolated from clinical cases in pigs [14–18]. Therefore, also for serotype 9 vaccines have been developed. The majority of these vaccines are autogenous whole bacterin vaccines, containing inactivated farm-specific isolates from clinical cases. Whether or not vaccines against serotype 9 are effective is, however, unknown.

From studies on the efficacy of serotype 2 vaccines it cannot be concluded that vaccines against serotype 9 are effective as well. The main reason is that differences between serotypes in immunogenic capacities may occur, as for example demonstrated in humans for infections with *Streptococcus pneumoniae* [19]. It has been shown that bacterial components, associated with virulence or induction of protective immunity, differ between *S. suis* serotype 2 and 9 strains [14,16,20]. Consequently, the efficacy of vaccines for serotype 9 strains needs to be examined either experimentally or in field studies.

Studies on vaccine efficacy have traditionally focused on protection against clinical signs after challenge infection of pigs with a *S. suis* strain [11–13,21–23]. This is a relevant outcome variable as farmers usually only want to prevent the occurrence of disease or suboptimal growth, and do not aim at reducing the probability of pigs of becoming colonized. However, reduction of number of colonized pigs or even eradication of the bacteria from a farm may be more effective in controlling the disease than reduction of clinical signs, and might also fit better in the 'high health' programmes that are being developed for the swine industry. Vaccines that are able to reduce the number of colonized pigs could contribute to improve the efficacy of this kind of programmes.

The aim of this study was, therefore, to quantify the efficacy of homologous whole bacterin vaccination of pigs upon *S. suis* serotype 9 transmission among pigs, and upon oro-pharyngeal *S. suis* colonization.

2. Materials and methods

2.1. Vaccine and inoculum

A Dutch field strain of *S. suis* serotype 9 (strain 7997), isolated from a clinically affected pig, was used. It contained genes for sullysin (*sly+*) and a larger variant of muramidase-released protein (*mrp**), but lacked the gene for extracellular factor (*ef-*). This combination was present in at least 80% of serotype 9 strains isolated from clinically affected pigs in seven European countries [16]. Strain 7997 was kindly provided by H.E. Smith (Central Veterinary Institute, CVI, Lelystad, The Netherlands), who used this strain successfully in animal experiments before (pers. comm. H.E. Smith).

Strain 7997 from an -80°C stock was cultured on Columbia agar plates containing 6% sheep blood (COLSH) (BioTrading, The Netherlands) overnight (ON) at 37°C and 5% CO_2 . One colony forming unit (CFU) was suspended in 10 ml Todd-Hewitt broth (TH) (BioTrading), and incubated for 3–4 h at 37°C until an optical density at 600 nm (OD_{600}) between 0.5 and 0.6 was reached. After ON storage at 4°C , this suspension was diluted tenfold in 90 ml TH and cultured for 2 h at 37°C resulting in an OD_{600} between 0.5 and 0.6. The bacterial concentration of this suspension was $2\text{--}3 \times 10^8$ colony forming units (CFU) per ml. For vaccine production, 100 ml suspension was washed twice in saline by centrifugation ($2000 \times g$ for 30 and 15 min, respectively, at 4°C) and finally suspended in 10 ml saline. This suspension was again centrifuged at $2000 \times g$ for 15 min. The bacteria in the resulting pellet were inactivated by suspending in 10 ml saline supplemented with 0.5% formalin, mixing for 15 min on a roller mixer at room temperature, followed by ON incubation at 4°C . Finally, adjuvant d,1- α -tocopherol acetate (Diluvac Forte[®]; Intervet, The Netherlands) was thoroughly mixed with the inactivated bacterial suspension (1:1 ratio). The adjuvant

α -tocopherolactate (Diluvac Forte[®]) was used before in a *S. suis* study with a sullysin vaccine [24], and in several bacterin studies with other pig pathogens (e.g. [25–27]). The bacterial concentration of the vaccine with adjuvant was $1\text{--}1.5 \times 10^9$ CFU/ml. The inoculum was prepared accordingly, up to the first washing step. Ten millilitres of the suspension with OD 0.5–0.6 was then washed twice in saline by centrifugation ($2000 \times g$ for 30 and 15 min, respectively, at 4°C) and finally suspended in 10 ml saline. The concentration of the inoculum was approximately $2\text{--}3 \times 10^8$ CFU/ml. The inoculum concentration and inoculation method were based on our purpose to create a colonization and transmission model, and not primarily a clinical or lethality model, and based on two pilot experiments carried out at CVI (pers. comm. H.E. Smith and N. Stockhofe-Zurwieden).

At 3 and 5 weeks of age, pigs were vaccinated by intramuscular (i.m.) injection of 2 ml vaccine in the neck as usually performed in the field. At 7 weeks of age, 25 of the in total 50 pigs were, under Stresnil[®] induced sedation, inoculated intranasally with 5 ml of the bacterial suspension, corresponding to $1\text{--}2 \times 10^9$ CFU/pig. Briefly, the inoculum was applied using a syringe with a sterile steel fork-like attachment with two plastic vents at the ends, which fitted in the pig nostrils. The inoculum was sprayed simultaneously in both nostrils during the inhalation period of 10 breathings.

2.2. Animal experiments

2.2.1. Animals and housing

Three similar experiments (I–III) were carried out sequentially, with 50 cross-bred Finnish Landrace \times Yorkshire pigs in total. To ensure *S. suis*-free status before challenge, caesarean derived/colostrum deprived (CD/CD) pigs were used, obtained from sows from the farrowing farm of the Faculty of Veterinary Medicine, Utrecht, The Netherlands. The caesarean sections were performed under complete anaesthesia. The sows were pre medicated with azaperone (4 mg/kg i.m.) and anaesthesia was induced with propofol (1–2 mg/kg intravenously). Additionally, the area of skin incision was infiltrated with 60–80 ml of lidocaine with adrenaline (Alfacaine cum adrenaline, Alfasan, The Netherlands). After all piglets were born, the sows, still under anaesthesia, were euthanized by intravenous injection of T 61[®] (Intervet, The Netherlands). Piglets were housed under positive pressure conditions, through high-efficiency particle arresting (HEPA) filters ventilated isolators. At the age of 4 weeks they were moved to an under negative pressure ventilated isolation unit with ground floor pens (12–15 pigs/pen) and a strict bio security regime. After challenge with *S. suis* serotype 9, at 7 weeks of age, pigs were housed pair wise in boxes in other, similar isolation units (for more details: see Section 2.2.2). The boxes had completely closed, multiplex walls (height: 80 cm), plasticised iron grid floor, rubber lying area, feeding trough, drinking nipple and two bite sticks. The total ground area per box was 1.2 m². In each isolation unit, four boxes were placed with an interspace of 80 cm (Fig. 1). In succession the pigs were fed with artificial milk (Sprayfo Porc Melk SPF; days 1–10), porridge (Sprayfo Porc Fresco; days 7–21) and pelleted concentrates: Sprayfo Porc Select (days 14–42) and Sprayfo Porc Tempo (days 35–77). All feeds, except Sprayfo Porc Tempo, contained the probiotic bacteria *Bacillus licheniformis* and *Bacillus subtilis*. The artificial milk also contained lactic acid bacterium *Enterococcus faecium*. All feeds were produced by Sloten B.V. (The Netherlands). The porridge powder and both concentrates were disinfected by gamma-irradiation prior to use (9.0 kGy Avg) (Isotron B.V., The Netherlands). Strict bio security protocols were followed to reduce the chance of natural infection with *S. suis*. Animal handlers were not allowed to have contact with other pigs on sampling days, changed clothes, and wore face masks, sterile gloves and hair covers. Moreover,

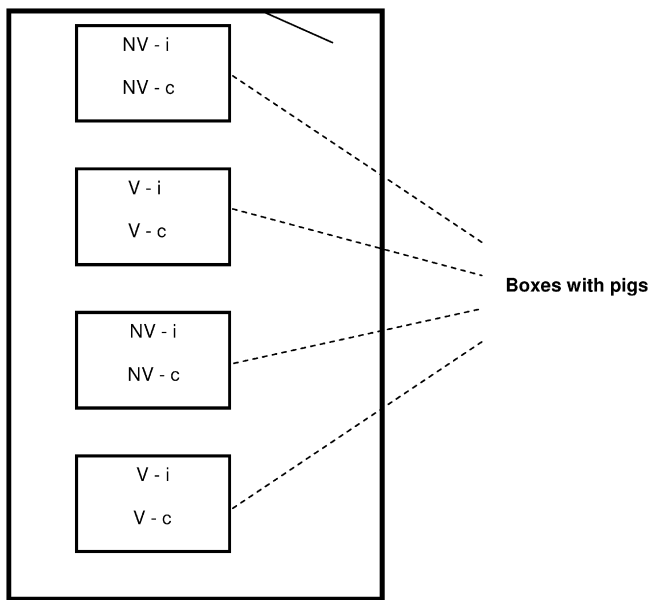


Fig. 1. Design of isolation unit (NV = non-vaccinated; V = vaccinated; i = inoculated pig; c = contact exposed pig).

all housing units and non-disposable materials were thoroughly cleaned, disinfected and dried. Both isolators and units, including all materials needed for the experiment, were gas-disinfected prior to entrance of pigs.

2.2.2. Experimental design

Pigs were randomly assigned to two treatment groups (i.e. vaccinated (V) vs. non-vaccinated (NV)) immediately before the first vaccination at the age of 3 weeks. The second vaccination was applied at 5 weeks of age. Two weeks later, at the age of 7 weeks, the transmission chain was started by inoculation of one randomly selected pig per pair, which was performed in a separate isolation unit. Two days post inoculation (DPI) each inoculated pig (i) was brought together with its pen mate. The use of a separate isolation unit for and the two days interval of separation after inoculation were chosen to prevent colonization of the contact-pigs (c) by the inoculum. In each isolation unit both V and NV pairs were present (Fig. 1). Clinical observations and rectal temperatures were recorded daily. If necessary, a pig was euthanized for animal welfare reasons. The remaining pigs were euthanized at the end of the experiments, which was for logistic reasons spread over 26–28 DPI. The experiments were approved by the Animal Ethical Committee of Utrecht University, in accordance with the Dutch law on experimental animals.

2.3. Sampling procedures and tests

2.3.1. Serum samples and antibody detection

Blood samples were collected from all pigs at 3 weeks (i.e. before the first vaccination) and 7 weeks (i.e. before inoculation) of age and were stored at -20°C until further processing. To check if vaccination was applied correctly, sera were tested in an indirect ELISA as described by Wisselink et al. [13], with slight modifications. Briefly we used the vaccine-suspension without adjuvant diluted 1:10 in coating buffer as coating antigen, Horse Radish Peroxidase (HRPO) conjugated goat anti-porcine IgG (H+L) (Sanbio B.V., The Netherlands) as secondary antibody and 3,3',5,5'-tetramethylbenzidine (TMB Liquid Substrate System, Sigma-Aldrich, The Netherlands) as substrate. The plates were

read at 450 nm by use of a MultiscanFC (Thermo Fisher Scientific, The Netherlands). Each serum was tested in triplo in a 1:40 dilution. Serum of a CDCD-pig that was neither vaccinated against nor infected with *S. suis* was used as negative control. The cut-off value that was chosen was the OD - value of twice the mean values of the 1:40 diluted negative control serum. From sera from a selection of 6 vaccinated pigs a serial dilution (two fold dilutions, from 1:20 to 1:1280) was made to estimate antibody titres.

2.3.2. Tonsil and saliva samples and antigen detection

For detection of *S. suis* colonization and shedding, tonsil brushing and saliva samples were collected from each pig at 0, 2–7, 9, 12, 15, 19, 22 DPI, and at the end of the experiment (at 26 or 27 or 28 DPI). The *S. suis* - free status before inoculation was checked in samples taken at 5 days before inoculation (tonsil) and 3 h before inoculation (saliva and tonsil). Tonsil brushing was performed as described by Swildens et al. [28] with slight modifications. Briefly a sterile steel wedge was put between the pig's teeth, and both palatine tonsillar areas were brushed for 3 s each using a sterile toothbrush. Saliva was sampled by turning round a swab (Cultiplast[®] CE0051) under the tongue for 5 s. The brush and swab heads were cut off with a sterile pair of wire cutters, put in separate sterile tubes containing 10 ml saline and transported to the laboratory. Tubes were mixed on a roller mixer (60 rotations/min) for 10 min and of the resulting suspension 100 μl was used for bacteriological culture after making 10 fold dilutions in saline (10^0 – 10^{-3} for saliva; 10^{-2} – 10^{-4} for tonsil brushing sample). Per dilution 50 μl was plated on COLSH agar plates, supplemented with 0.2 $\mu\text{g}/\text{ml}$ crystal violet and colistin/oxolinic acid (X013, Lab M, United Kingdom) according to manufacturer's instructions. After 18–24 h incubation at 37°C and 5% CO_2 , plates with 10–200 colonies were selected, suspected *S. suis* colonies were counted, and per selected plate two *S. suis* suspected colonies were picked, subcultured and tested for amylase-activity as described by Devriese et al. [29]. Amylase-positive isolates were suspended in 0.5 ml TE buffer pH 7.5 (10 mM), from which DNA was isolated using InstaGene[™] Matrix (Biorad, The Netherlands) according to manufacturer's instructions. Samples were stored at -20°C . From each pig *S. suis* suspected, amylase positive isolates obtained during the first days of infection and the last day of the experiment (for details: see Tables 2 and 3) were confirmed as being *S. suis* serotype 9 by a polymerase chain reaction (PCR) on the *cps9H* gene [30], modified to real-time. The described protocol was adapted to a real-time PCR protocol as follows: 12.5 μl Premix Ex Taq[™] (TaKaRa Bio Inc., USA), 0.5 μl forward primer (10 μM) (5'-CAAAGTTAGTTCAGGAAGGAATAGTCT-3', corresponding to position 4321–4347 of the *cps9H* gene) (Invitrogen, The Netherlands), 0.5 μl reverse primer (10 μM) (5'-CCGAAGTATCTGGGCTACTG-3'; position 4494–4475 in the *cps9H* gene [30]) (Invitrogen), 1.0 μl FAM-labelled probe (5 μM) (5'-FAM-TTTCAGATCAAGATGATATTTGGGACT-MGB-BHQ-3'; position 4361–4387 of the *cps9H* gene) (Biolegio, The Netherlands), 0.5 μl PCR-grade H_2O (Roche Diagnostics, The Netherlands) and 10 μl of sample template was added to a total 25 μl /reaction. DNA-amplification was carried out in a Bio-Rad iQ[™]5 Single Color Real-Time PCR Detection System (Biorad), using 96-wells plates (Biorad). With each run, negative controls (Milli-Q, *S. suis* serotype 2 and *E. faecium*) and a positive control (*S. suis* serotype 9 strain 7997) were tested. The PCR conditions were a hot start at 95°C for 1 min, 45 cycles consisting of denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and amplification at 72°C for 30 s. Also colonies with other morphology were picked and amylase tested. Most of these isolates were amylase negative and were identified as *E. faecium* (i.e. the probiotic feed bacterium) with API 20 Strep (bioMérieux, France) according to the manufacturer's instructions. Some were

Table 1
Bacteriological culture results clustered for separate experiments.

Experimental conditions		N positive ^a /N total (pigs)	Onset of tonsilar colonization (DPI) ^b	Quantitative tonsil colonization (CFU/sample) ^c	Onset of saliva shedding (DPI) ^b	Quantitative saliva shedding (CFU/sample) ^c	Quantitative in tonsilar tissue (CFU/g) ^b
<i>Experiment I</i>							
Vaccinated	Inoculated	3/3	2.00	1.84 × 10 ⁶	2.00	2.11 × 10 ⁵	1.35 × 10 ⁶
Vaccinated	Contact	3/3	3.00	6.16 × 10 ⁵	3.00	1.87 × 10 ⁵	8.26 × 10 ⁶
Non-vaccinated	Inoculated	2/2	2.00	1.21 × 10 ⁶	2.00	3.63 × 10 ⁵	2.52 × 10 ⁶
Non-vaccinated	Contact	2/2	3.50	1.66 × 10 ⁶	3.50	1.83 × 10 ⁵	1.66 × 10 ⁷
<i>Experiment II</i>							
Vaccinated	Inoculated	6/6	2.00	2.37 × 10 ⁶	2.00	1.99 × 10 ⁵	2.62 × 10 ⁶
Vaccinated	Contact	6/6	3.17	6.52 × 10 ⁵	3.17	2.64 × 10 ⁵	2.67 × 10 ⁶
Non-vaccinated	Inoculated	4/4	2.00	8.54 × 10 ⁵	2.25	4.68 × 10 ⁵	4.65 × 10 ⁶
Non-vaccinated	Contact	4/4	3.50	6.37 × 10 ⁵	3.50	1.91 × 10 ⁵	2.36 × 10 ⁶
<i>Experiment III</i>							
Vaccinated	Inoculated	4/4	2.25	9.41 × 10 ⁵	2.25	6.11 × 10 ⁵	2.68 × 10 ⁶
Vaccinated	Contact	4/4	3.25	3.79 × 10 ⁵	3.25	1.57 × 10 ⁵	1.34 × 10 ⁶
Non-vaccinated	Inoculated	6/6	2.17	5.51 × 10 ⁵	2.00	1.87 × 10 ⁵	4.29 × 10 ⁶
Non-vaccinated	Contact	6/6	3.17	4.25 × 10 ⁵	3.33	1.48 × 10 ⁵	3.30 × 10 ⁶

N = number; DPI = days post inoculation; CFU = colony forming units of *S. suis* serotype 9.

^a 'Positive' means *S. suis* serotype 9 was detected in at least one tonsil brushing and/or saliva swab sample during the experimental period.

^b Mean values per animal category.

^c Mean daily values per animal category based on sampling days 2–7, 9, 12, 15, 19, 26 DPI for inoculated pigs and on 2–7, 10, 13, 17, 20, 24 days post contact (DPC) for contact exposed pigs, i.e. days after housing the inoculated and contact exposed pig together (0 DPC = 2 DPI).

amylase-positive and were identified as *Aerococcus viridans*. All pigs were necropsied and samples for bacteriological culture were taken from macroscopically affected organs, if present. Additionally palatine tonsilar plates were removed, submerged in boiling water (5 s), and squeezed in a Stomacher® macerator (Laméris, The Netherlands) for 10 min. This specimen was further handled as described for saliva samples, except for the dilutions that were plated (10⁻³–10⁻⁶).

2.4. Quantification of transmission

2.4.1. Transmission

Transmission of *S. suis* was quantified using a stochastic SI (susceptible-infectious) model [31,32]. Pigs were classified as being infectious (I) as from the moment that PCR-positive colonies picked from saliva and/or tonsil sample cultures were found. The S-category consisted of contact exposed pigs that were not

Table 2
Individual bacteriological and clinical results of non-vaccinated pigs.

Exp. nr.	Pig nr.	Pair nr.	Status	Days post inoculation (DPI)													Tonsil tissue	
				1	2	3	4	5	6	7	9	12	15	19	22	26–28 (end)		
I	13	2	ino	+/* ^a	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	2		contact	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	12	4	ino	+/* ^a	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
6	contact		-/-	+/*	+/* ^b	+/* ^b	+/* ^b	+/* ^b	+/* ^b	+/* ^b	+/* ^b	+/* ^b	+/*	+/*	+/*	+/*	+	
II	32	6	ino	+/* ^{a,b}	+/*	+/*	+/*	+/*	+/*	+/* ^b	+/* ^b	+/*	+/*	+/*	+/*	+/*	+/*	+
	39		contact	-/-	+/*	+/*	+/*	+/* ^b	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	20	8	ino	+/* ^a	+/*	+/* ^a	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	30		contact	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	46	12	ino	+/* ^a	+/*	+/*	+/* ^a	+/* ^a	+/* ^a	+/* ^d	+/* ^d	† euth	+/*	+/*	+/*	+/*	+/*	+
	33		contact	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	34	14	ino	-/+	+/*	+/* ^a	+/* ^b	+/*	+/*	+/*	+/*	+/*	-/+	+/*	+/*	+/*	+/*	+
45	contact		-/-	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+	
III	65	16	ino	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	69		contact	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	77	18	ino	+/* [*]	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	60		contact	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/* ^b	+
	61	20	ino	+/* ^{a,b}	+/* ^b	+/* ^c	+/* ^{a,c}	+/* ^{a,c}	+/* ^{a,c}	+/* ^b	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	71		contact	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	75	21	ino	+/*	+/*	+/*	+/* ^b	+/*	+/*	+/* ^b	+/*	+/*	+/*	+/*	+/*	+/*	+/* ^b	+
	72		contact	-/-	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	67	22	ino	+/* ^b	+/*	+/* ^c	+/* ^c	+/* ^b	+/* ^b	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+
	66		contact	-/+	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	-/+	+/*	+
59	24	ino	+/*	+/*	+/*	+/*	+/* ^b	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/* ^c	+	
73		contact	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+/*	+	

exp. = experiment; nr. = number; end = end of experiment; ino = inoculated pig; contact = contact exposed pig; † = found dead; † euth = euthanized for animal welfare reasons. * '+/-' = saliva+tonsil-, i.e. *S. suis* serotype 9 isolated from saliva, but not from tonsil sample; a bold '+' character indicates that isolates of this sample were tested in the *cps9H*-PCR-test, and found positive. Superscript letters a, b, c, d or e indicate when a specific pig showed clinical signs:

^a Body temperature ≥ 40 °C.

^b Lameness, degree 1 (i.e. avoiding movement of leg(s)).

^c Lameness degree 2 (i.e. avoiding bearing weight on leg(s)).

^d Neurologic signs.

^e Pig had mild locomotory problems before day of inoculation (DPI = 0).

Table 3
Individual bacteriological and clinical results of vaccinated pigs.

Exp. nr.	Pig nr.	Pair nr.	Status	Days post inoculation (DPI)												Tonsil tissue			
				1	2	3	4	5	6	7	9	12	15	19	22		26–28 (end)		
I	4	1	ino		+/+ ^a	+/+	+/+	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-		
	8		contact		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+	
	3	3	ino	a	+/- ^a	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	+/+	+/+	+
	15		contact		+/-	+/-	+/+	+/+	+/+	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
	10		ino		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
II	5	5	ino		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+	
	27		contact		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
	18	7	ino	a	+/+	+/+	+/+	†	+/+	+/+	+/+	+/+	+/-	+/+	+/+	+/+	+/+	+	
	22		contact		+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
	25	9	ino	a	+/+	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+	
	16		contact		-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/+	+/+	+	
	40 ^e		ino		+/+ ^b	+/+	+/+ ^b	+/+ ^b	+/+ ^b	+/+ ^b	+/+ ^b	+/+	+/+	+/+	+/+	+/-	+/+	+/+	+
	20	10	contact		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
	23		ino		+/+ ^{a,b}	+/+ ^{a,d}	+/+ ^d	+/+ ^c	+/+ ^c	+/+	+/+	+/+	+/+ ^{a,c}	+/+ ^b	+/+ ^d	+/+ ^d	+/+ ^d	+/+	+
	44	13	contact		-/+	+/+	+/+	+/+	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+	
	42		ino		+/+	+/+ ^{a,b}	+/+ ^b	+/+	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+	
	24		contact		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
	41	15	ino		+/+ ^a	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
	17		contact		+/+	+/+	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
	III	53	17	ino	a	+/+ ^{a,d}	+/+ ^{a,d}	+/+ ^{a,d}	+/+ ^{a,d}	+/+ ^{a,d}	† euth	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
		55		contact		-/-	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+ ^b
		52	19	ino	a	+/+ ^a	+/+	+/+ ^{a,d}	+/+ ^{a,d}	† euth	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
58		contact			+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
54		23	ino		+/-	+/+	+/+	+/+ ^c	+/+	+/+	+/+	+/+	+/+ ^c	+/+	+/+ ^b	+/+ ^b	+/+	+	
56			contact		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
51		25	ino	a	-/+	+/+	+/+ ^c	+/+ ^{a,c}	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+	
57			contact		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+

See captions below Table 2 for more information.

positive for *S. suis* on the sampling day. The SI-model assumes that susceptible pigs can become infected and that infectious pigs will stay infectious during the rest of the sampling period [32,33]. In this model the transmission rate parameter β was defined as the average number of secondary infections caused by one typical infectious individual in a susceptible population per unit of time. Consequently, β is a function of the infectivity of infectious pigs and the susceptibility of susceptible pigs. To calculate β we used an algorithm described by Velthuis et al. [32]. Briefly, infection parameter β was estimated from the number of new cases (C) per day within a period between two subsequent samplings, which follows a binomial distribution, using the following relation between the number of susceptible animals (S), the number of infectious animals (I) and the total number of animals (N):

$$C = S(1 - e^{-\beta I/N})$$

The effect of vaccination on infection parameter β was tested using a GLM with a complementary-Log-Log link function and $\text{Log}I/N$ ($=0.5$) as offset variable [32,34,35]. For evaluation of the effect of vaccination on transmission we tested the null hypothesis of having no effect of vaccination ($H_0: \beta_v = \beta_{nv}$) against the two sided alternative $H_a: \beta_v \neq \beta_{nv}$. In these formulas β_v is the transmission parameter in a vaccinated population and β_{nv} is the transmission rate in a non-vaccinated (control) population. The calculation of β was performed in R (version 2.12.1).

2.4.2. Statistical analysis

S. suis serotype 9 counts (CFU) of saliva, tonsil and tonsillar tissue samples were ^{10}Log transformed to normalize the data ($^{10}\text{Log}(\text{CFU})$). As a measure for infectivity of the inoculated pigs in the time period that transmission was established within their pair (i.e. from 2 DPI until, and including, the day the corresponding contact was found to be positive for the first time), the mean of its daily saliva $^{10}\text{Log}(\text{CFU})$ of *S. suis* was calculated. This measure was used to evaluate the effect of vaccination on *S. suis* shedding in saliva. The same calculation was performed for tonsil colonization. To facilitate comparison of the quantitative *S. suis* levels in saliva on the first

days after colonization between vaccinated and non-vaccinated, but also between inoculated and contact pigs, per pig the mean of the $^{10}\text{Log}(\text{CFU})$ shedding in saliva for the total experimental period and for its first four *S. suis* positive days was calculated. For tonsil colonization the same method was used. We chose to analyse the period of the first four *S. suis* positive days separately, because the transmission chain was completed (i.e. all contacts were *S. suis* positive) within a period of 4 days. For samples where no *S. suis* was detected, the detection level of the test procedure (i.e. 2×10^3 CFU/sample) was used in calculating the mean levels. It did not affect the results if we had used a value of 2 CFU/sample instead for analysis (data not shown). The presence of *S. suis* serotype 9 in palatine tonsillar tissue was expressed as $^{10}\text{Log}(\text{CFU})$ per gram tissue ($^{10}\text{Log}(\text{CFU})/\text{g}$). Data from pigs that died during the experiment were included in all calculations; exclusion did not influence the results (data not shown). To evaluate the effect of vaccination on colonization and shedding a two-sample *t*-test was performed on the categories V-i vs. NV-i and V-c vs. NV-c, when assumptions of normality of distribution (with Kolmogorov Smirnov test) and equality of variances (with Levene's test) were satisfied. The Bonferroni approach was used to correct for multiple comparisons. The null hypothesis of having no effect of vaccination (equality of means) was tested against the two sided alternative hypothesis. Additionally the same procedure was performed for evaluation of the effect of the pig being inoculated (i) or contact exposed (c) on colonization and shedding (V-i vs. V-c, NV-i vs. NV-c). For statistical analysis of clinical data a Chi-squared test was performed. All shedding and colonization data were analysed using SPSS 16.0.2. The differences in outcome variables were considered significant if *P* values were less than 0.05.

3. Results

3.1. Colonization and shedding

The bacteriological culture results of the three similar experiments are shown in Tables 1–3. Before inoculation no *S. suis* was

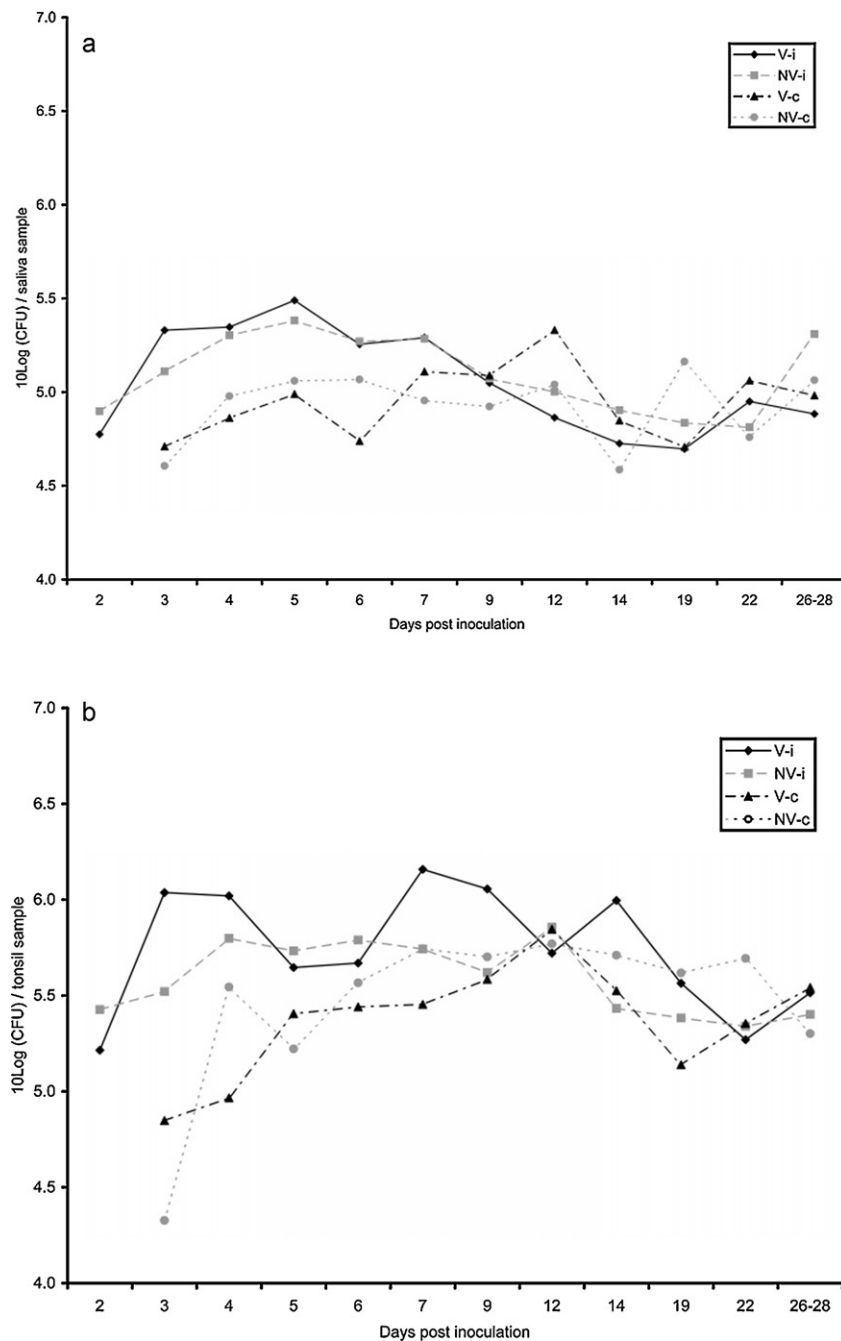


Fig. 2. Mean numbers of *S. suis* serotype 9 colony forming units (CFU) detected in saliva (a) and tonsil brushing samples (b). Mean numbers are shown separately for vaccinated and non-vaccinated inoculated pigs (V-i and NV-i), and also for vaccinated and non-vaccinated contact exposed pigs (V-c and NV-c) (V = vaccinated; NV = non-vaccinated; i = inoculated pig; c = contact exposed pig; CFU = colony forming units).

isolated. After inoculation, all inoculated pigs were *S. suis* positive within two days. All contact pigs were positive within two days after exposure to inoculated pigs. All positive pigs remained positive during the rest of the experimental period.

The patterns of *S. suis* shedding in saliva and colonization on palatine tonsils are shown in Fig. 2. In both intervention groups the mean levels of *S. suis* during the period of first four *S. suis* positive days did not differ significantly between the inoculated and contact piglets, both in saliva ($P=0.60$ for NV; $P=0.14$ for V) and in tonsil samples ($P=0.61$ for NV; $P=0.16$ for V). The same was observed for the mean levels of saliva shedding ($P=0.34$ for NV; $P=0.10$ for V) and tonsil colonization ($P=0.39$

for NV; $P=0.10$ for V) calculated for the whole experimental period.

Levels of colonization and shedding did not differ significantly between vaccinated and non-vaccinated pigs. The mean levels of *S. suis* detected in saliva or tonsil samples taken from vaccinated inoculated pigs during the time period that transmission was established within their pair did not differ from non-vaccinated ones ($P=0.63$ for saliva; $P=0.98$ for tonsil). Mean values of saliva shedding and tonsil colonization of *S. suis* during the first four *S. suis* positive days did also not differ between the intervention groups, both for the inoculated ($P=0.99$ for both saliva and tonsil) and the contact pigs ($P=0.47$ for saliva; $P=0.92$ for tonsil). The same was

observed for the mean levels of saliva shedding and tonsil colonization calculated for the whole experimental period, also both in inoculated ($P=0.99$ for both saliva and tonsil) and contact pigs ($P=0.99$ for saliva; $P=0.94$ for tonsil).

The content of *S. suis* per gram palatine tonsillar tissue did neither differ between vaccinated and non-vaccinated pigs ($P=0.36$ for inoculated and $P=0.82$ for contact pigs), nor between inoculated and contact pigs within the same intervention group ($P=0.61$ for V; $P=0.80$ for NV).

The qualitative bacteriological culture results from 491 tonsil and saliva samples of 521 sampling moments in individual pigs were equal, and in 30 samplings *S. suis* was not isolated from either saliva or tonsil brush samples. For 18 *S. suis* positive tonsil samples, the saliva sample taken from the same pig at the same time was negative. For 12 *S. suis* positive saliva samples, the tonsil brushing sample taken at the same time was negative (Tables 2 and 3).

3.2. Transmission

Transmission of *S. suis* serotype 9 was established in all pairs within three days after bringing inoculated and contact pigs together. The transmission rate was 5.27/day for the vaccinated group (β_v) and did not differ from the transmission rate in the non-vaccinated group (β_{nv}), which was 2.77/day ($P=0.18$).

3.3. Clinical signs

Clinical signs that were observed after challenge with *S. suis* serotype 9 are shown in Tables 2 and 3. In the non-vaccinated group 11 out of 12 inoculated (91.7%) and 2 out of 12 contact pigs (16.7%) showed clinical signs. In the vaccinated group 12 out of 13 inoculated (92.3%) and no contact pigs showed clinical signs. The morbidity of the vaccinated and non-vaccinated groups was not significantly different for both the inoculated ($P=0.50$) and the contact pigs ($P=0.43$). Within each intervention group the morbidity in inoculated pigs was significantly higher than in contact pigs ($P<0.001$ for both V and NV). Ten non-vaccinated pigs and 11 vaccinated pigs, showed temperatures $>40^\circ\text{C}$. Lameness was observed in 9 non-vaccinated (6 inoculated and 3 contact) and in 6 vaccinated pigs (5 inoculated and 1 contact). Neurologic signs were seen in 1 non-vaccinated and 2 vaccinated inoculated pigs.

One vaccinated pig died; one non-vaccinated and two vaccinated pigs were euthanized. In all four *S. suis* serotype 9 was isolated in pure culture from affected meninges, brain tissues or joints. *S. suis* was also isolated at necropsy from several joints of pigs that were sacrificed at the end of the experiment (pig nos. 23, 55, 75).

Before vaccination (i.e. at 3 weeks of age) none of the piglets showed antibody titres against *S. suis* serotype 9. At challenge all vaccinated pigs, except no. 44, showed antibody titres, whereas none of the non-vaccinated pigs did. In all vaccinated pigs from which serial serum dilutions were tested ($N=6$), the OD values at the highest serum dilution (i.e. 1:1280) were above cut off level. All other vaccinated pigs showed comparable high OD levels in 1:40 diluted sera (about 2.0), except for pig nos. 8 and 15 which had levels of 1.0 and 1.1, respectively.

4. Discussion

The aim of this study was to evaluate the efficacy of vaccination on transmission of *S. suis* serotype 9 among pigs and on colonization after challenge with a homologous *S. suis* strain. Vaccination did not significantly reduce the transmission rate of *S. suis* between pigs, and all vaccinated and non-vaccinated contact pigs became colonized. The transmission rate did not differ significantly between both groups; β was 2.77/day, and 5.27/day for non-vaccinated and vaccinated pigs, respectively, indicating that vaccination could not

slow down the spread of the pathogen. The vaccine was also not able to reduce oro-pharyngeal *S. suis* levels and there were no indications that vaccination induced protection against clinical signs after challenge infection. Our findings suggest that homologous whole bacterin vaccination of piglets might not be a very useful measure to prevent colonization with *S. suis* serotype 9.

Pigs vaccinated with the bacterin developed an IgG antibody response in all but one pig, indicating that the vaccine indeed induced an immune response. Nevertheless, there were no indications that the vaccine induced protection against colonization or against clinical signs. An explanation for the absence of protection against colonization might be that antibodies or other immunological factors did not reach the mucosal surface in the tonsillar areas. Mice studies with *S. pneumoniae* showed the role of specific cellular immunity or IgA in controlling mucosal colonization [36,37], but for *S. suis* it remains unknown what specific factors are important to obtain protection against colonization on mucosal surfaces.

The results with respect to protection against clinical signs differed from several vaccine studies with *S. suis* serotype 2, in which protection against clinical infection with a homologous strain was demonstrated (e.g. [11,13]), but this finding was absent in a recent study with an *S. suis* serotype 2 strain [38]. The difference in outcome between studies could be caused by differences in host status (e.g. age), vaccine composition (e.g. adjuvant), vaccination schedule, inoculation method, bacterial strain etc. Our inoculation method was performed to achieve mucosal colonization and transmission, and not mortality or clinical signs. We therefore did not perform i.v. inoculation or pre-treatment [39–42] to facilitate colonization.

Another explanation could be the differences in structural bacterial components. Serotype 9 differs from most virulent serotype 2 strains in presence or structure of several components that were associated with immune responses and clinical protection against serotype 2, like CPS, EF or MRP [13,14,16,30,43]. Since in some serotype 2 studies with bacterin induced clinical protection most of the secreted protein EF was probably lost by the washing steps in the bacterin production protocol [13,43], the lack of EF is a less likely explanation for clinical differences between serotype 2 bacterin studies and this serotype 9 study. From another virulence factor, membrane protein MRP, it is known that it is highly immunogenic [13,43] and its clinical protective efficacy was indicated after application of a vaccine composed of both EF and MRP [43]. In contrast, clear protection against *S. suis* serotype 2 challenge was not observed after vaccination with purified MRP [43], or after application of MRP containing murein associated protein (MAP) [11]. Therefore contribution of MRP to a clinical protective immune response is not clear [12], and the differences in MRP structure between most virulent serotype 2 and serotype 9 strains seems not to be a likely explanation for clinical differences observed in bacterin studies. However, differences in CPS might on this point be of importance. Although pure CPS derived from *S. suis* is poorly immunogenic [44] and antibodies directed against CPS seem not to be important for opsonophagocytosis of *S. suis* serotype 2 [11,44], its role in induction of clinical protective immunity after vaccination against *S. suis* infections could however still be of importance [11–13,45–48]. Differences between serotypes in immunogenic capacities of pure capsular polysaccharides have been demonstrated before, for example for *S. pneumoniae* in humans [19]. Although some progress is made for serotype 2 (e.g. association between opsonising antibodies and clinical protection), the exact mechanism of protective immune responses and differences between serotypes 2 and 9 remain unclear.

As a measure of infectiousness we used detection of *S. suis* in saliva and in tonsil brushing and tissue samples [2,3,49], and this seemed to be a reliable and robust measure. Whether or not this is a suitable measure for infectiousness cannot be made clear as all pigs

became colonized, regardless of the test result, and transmission could not be linked to level of shedding. In a study with *Actinobacillus pleuropneumoniae* [49] differences in levels of bacteria were observed between sample types. The authors concluded that the best indicator to classify a pig as infectious was when bacteria were detected in tonsil tissue at necropsy, even better than successive positive tonsil swabs [49]. We did not find such differences; most saliva and tonsil samples showed the same test result (positive or negative), and all but one tonsil tissue samples were positive. A practical implication of this is that it might be preferable to detect *S. suis* serotype 9 colonized pigs using the less effort requiring method of saliva sampling in comparison to the more invasive tonsil brushing sampling method. Collecting saliva by a simple method using bite-ropes could be proposed for *S. suis* serotype 9 monitoring in groups of pigs.

S. suis could not be isolated from tonsils and saliva for all pigs at all samplings. This may be due to intermittent shedding or by competition on the mucosal level between *S. suis* and the resident flora, e.g. *E. faecium*. Individual differences might also cause differences in shedding patterns. It remains however unclear what the exact explanation is, but intermittent shedding is also observed during the course of other bacterial diseases.

When quantifying transmission, it is assumed that inoculated and contact-infected individuals respond similarly with respect to susceptibility and infectiousness [32,50]. As numbers of *S. suis* colonized on tonsils and shed in saliva were similar in inoculated and contact pigs, it seems reasonable to assume that pigs did not differ in susceptibility and infectiousness, and the model to estimate the transmission rate parameter could be applied. The experimental model presented here would be useful for testing other interventions for their effect on *S. suis* serotype 9 transmission. Although shedding patterns were comparable between inoculated and contact-colonized pigs, the incidence of severe clinical signs did not. Clinical signs in contact infected pigs, either vaccinated or not, were sporadically seen and mild, whereas inoculated pigs frequently showed clinical signs. Similar observations were reported in an earlier study with *S. suis* serotype 2, although in that study inoculated pigs were infected intravenously [51]. Also in other transmission studies with other pathogens this kind of differences have been observed [52,53]. Pigs were randomly allocated to inoculation, which implies that the presence of individual differences in susceptibility is not a likely explanation. Possibly, these differences in clinical signs were caused by difference in infection dose between inoculated and contact pigs. It is, however, not likely that these clinical differences influenced transmission, because the sites where colonization of *S. suis* mostly causes clinical damage, for example joints and brain tissue [3,54], are most likely not related to *S. suis* shedding to other pigs. It seems therefore reasonable to assume homogeneity for susceptibility and infectiousness, indicating that the design to quantify transmission is correct.

In conclusion, this study suggests that whole bacterin vaccination of piglets might not be a promising tool to control the spread of *S. suis* serotype 9 among pigs, as it is not able to reduce either horizontal transmission or mucosal colonization levels of *S. suis* after intranasal challenge.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.12.035.

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