Papers

Use of an avirulent live *Salmonella* Choleraesuis vaccine to reduce the prevalence of *Salmonella* carrier pigs at slaughter

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This study evaluated the use of an avirulent live *Salmonella* Choleraesuis vaccine to reduce the seroprevalence and number of *Salmonella* carrier pigs at slaughter. Seven batches of 500 pigs were included in each of the two study groups: the vaccinated group (VG) that was orally vaccinated and the control group (CG) that received a placebo on the first day of life. The groups were managed in a three-site system and followed up from birth to slaughter. Blood samples (n=378) were collected from each VG and CG to monitor the on-farm seroprevalence in both groups. Mesenteric lymph nodes and blood from animals (n=390) belonging to each group were collected at slaughter. At the first day of life, the seroprevalence in control batches ranged from 77.9 to 96.3 per cent, while in vaccinated batches, it ranged from 66.6 to 92.6 per cent. At weaning (21 days of age), the number of seropositives decreased in both groups (mean of 12 and 3.7 per cent for CG and VG, respectively). At slaughter, batches of VG had a significantly (P<0.0001) lower seroprevalence (46.6±5 per cent) and isolation of *Salmonella* from lymph nodes (33.1±5 per cent) compared with CG batches (79.7±4 per cent and 59.5±5 per cent, respectively). The results indicate that administration of a *Salmonella choleraesuis*-attenuated vaccine on the first day of life decreases *Salmonella* isolation and seroprevalence in pigs at slaughter.

Salmonella enterica is one of the major foodborne pathogens transmitted by pork, and a decrease in the number of carrier pigs at slaughter is considered the first step in achieving *Salmonella*-free pork products (Berends and others 1996). *Salmonella* control has mainly been based on the measures to improve postharvest hygiene and good management practices on farms (Mousing and others 1997, Alban and others 2002). In Denmark, these measures led to a reduction in the number of herds with high *Salmonella* seroprevalence, as well as in the number of human salmonellosis cases associated with the consumption of pork (Alban and Stärk 2005); however, the costs estimated for this programme were considered high (Goldbach and Alban 2006).

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Different strategies have been investigated to control *Salmonella* in the pig industry including the addition of organic acid to feed (Creuss and others 2007) or water (De Busser and others 2009) and the administration of probiotics (Casey and others 2007) or prebiotics (Martín-Peláez and others 2010) to the pigs. Although the addition of probiotics and acids to the feed has been shown to decrease the faecal excretion of *Salmonella* (Casey and others 2007, Creuss and others 2007), their effects may be restricted to a specific age group or their use might only be a strategic measure for the preslaughter.

Vaccination may be a viable approach for shortening the time required to decrease the number of herds with a high infection rate via the increase of resistance to *Salmonella* in susceptible pigs (Haesebrouck and others 2004, Boyen and others 2008). A long-lasting immunity to infection relies upon the development of T- and B-cell-specific immunity (Mastroeni and others 2000). Killed *Salmonella* whole-cell vaccines have been used in animals with variable results, probably due to their poor ability to induce cell-mediated immunity in vaccinated animals (Xu and others 1993, Yamane and others 2000, Davies and Breslin 2003). On the other hand, attenuated live vaccine strains have been shown to offer better protection against *Salmonella* infection due to the cellular immune response and the induction of mucosal IgA production (Haesebrouck and others 2004). Live vaccine strains can be divided into three types (Mastroeni and others 2000): strains that are attenuated without the attenuation being localised or characterised; strains with a mutation in genes that are important for bacterial metabolism, such as *aroA* (Lumsden and Wilkie 1992); strains in which specific virulence genes are removed, for example, the *spv* genes located on the *Salmonella* virulence plasmid (Kramer and others 1992) or strains with defined knockout mutation (Boyen and others 2009).

The SC-54 attenuated strain of *S enterica* serovar Cholearaesuis variety Kurzendorf was obtained after repeated passages of a viru-

lent strain in swine neutrophils, resulting in the loss of the *spv* gene from the virulence plasmid (Kramer and others 1992, Roof and others 1992). Trials conducted on artificially inoculated mice and pigs demonstrated that the vaccine derived from SC-54 strain is safe and able to protect animals from infection and clinical disease (Kramer and others 1992, Baum and others 1997). Moreover, the use of this vaccine to control *Salmonella* carriers yielded promising results in trials conducted with artificially infected pigs (Letellier and others 2001), calves (Fox and others 1997) and dairy cows (House and others 2001).

In Brazil, studies have shown a high prevalence of carrier pigs at slaughter (Bessa and others 2004, Schwarz and others 2009), highlighting the fact that efficient and cost-effective measures, which can be associated with good management practices in farms, are needed to achieve *Salmonella* control. Thus, this study evaluated the use of the vaccine produced with the SC-54 strain in a herd with a high *Salmonella* prevalence with the aim of reducing the number of *Salmonella* carriers at slaughter.

Materials and methods

Location of the trial

The trial was conducted in a three-site vertical integration company with a history of a high prevalence of pigs carrying *S enterica* in the mesenteric lymph nodes (85 per cent) and a high rate of seropositives (77.8 per cent). The most prevalent serovars in this herd were *S* Brandenburg, *S* Typhimurium and *S* Agona (Schwarz and others 2009).

Vaccine

The animals were vaccinated with the attenuated live vaccine EnterisolSC-54 (Boehringer Ingelheim Vetmedica GmbH) containing the *Salmonella* Choleraesuis variety Kurzendorf SC-54 strain attenuated via several passages in pig neutrophils (Roof and others 1992).

Study design and management of the animals

A controlled, blinded clinical trial was designed to assess the efficacy of EnterisolSC-54 orally administered to piglets. The experiment was started in the breeding site, located in a farm with a 1000 sow inventory housed in two barns. The facilities and management practices in both barns were similar. Sows were maintained in individual crates in all phases of production. Breeding and gestation rooms were managed with a continuous flow of sows. *Salmonella* seroprevalence in sows housed in this farm was determined before the start of the trial and a similar seroprevalence (98 to 100 per cent) was observed in both barns.

Since a live vaccine was tested, the vaccinated group (VG) and control groups (CG) could not be housed in the same barn; thus, one sow barn was randomly selected for performing the vaccination, while the other barn housed the controls. For the same reason, the nursery and finishing units exclusively received pigs from either the VG or CG. Pigs not entered into the study were co-mingled with experimental pigs from both VG and CG. Piglets born to sows housed in one barn were VG, while piglets born in the same week to sows housed in the other barn constituted the CG. Each group (VG and CG) included seven batches of 500 piglets born within a one-week interval over seven consecutive weeks, making a total of 3500 pigs in VG and CG. Both groups were managed according to the company's standard practices in all three sites and followed up side by side from birth to slaughter. Piglets in all sites received chlorinated water, which was periodically monitored for quality by the company. Pelleted diets with a composition and nutrient contents in compliance with the recommendations for each stage were manufactured in the company's feed mill. The diet in both groups was not supplemented with acids, probiotics or prebiotics.

Piglets in VG received 2 ml of the vaccine orally on the first day of life and iron dextran was routinely administered to animals of that age. They were notched in their right ear and did not receive antimicrobials until three days after vaccination. Animals that required treatment with antimicrobial drugs in the first week of life were excluded from the trial. Between five and six piglets were excluded from each vaccinated batch. The CG included pigs of the same age that received the same management, except for the vaccine. They were identified with a notch in the left ear. The employees at the farms and at the laboratory were blind to the treatment groups (VG or CG).

At 14 days of age, the piglets started to receive feed with zinc oxide and colistin added to it. Animals from VG and CG were weaned at 21 days of life and housed in nursery units with similar management practices and health status. The nursery farms typically comprised one barn with two rooms, housing a mean of 1000 pigs. Each room had approximately 25 pens with 20 pigs per pen. The nursery facilities comprised a partially slatted floor and a solid partition, nipple drinkers and common feeders. The farms were submitted to all in/all out (AIAO) management by room, with an empty period of four days after cleaning and disinfection with quaternary ammonium compounds. The diets were supplemented with colistin, and two ten-day pulses of amoxicillin and florfenicol were administered to the animals on the first and third week after housing.

At 56 days of age, VG and CG were housed in finishing farms comprising one barn with a single room. The finishing farms housed between 300 and 450 pigs in 20 to 30 pens (15 pigs per pen), with partially slatted floors, solid partition, nipple drinkers and common feeders. These farms were also subjected to AIAO management by farm, with an empty period of seven days after cleaning and disinfection with quaternary ammonium compounds. A total of 13 finishing farms were needed for the placement of each experimental group; the seven nursery barns filled 13 finishing barns. Three ten-day antimicrobial pulses were administrated to the finishing pigs: tiamulin/doxycycline (first week after placement), florfenicol (eighth week after placement) and tiamulin/doxycycline (12th week after placement).

At 150 days of age, the pigs were transported to the same slaughterhouse in trailers that were cleaned and disinfected daily with quaternary ammonium compounds. The length of transportation was up to three hours and the holding period between five and six hours. The holding pens were cleaned and disinfected with peracetic acid before placement of the pigs. A total of 13 batches (one per finishing farm) of VG and CG were slaughtered. Slaughtering was conducted over seven different days and up to two batches each of VG and CG were slaughtered each day. The slaughter batches were kept separated in the holding pens.

Sample collection

At each site (farrowing, nursery and finishing), sampling for serology and bacteriological cultures was conducted in all batches in order to monitor the exposure to *Salmonella* and the seroprevalence in VG and CG.

In each batch, blood from 54 piglets in VG and 54 in CG was sampled at the first, 21st and 49th day of age. This sample size was sufficient (95 per cent confidence interval) to detect at least one positive sample when considering a 5 per cent minimum expected prevalence in the herd (Toma and others 1999). These sampling times were chosen in order to estimate the seroprevalence resulting from passive immunity before vaccination (day 1), the decrease in the passive antibody titer (day 21) and seroconversion in the nursery period (day 49).

Before the housing in the nursery, environmental samples were collected to monitor residual *Salmonella* contamination. One drag swab was taken from the solid area of the floor in 10 pens per room and processed in pools of five swabs. At 49 days of age (nursery) and at 143 days of age (finishing), faecal samples were collected from 10 animals in each batch in order to detect faecal shedding before being transported to the finishing farms and the slaughterhouse, respectively. In each sampling event, five pens (four on the corners and one in the middle of the barn) were chosen, and two animals were sampled in each pen. Faecal samples were processed in pools of two samples, making a total of five pools per batch in each group (VG and CG).

At slaughter, blood samples and mesenteric lymph nodes were taken from 30 animals from the 13 batches of both VG and CG. This sample size allowed the estimation of herd prevalence, given an expected prevalence of 65 per cent (Kich and others 2005) and a relative precision of 15 per cent (Toma and others 1999).

Sample processing

Serum samples were submitted to an indirect ELISA-LPS test to detect IgG anti-*Salmonella* antibodies. The test is based on somatic antigens 1, 4, 5 and 12 of *Salmonella*, with an optical density cut-off point of 0.169. At this cut-off point, the test has a sensitivity of 0.92 and a specificity of 1.0 (Kich and others 2007). Pooled faeces (25 g), drag swabs

TABLE 1: Seroprevalence in vaccinated and control groups at birth (first day of life), at weaning (21 days of age) and at nursery (49 days of age)

	Seropositives % (95% CI) Birth	Weaning	Nursery
Control group	89.1 (85.1-93.1)	12.0 (9.0-15.0)	2.0 (0-6.0)
Vaccinated group	82.2 (78.2-86.2)	3.7 (0-8.7)	0

and individual lymph node (25 g) samples were processed using a *Salmonella* isolation protocol comprised of pre-enrichment in 1 per cent buffered peptoned water, selective enrichment (tetrathionate broth and Rappaport-Vassiliadis broth; Merck; 42°C, 24 hours) and isolation on solid medium (xylose-lysine-tergitol 4 agar and Brilliant Green-phenol red-lactose-sucrose agar; Bencton-Dickson and Company; 37°C, 24 hours), as previously described (ISO6579). Isolates identified as *Salmonella* species were shipped to Fundação Instituto Oswaldo Cruz for serotyping.

Statistical analysis

All statistical analyses were performed using commercial software (SAS 9.1.3; 2009). Logistic regression was used to test the relationship between vaccination and seroprevalence or *Salmonella* isolation from the mesenteric lymph nodes at slaughter. Overdispersion was corrected by Williams' method. p-Values <0.05 were considered significant. Spearman's correlation was calculated between frequency of *Salmonella* presence in lymph nodes and the batch serology at slaughter.

Results

Seroprevalences ranging from 77.9 to 96.3 per cent were found in batches of one-day-old piglets belonging to the CG, while in vaccinated batches frequencies of seropositive piglets varied between 66.6 and 92.6 per cent. On day 21, a prominent decrease in the frequency of seropositives was observed in the batches of both groups (Table 1). No statistical difference between groups was observed in seroprevalence ($P>0.05$).

Five nursery facilities had environmental samples that were positive for *Salmonella* before the allocation of pigs, and both groups were exposed to a similar challenge (two of four positive farms in VG and three of four in CG). At 49 days of age, only one batch of VG had positive faecal samples. This group was housed in one of the farms that was positive on environmental sampling. All sampled animals from VG were serologically negative on this sampling event, while three batches of CG presented seropositive pigs in frequencies ranging from 3.7 to 5.5 per cent.

At the finishing phase (143-day-old pigs), most batches belonging to both experimental groups had at least one *Salmonella*-positive faecal pool. However, the number of positive pools was lower in batches of VG (median=1) compared with CG (median=3).

At slaughter, the isolation of *Salmonella* from mesenteric lymph nodes in VG (129/390; mean=33.1 per cent; IC95=28.1 to 38.1 per cent) was significantly ($P<0.0001$) less frequent than in CG (232/390; mean=59.5 per cent; IC95=54.5 to 64.5 per cent). The *Salmonella* serovars identified in the samples from both groups were similar, and *S* Agona (33 per cent), *S* Panama (27 per cent), *S* Ohio (11 per cent), *S* Schwarzengrund (11 per cent) and *S* Typhimurium (5 per cent) were the most prevalent. The seroprevalence at slaughter was also significantly ($P<0.0001$) lower in VG (174/390; mean=44.6 percent; IC95=39.6 to 49.6 per cent) than in CG (311/390; mean=79.7 per cent; IC95=74.7 to 83.7 per cent). Vaccinated batches therefore had a lower chance of having pigs at slaughter that were positive for *Salmonella* isolation from lymph nodes (OR=0.33; CI95=0.19 to 0.57) and in ELISA testing (OR=0.20; CI95=0.09 to 0.42).

Discussion

This study demonstrated that the vaccine based on the attenuated strain SC-54 was able to significantly reduce the seroprevalence and the number of *Salmonella*-carrier pigs in mesenteric lymph nodes at slaughter. These results agree with those of previous studies where a reduction in the number of positive animals was found at slaughter, even though they adopted different vaccination protocols and evaluation parameters. The SC-45 strain was tested in previous studies that evaluated the administration of the vaccine via drinking water to pigs allocated in finishing farms (Baum and others 1997, Kolb and others 2002). In one trial, a statistically significant reduction in *Salmonella* isolation was achieved for serogroups B and C1 but not for serogroups C2 or E (Baum and others 1997). In the second trial, a significant ($p=0.02$) reduction in the frequency of *Salmonella* isolated from carcasses in the VG was observed in a herd that previously presented a prevalence of 26.2 per cent positive carcasses (Kolb and others 2003).

In our study, oral administration of the SC-54 strain vaccine to one-day-old piglets was evaluated in a vertically integrated system with a history of high (>70 per cent) seroprevalence and *Salmonella* isolation at slaughter. Vaccinated and control batches, following a longitudinal study, showed a similar pattern of transmission and seroconversion. One-day-old piglets of both groups presented a high seroprevalence, possibly caused by the transfer of maternal antibodies, as described by Funk and others (2001) and Chiu and others (2006). In this sense, piglets in both groups had been delivered by seropositive sows and both presented antibody concentrations that gradually decreased from birth until the 21st day of age.

The disappearance of colostral antibodies coincided with transfer to the nursery, where stress factors are responsible for increasing susceptibility to infection in pigs (Funk and others 2001, Fosse and others 2009). In addition, the residual environmental contamination of the nursery facilities observed in the study and the co-mingling with non-vaccinated pigs could have contributed to *Salmonella* transmission. Despite this, after 28 days (49-day-old pigs) in the nursery, only three batches were detected with a (low) prevalence of seropositive pigs (2 per cent, IC95=0 to 6 per cent) and only one cohort from the VG group was shedding *Salmonella*, indicating that most of the pigs were either negative for *Salmonella* or the animals were still in the initial stages of infection. At the finishing site, the pigs started to shed *Salmonella* in their faeces, in line with the amplification in the number of infected animals often observed at this stage (Funk and others 2001, Lo Fo Wong and others 2004). However, the VG showed a lower number of positive faecal pools, which may have resulted from a lower number of infected animals due to the protection conferred by vaccination. Although a poor correlation between individual *Salmonella* ELISA tests and bacteriology on an individual pig basis has been reported (Christensen and others 1999, Davies and others 2003), the seroprevalence at slaughter has demonstrated a strong correlation with the presence of *Salmonella* in caecal contents and on the carcass surface in pig batches (Sørensen and others 2004). Thus, programmes to control *Salmonella* have aimed to reduce seroprevalence in herds to levels below 40 per cent, as well as the eradication of herds with seroprevalence above 70 per cent in order to decrease the hazard of carcass contamination at slaughter (Mousing and others 1997, Alban and Stärk 2005). In our study, all batches vaccinated with the SC-54 strain presented seroprevalences below 70 per cent, whereas the control cohorts presented a significantly ($P<0.0001$) higher number of seropositive pigs. A moderate correlation ($r=0.506$) was observed between seroprevalence and *Salmonella* isolation from mesenteric lymph nodes, and the VG showed a significant reduction ($P<0.0001$) in the frequency of *Salmonella* isolation from mesenteric lymph nodes, demonstrating that the vaccination had a protection effect. Veterinary vaccine challenge studies should provide evidence for vaccine efficacy, presenting internal controls and replicating field conditions (Denagamage and others 2007). The authors conducted a clinical trial that followed up seven batches of vaccinated pigs and a similar group of control pigs from birth to slaughter in a multiple-site system, which had a history of high *Salmonella* prevalence. This herd presented a significant reduction in seroprevalence and the number of *Salmonella* lymph node carriers in vaccinated batches at slaughter, which supports studies that proposed the use of vaccines for the control of *Salmonella* infection (Maes and others 2001, Haesenbrouck and others 2004, Boyen and others 2008). Live-attenuated vaccines are more suitable for induction of cell-mediated immunity required for an effective protection against facultative intracellular bacteria, such as *Salmonella* species. However, mucosal and serum antibodies against somatic antigens also play a role in host immunity (Haesenbrouck and others 2004), and a low level of cross-protection is observed between antibodies induced by different *Salmonella* serovars (Wallis 2001). Therefore, the further

development of a vaccine including an attenuated *S* Typhimurium strain may be able to induce an even better protection in pigs.

Another issue about the use of vaccines has been the induction of antibodies that interfere with the serological tests adopted by monitoring programs (Leyman and others 2011). In the present study, no seroconversion after vaccination was noticed in the ELISA test based on somatic antigens 1, 4, 5 and 12 of *Salmonella*. It may be related to the fact that somatic antigens of *S* Choleraesius (O:6, 7) are not included in the adopted test. However, low frequencies of seropositive pigs have also been reported after the administration of the SC-54 strain vaccine, when tests that included somatic antigens 6 and 7 were used (Maes and others 2001).

In conclusion, in herds with a high prevalence of *Salmonella*, vaccination with the SC-54 strain can be considered as an additional management tool to reduce the number of carriers in a shorter period of time, even though changes in management and the correction of risk factors remain essential for achieving the target of a low prevalence level in *Salmonella* control programmes.

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