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Survival and Transmission of *Salmonella enterica* Serovar Typhimurium in an Outdoor Organic Pig Farming Environment

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It was investigated how organic rearing conditions influence the *Salmonella enterica* infection dynamics in pigs and whether *Salmonella* persists in the paddock environment. Pigs inoculated with *S. enterica* serovar Typhimurium were grouped with *Salmonella*-negative tracer pigs. Bacteriological and serological testing indicated that organic pigs were susceptible to *Salmonella* infections, as 26 of 46 (56%) tracer pigs turned culture positive. An intermittent and mainly low-level excretion of *Salmonella* ($<$100 cells g^{-1}) partly explains why the bacteriological prevalence appeared lower than the seroprevalence. *Salmonella* persisted in the paddock environment, as *Salmonella* was isolated from 46% of soil and water samples (n = 294). After removal of pigs, *Salmonella* was found in soil samples for up to 5 weeks and in shelter huts during the entire test period (7 weeks). Subsequent introduction of *Salmonella*-negative pigs into four naturally *Salmonella*-contaminated paddocks caused *Salmonella* infections of pigs in two paddocks. In one of these paddocks, all tracer pigs (n = 10) became infected, coinciding with a previous high *Salmonella* infection rate and high *Salmonella* excretion level. Our results showed that pigs reared under organic conditions were susceptible to *Salmonella* infections (just like conventional pigs) and that *Salmonella* persisting in the paddock environment could pose an infection risk. A driving force for these infections seemed to be pigs with a high *Salmonella* excretion level, which caused substantial contamination of the environment. This suggests that isolation of animals as soon as a *Salmonella* infection is indicated by clinical symptoms of diarrhea could be a means of reducing and controlling the spread and persistence of *Salmonella* in outdoor organic pig production environments.

Organic pig production is a growing alternative to conventional and often large-scale pig production and aims at improving animal welfare and providing growth conditions under which the pigs can express natural behaviors. The main differences between organic and conventional production are that the pigs have access to outdoor areas, farrowing takes place outdoors, and piglets live together with the sow for a minimum of 7 weeks before weaning, compared to 3 to 4 weeks in conventional pig production (2).

Outdoor pig production may imply increased exposure to pathogens that persist in the environment or are transmitted through wildlife. The pathogens may not influence the welfare of the pigs, but zoonoses such as *Salmonella enterica* can subsequently be transferred to humans through consumption of contaminated pork. Several investigations have demonstrated that *Salmonella* infections in conventional pig farms are able to persist in the herd environment for several months and even years (5, 9, 19, 46). Even though it is difficult to differentiate between the persistence of pathogens in pigs caused by subclinically infected animals and infection originating from the environment, isolations of *Salmonella* from soil, slurry, manure, and equipment indicate that a contaminated environment may constitute an important source of infection (4, 18).

Organic pig farms often produce less than 200 pigs per year and are therefore not included in the Danish national surveillance program, which monitors the *Salmonella* seroprevalence in pigs (meat juice) at slaughter (1). Thus, limited data on *Salmonella* infection rates in organic pig production are available. Nevertheless, a comparison of the *Salmonella* seroprevalences in Danish pig herds showed that there was a higher risk of meat juice samples from both organic and free-range herds being seropositive than from samples from conventional herds (odds ratio = 1.7; P = 0.0001) (54). The result was significant for the free-range herds (P = 0.001) but not for organic herds, due to a limited number of samples. Similar results were obtained in a Dutch study in which the *Salmonella* seroprevalence in free-range finishers was significantly higher (44.6%) than in intensive conventional indoor production of finishers (24.5%) (51).

Information about the time of establishment of a *Salmonella* infection, its duration, and the level of infection in individual animals would help to illuminate the infection dynamics in a herd and thus the potential contamination risk at the time of slaughter. Due to the differences between organic and conventional pig production, the current information on *Salmonella* dynamics in conventional pigs may not apply to organic and other alternative pig production systems. Little is known about the effect of organic rearing with respect to susceptibility to infections such as salmonellosis. Perhaps the late weaning; the organic feed, including roughage, that potentially alters the microbial composition of the gut; and the lower animal density diminish the levels of *Salmonella* in organic pigs. Since many of the normal measures taken to prevent and control *Salmonella* infection in indoor systems do not apply in outdoor systems,

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obtaining information about potential risk factors is important in limiting the risk of *Salmonella* infections in these systems.

The aim of the current experimental study was to examine the *Salmonella* infection dynamics in organic pigs raised outdoors. Noninfected tracer pigs were grouped with pigs inoculated with different concentrations of *Salmonella* to determine the transmission of *Salmonella* between animals. Furthermore, the establishment of *Salmonella* in the pasture environment was studied, including its impact on *Salmonella* infection of new tracer animals introduced into the pasture.

MATERIALS AND METHODS

Pigs and field sites. On three occasions 56, 56, and 51 organic pigs with average weights of 16.9 ± 4.0 kg, 12.7 ± 2.4 kg, and 20.6 ± 3.9 kg, respectively, were purchased from a Danish organic farmer at the time of weaning (7 weeks old). Upon arrival at the university research farm in Taastrup, Denmark, rectal fecal and blood samples were collected from the pigs (zero samples) to determine their *Salmonella* status by microbiological culture and enzyme-linked immunosorbent assay (ELISA) (8). To avoid parasite contamination of the experimental pastures, the pigs were treated with fenbendazole, 10 g active ingredient per 56 animals, administered with the feed for 2 days. The pigs were fed ad libitum with organic pelleted feed and pea/barley silage as roughage.

Six experimental outdoor paddocks, named A, B, C, D, E, and F, were set up on a 3-year-old mixture of grass and clover not previously grazed. The rectangular paddocks (50 m^2 per pig) were enclosed with electric fence and placed 2 meters apart to avoid direct contact between the animals. In each paddock, the pigs had free access to an insulated hut with straw bedding, water cups, wallowing area, and a feed dispenser. Before initiation of a new experiment, the old straw bedding in the huts was removed and slaked lime was spread on the surface ground to diminish the survival of *Salmonella*; however, the efficacy of this procedure was not assessed.

Experimental design. In three successive experiments, referred to as periods 1 to 3, a total of 163 organic weaning pigs (*Salmonella* negative) were distributed among the six paddocks. Four paddocks (C to F), each with 10 pigs, were used for *Salmonella* infection experiments, and two paddocks (A and B), each with 8 pigs, served as *Salmonella*-negative control paddocks (Table 1). Each of the three experimental periods lasted for 6 weeks and were carried out from late April to the beginning of September 2003.

Seven *Salmonella*-negative pigs, designated tracer pigs, were grouped with three pigs artificially inoculated orally with an *S. enterica* serovar Typhimurium DT12 test strain to study the transmission of *Salmonella* from infected pigs to the tracer pigs. Two groups of pigs were inoculated with 7.4×10^7 cells (referred to as low dose) in paddocks C and D (in the first of the three experimental periods), and five groups of pigs were inoculated with 3.2×10^9 cells (referred to as high dose) in paddocks E and F (first experimental period) and paddocks C, D, and E (third experimental period) (Table 1). The bacterial cells were given to each pig via a gastric tube, using a volume of 10 ml physiologic saline (0.9% NaCl) solution. Inoculation experiments were carried out in both the first and the third period, and *Salmonella* test strains with two different resistance markers, as described below, were used to permit differentiation of *Salmonella* organisms isolated from the two rounds of inoculation.

The infectivities of *Salmonella*-contaminated paddock environments were studied in the second experimental period by introducing 10 *Salmonella*-negative tracer pigs into paddocks C, D, E, and F at 1 day after termination of the first period. In case the pigs became *Salmonella* infected in the second period, new tracer pigs were introduced into the paddock in the third period to examine whether the contaminated environment continued to be infective. The paddocks in which no or little *Salmonella* infection occurred in the second period were used for new inoculation experiments in the third period (Table 1).

***S. enterica* serovar Typhimurium DT12 test strain.** A Danish strain of *Salmonella* serovar Typhimurium DT12, originally isolated from pigs with clinical signs of salmonellosis, was grown on rifampin- and nalidixic acid-containing nutrient agar (Oxoid Limited, Basingstoke, United Kingdom) to select for single rifampin or double rifampin and nalidixic acid resistance. These strains (*Salmonella* serovar Typhimurium DT12 rifampin^{res} and *Salmonella* serovar Typhimurium DT12 rifampin/nalidixic acid^{res}) were cultured as described previously (40), harvested, and diluted to approximately 10^6 and 10^8 CFU/ml. The inoculation doses were determined by serial dilutions and growth on brilliant green agar (BGA) (Oxoid).

Sampling of feces, blood, and environmental samples. Rectal fecal samples (5 g) and blood samples (5 ml) of each pig and seven samples from each paddock environment were collected once per week for 6 weeks in each period. A 50-ml water sample was collected from the water cup (environmental sample 1). Soil samples (>30 g) were collected as pools of five small samples of top-surface soil (1 to 5 cm) from each of the six following locations in the paddock: rear end of the paddock (environmental sample 2), intersection of hut/water cup (environmental sample 3), defecation area (environmental sample 4), feeding area (environmental sample 5), hut (environmental sample 6), and mud hole (environmental sample 7). The first samples were collected 3 days postinoculation or 4 days after introduction of tracer pigs into the contaminated environment (second period). The sampling of environmental samples continued for 7 weeks after the paddocks had been vacated. The samples were transported to the laboratory at ambient temperature (blood and soil) or on ice (feces) and stored at 4°C until testing the next day. To avoid cross-contamination between animals, materials, and samples, only disposable or disinfected equipment was used for collecting samples, as well as good hygiene practices.

TABLE 1. Study design of *Salmonella* infection in an organic outdoor pig farming environment

Period[a]	No. of pigs in paddock[c]:					
	A[b]	B[b]	C	D	E	F
(1) 2 May–10 June	8	8	7*	7*	7†	7†
(2) 12 June–21 July	8	8	10	10	10	10
(3) 23 July–1 Sept	8	7	5†	6†	7†	8

[a] Three periods that aimed to study (1) transmission of *Salmonella* from artificially infected pigs to tracer pigs in paddocks C to F, (2) transmission of *Salmonella* from a contaminated paddock environment to new tracer pigs in paddocks C to F, and (3) repetition of period 2 in paddock F and repetition of period 1 in paddocks C to E.

[b] *Salmonella*-negative control paddocks and pigs.

[c] *, indicated number of tracer pigs plus three pigs inoculated with 7.4×10^7 *Salmonella* cells (low dose); †, indicated number of tracer pigs plus three pigs inoculated with 3.2×10^9 *Salmonella* cells (high dose).

Isolation and enumeration of *Salmonella* organisms. The detection of *Salmonella* in fecal samples (5 g) was done as described by Baggesen et al. (8) with minor modifications. A qualitative assessment with a detection limit of 1 CFU per 5 g was used for the control pigs and the samples collected upon arrival at the farm for screening of *Salmonella* (zero samples), whereas the level of *Salmonella* excretion was examined by a semiquantitative approach for all other pigs. The fecal materials were diluted 100-fold in buffered peptone water (Merck KGaA, Darmstadt, Germany) supplemented with novobiocin (Sigma-Aldrich Co., St. Louis, MO) (BPW-N) (22 μg/ml) to assess the *Salmonella* excretion levels in the pigs (30). Dilutions were incubated overnight and spotted onto modified semisolid Rappaport-Vassiliadis agar (Oxoid). After overnight incubation, material from presumptive *Salmonella* swarming zones was subcultured onto BGA. The test strain was identified by slide agglutination of colonies from BGA plates with polyclonal O5 antiserum and confirmation of the resistance markers by inoculation onto nutrient agar with and without rifampin or rifampin/nalidixic acid (50 mg/ml). Nonresistant *Salmonella* strains were identified by serotyping by agglutination using polyclonal sera (Statens Seruminstitut, SSI, Copenhagen, Denmark) according to the Kauffmann-White scheme (29, 43).

Survival of *Salmonella* in the paddock environment was assessed qualitatively by pre-enrichment of 25 g soil in BPW-N (1:9). The water samples were filtered onto a 0.45-μm filter (Millipore, Billerica, MA) by use of vacuum, and filters were pre-enriched in 9 ml of BPW-N. Overnight incubation and further isolation of *Salmonella* organisms were done as described above.

Postmortem examinations of pigs. Pigs with health problems were excluded before the end of the trial and subjected to postmortem bacteriological examinations of liver, spleen, intestinal wall, cecum content, and mesenteric and ileocolic lymph nodes, as described by Baggesen et al. (8).

Enumeration of presumptive *E. coli* in the paddock environment. Detection of presumptive *Escherichia coli* was based on standard conventional bacteriological methods and performed as described previously (15). No attempt was made to discriminate between pathogenic and nonpathogenic *E. coli* organisms. The water and homogenized soil samples (5 g) were 10-fold serially diluted (10^{-1} to 10^{-5}), and 1 ml from each of the soil dilutions was then mixed with 5 ml of molten tryptone soya agar (BD, Franklin Lakes, NJ) with a temperature of 45°C. Plates were preincubated at room temperature for 1 to 2 h, after which 10 to 15 ml of violet red bile agar (Oxoid) was poured onto the surface. Inoculated plates were incubated overnight at 44°C. The water dilutions were filtered through a

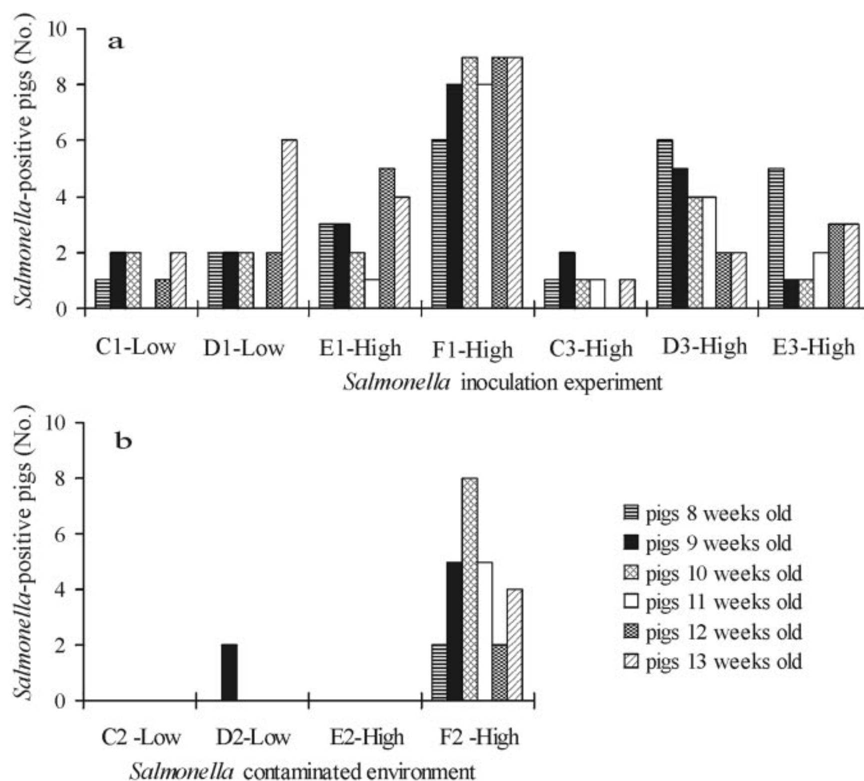

FIG. 1. (a) *Salmonella* serovar Typhimurium culture-positive organic pigs found during 6 weeks after inoculation of 3 of 10 pigs with a low dose (paddocks C and D in period 1) or a high dose (paddocks E and F in period 1 and C to E in period 3) of *Salmonella* serovar Typhimurium cells. (b) Transmission of *Salmonella* serovar Typhimurium to tracer pigs after introduction into the *Salmonella*-contaminated environment of paddocks C to F in the second experimental period.

0.45-μm filter (Millipore), and the filters were incubated on membrane lauryl-sulfate agar (Oxoid) overnight at 44°C. Plates with 10 to 100 colonies typical of thermotolerant coliform bacteria were counted, and 5 colonies per plate were confirmed as presumptive *E. coli* by testing for production of gas in lactose tryptone lauryl-sulfate broth (Oxoid) and indole by addition of Kovács indole reagent (Merck KGaA). The results were expressed as number of presumptive *E. coli* organisms per gram, and the detection level of the method was 10 CFU g^{-1}.

Serology. Blood serum from the pigs was tested by ELISA based on O:1,4,5,6,7,12 lipopolysaccharide from different *Salmonella* types, designated mix-ELISA (40). This test permits detection of the most common serotypes, including the test strain (O:4,5,12). The sample optical density (OD) readings (490 nm) were transformed to calibrated OD%, with a positive cutoff at 10 OD% (40).

Statistical analysis. Due to the low numbers of pigs at each location, nonparametric methods were used. We used the Wilcoxon rank sum test (32) with a one-sided alternative in all analyses. The effect of the *Salmonella* dose was investigated by comparing the low-dose group with the high-dose group. This was done for both bacteriological and serological results. The frequencies of tracer pigs becoming infected with *Salmonella* in the study period were compared, as well as the "waiting time to infection," i.e., the number of weeks after inoculation until infection was detected in tracer pigs.

The number of times that *Salmonella* was isolated at the various locations in the paddock was investigated, and we investigated whether one location might be considered an outlier compared to the others with regard to this number by fitting a normal distribution to the others and adjusting for the most extreme case not being included (through simulation). We then calculated the probability that the most extreme case could be as different from the rest as was observed. To investigate whether a different (smaller) number of *Salmonella*-positive environmental samples at specific locations was related to a different (smaller) load of feces (with *E. coli* as indicator), we fitted a second-order polynomial to the *E. coli* numbers for each of the locations as a function of time and compared the results graphically.

We investigated the survival of *E. coli* in the pasture environment by fitting a standard log-linear regression model to the *E. coli* numbers as a function of time, from the day on which the paddocks were vacated until decay set in. The regression model was used to estimate decimation time (T_{90}) values both for the average of the locations in the paddock and for the most extreme location (i.e., with the lowest decay rate).

All calculations where carried out with Splus software, version 6.1.

RESULTS

Establishment of *Salmonella* infection. Three pigs in each group ($n = 3 \times 7$) were inoculated with a low or high dose of *Salmonella* serovar Typhimurium DT12 cells and grouped together with five to seven tracer pigs ($n = 46$) to study the possible spread of *Salmonella* between outdoor organic pigs. Two groups of pigs were given a low dose of *Salmonella* (in the first experimental period), and five groups were given a high dose (in the first or third experimental period). One of the pigs from the third batch was found to be *Salmonella* positive (serovar Typhimurium, unspecific phage type) when tested after arrival at the research farm (zero samples). This pig was excluded from the experiment but may have contributed to the slight seroresponse (<18 OD%) found in three otherwise culture-negative pigs (data not shown). The frequency of *Salmonella*-positive animals in the groups and the number of times *Salmonella* was isolated from each pig during the 6-week period showed large variations (Fig. 1a). The inoculated pigs ($n = 21$) showed the highest level of *Salmonella* excretion (75%) immediately after the challenge with *Salmonella*, whereas the number of *Salmonella*-positive tracer pigs increased from 20 to 41% over the 6 weeks (Fig. 2).

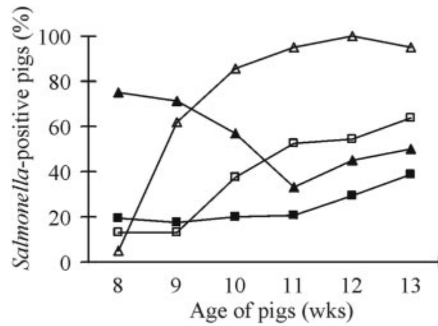

FIG. 2. Overall percentages of *Salmonella*-positive pigs over time after artificial inoculation of 3 of 10 pigs (seven groups) based on bacteriological *Salmonella* serovar Typhimurium status for inoculated pigs (▲) and tracer pigs (■) and on the antibody level (ELISA serology) for inoculated pigs (△) and tracer pigs (□).

TABLE 2. Frequency of tracer pigs testing *Salmonella* positive based on results by two diagnostic methods

Diagnostic method	No. positive/total in indicated *Salmonella* inoculation experiment[a]						
	Low dose		High dose				
	C-1	D-1	E-1	F-1	C-3	D-3	E-3
Bacteriology	2/7	5/7	3/7	7/7	1/5	5/6	3/7
Serology[b]	1/7	2/7	5/7	7/7	4/5	6/6	4/7

[a] Number of *Salmonella*-positive pigs (positive in at least one of six samples) out of the total number of tracer pigs in each group is shown, reflecting transmission of *Salmonella* from artificially infected pigs (low or high dose of *Salmonella* cells) to tracer pigs in paddocks C to F in periods 1 and 3 (see Table 1).
[b] ELISA method.

The three inoculated pigs in each group were *Salmonella* positive on 5 to 16 out of 18 occasions irrespective of the inoculum dose. The inoculated pigs mostly had intermittent excretion of *Salmonella*, although three pigs remained *Salmonella* culture negative and five pigs culture positive throughout the trial (6 weeks). The *Salmonella* excretion level in the inoculated pigs was generally low, as 72% of the positive samples (n = 68) contained <100 cells g^{-1}. Only two pigs excreted $>10^4$ cells; one, an inoculated pig from paddock F, showed clinical symptoms of salmonellosis, with an excretion level of $>10^6$ cells g^{-1} on two occasions before it died (week 4). *Salmonella* serovar Typhimurium DT12 rifampin^{res} was isolated from this pig in liver, spleen, lung, intestinal wall, cecum content, and mesenteric and ileocolic lymph node samples following postmortem bacteriological examination. In addition, four pigs were excluded before the end of the inoculation trials in the first and third periods due to general poor health (n = 3) and a broken leg (n = 1), and *Salmonella* spp. were isolated at necropsy in three of these.

Transmission of *Salmonella* to tracer pigs. Twenty-six of the 46 tracer pigs (56%) grouped with the inoculated pigs tested *Salmonella* culture positive (test strain) at least once during the 6 weeks. A pig with at least one positive finding was termed a *Salmonella* culture-positive pig, and a similar definition was used for ELISA-positive pigs. The numbers of *Salmonella* culture-positive tracer pigs in the five high-dose groups varied from a single pig (C3) to all seven tracer pigs (F1), while half of the tracer pigs became *Salmonella* culture positive in the two low-dose groups (Table 2). The mean frequencies of *Salmonella* culture-positive tracer pigs were 0.50 in the low-dose groups and 0.58 in the high-dose groups. The low-dose and high-dose groups were not found to be significantly different (P = 0.42). In contrast to the results of bacteriological analyses, the mix-ELISA test results indicated that the frequencies of seropositive tracer pigs were significantly different (P = 0.04) between the low- and high-dose groups, with mean frequencies of 0.21 and 0.82, respectively (Table 2).

The *Salmonella* seroprevalence in the pigs was generally higher than their bacteriological status (Fig. 2 and 3). Overall, 22 out of 94 tracer pigs tested seropositive without being *Salmonella* culture positive. Eight culture-positive tracer pigs were not seroresponding, but half of these were not culture positive until the last week and were therefore unlikely to seroconvert within the time span of the study. Two of the nonresponding pigs excreted <100 CFU g^{-1} once in the second week, one pig excreted <100 CFU g^{-1} in weeks 3 and 4, and one pig was positive on three occasions ($<10^4$ CFU g^{-1} the last week) before being killed due to poor health.

Measuring the number of weeks (1 to 6) before a tracer pig tested *Salmonella* culture positive for the first time showed that the seven *Salmonella*-positive tracer pigs from the low-dose groups were all *Salmonella* negative until the last week (week 6), except for one (negative until week 5) (mean, 5.9 weeks), whereas most of the high-dose tracer pigs (n = 19) became infected during the first weeks (mean, 2.2 weeks). The number of weeks passed for the high-dose groups was significantly lower than that for the low-dose groups (P = 0.0001). Furthermore, *Salmonella* was isolated more than twice in 31% of the tracer pigs (n = 32) from the high-dose groups. The infection level seemed to vary under apparently similar conditions, as 33 out of the 65 (50.8%) positive fecal samples (n = 268) came from tracer pigs in paddock F (high dose), while the other four high-dose groups together accounted for 38.5% of the positive samples. The level of *Salmonella* excretion in the tracer pigs was mainly low, as 93.8% of the positive samples (n = 65) contained <100 cells g^{-1} feces.

In the third test period, the *Salmonella* serovar Typhimurium DT12 rifampin/nalidixic acid^{res} test strain was de-

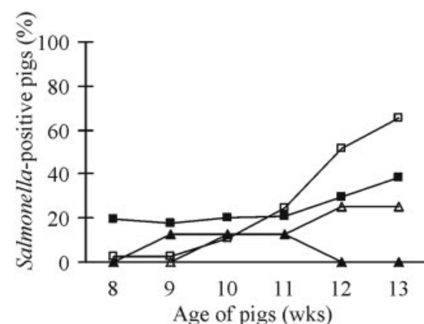

FIG. 3. Overall percentages of *Salmonella*-positive pigs after introduction of tracer pigs into a *Salmonella*-contaminated environment in the second period for paddocks C to F, based on bacteriological (■) and serological (□) status, and in the third period in paddock F, based on bacteriological (▲) and serological (△) status.

TABLE 3. Occurrence of the *Salmonella* serovar Typhimurium DT12 test strain and other *Salmonella* serotypes in pigs and the environment of paddocks A to F in three periods

Period[b]	Wk	No. of *Salmonella*-positive samples or other *Salmonella* serotypes in paddock[a]:											
		A[c]		B[c]		C		D		E		F	
		Pigs	ENV	Pigs	ENV	Pigs	ENV	Pigs	ENV	Pigs	ENV	Pigs	ENV
1	1					1		2	1	3	4	6	5
	2					2	1	2	1	3	6	8	6
	3					2	1	2	1	2	4	9	7
	4						1		2	1	6	8	7
	5					Gol		2	3	5	6	9	6
	6					2	3	6	3	4	5	9	6
2	1					107	Ana		1 170		5	2	6
	2				Ana	New 107	Ana	2	2 170		4	5	6
	3		Rea			107	New		1 170		2	8	6
	4	Gol	109 ND (3)			107	1 107 Sta		1 170 Uga		3	5	6
	5	Der Der				Liv 41	41 170		Uga		3 170	2	6
	6	Der				41	Liv		41		2	4	7
3	1	New	New New Ohio			1 107	3	6	6	5	2		5
	2		New New			2	2 New	5	7	1 107	3	1	4
	3		New 109	RDNC 109	109	1 Ind	2	4	4 RDNC	1 107	1	1	4
	4	3.10:	New	1		1 4.12:	Liv	4 4.12: Ago	5	2	1	1	5
	5		1				1	2	3	3			4
	6				Ohio	1	3 NT	2	4 109	3 109	2 109 Uga		4

[a] The numbers refer to the number of times the *Salmonella* test strain was detected on each sampling occasion. Additional detection of other *Salmonella* serotypes is shown with the following abbreviations: Ana, Anatum; Ago, Agona; Der, Derby; Gol, Goldcoast; Ind, Indiana; Liv, Livingstone; New, Newport; Rea, Reading; Sta, Stanley; Uga, Uganda; 4.12:, 4.12:d:−; 3.10:, 3.10:−:1.5; NT, not typeable; ND, not serotyped. 41, 107, 109, and 170 are phage type (DT) numbers of serovar Typhimurium; RDNC, unspecific phage type.

[b] Three experimental periods each lasting 6 weeks, running from late April to the beginning of September.

[c] Paddocks A and B are *Salmonella*-negative control paddocks.

tected once in the environment of *Salmonella*-negative control paddock A (mud hole) and once in a pig from paddock B. Additionally, a rifampin^{res} (second period) or rifampin/nalidixic acid^{res} (third period) *Salmonella* serovar Typhimurium organism of phage type 109 (the test strain is DT12) was found in the environment of paddock A once in each of the last two periods and once in paddock B in the third period (Table 3). The doubly resistant strain was isolated from the pig in paddock B that also tested positive for the test strain (two subsequent weeks) (Table 3). In addition, Table 3 shows the detection of serotypes of *Salmonella* besides the test strain (on 23 occasions in pigs and 40 occasions in environmental samples), which has been described previously (29).

Survival of *Salmonella* in the pasture environment. The ability of *Salmonella* to persist in the nonhost environment was indicated by the detection of *Salmonella* in 6 to 29 out of the 42 environmental samples from each of the two low- and five high-dose inoculation experiments (Fig. 4a). There was no indication of a relationship between the inoculum dose and number of positive environmental samples.

The overall numbers of times *Salmonella* was isolated from each of the seven locations (1, water cup; 2, rear end of paddock; 3, intersection of hut/water cup; 4, defecation area; 5, feeding area; 6, hut; 7, mud hole) in the paddocks were 34, 9, 35, 36, 31, 39, and 39, respectively. Location 2, in the rear end of the paddock opposite the hut (9 positive samples), had significantly fewer *Salmonella*-positive samples ($P < 0.0001$) than the other locations (mean, 35). This was apparently not due to a lower level of fecal contamination at this more distant location, based on enumeration of presumptive *E. coli* organisms serving as feces indicator. An approximation to the development over time by a second-order polynomial did not detect abnormal behavior at location 2 (results not shown). No *E. coli* organisms were detected in the paddock environment before introduction of the pigs. Four weeks after introduction of pigs, the levels of *E. coli* in the environmental samples had increased to and remained at a range of approx-

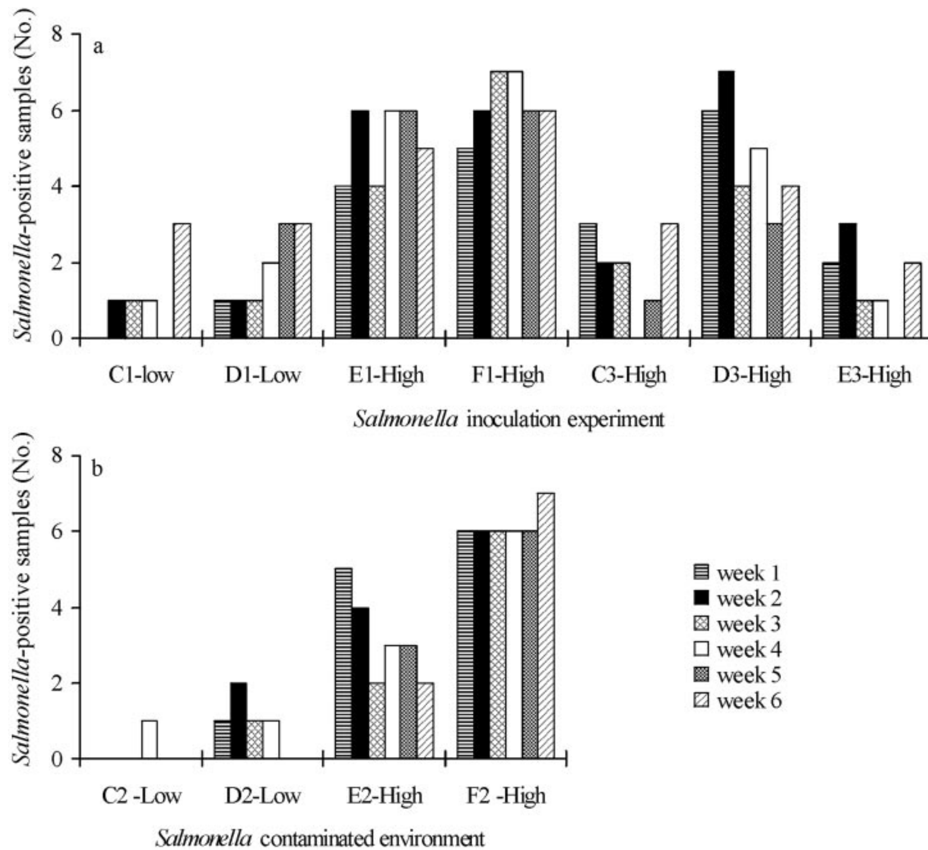

FIG. 4. (a) *Salmonella* serovar Typhimurium culture-positive environmental samples found during 6 weeks after inoculation of pigs with a low or high dose of *Salmonella* serovar Typhimurium cells in paddocks C to F in period one or three. (b) Survival of *Salmonella* serovar Typhimurium in the environment of paddocks C to F in the second experimental period.

imately 3 to 7 $\log_{10}$ CFU g^{-1} soil, independent of the sample location (data not shown).

After removal of the pigs, the inoculum strain was isolated a total of 11 times ($n = 196$) from the paddock environment within 5 weeks, and two of the huts were contaminated until the examinations were terminated after 7 weeks. The decline in *E. coli* numbers within these 7 weeks was found to be an approximate log-linear reduction, with a T_{90} of 3.4 for all locations and a T_{90} of 8.4 for the hut (data not shown). Via an extrapolation of this log-linear reduction, a level of 1 CFU was found to be reached after 19 weeks, on average, for the seven locations in the paddock and after 48 weeks in the huts.

Infectivity of the contaminated paddocks. New tracer pigs ($n = 40$) were introduced into the paddocks (C to F) at 1 day after the pigs from the first period were removed to ensure the highest possible level of *Salmonella* organisms remaining in the environment. *Salmonella* infections were almost limited to tracer pigs in paddock F (high dose), where all 10 pigs excreted *Salmonella* at least once during the 6 weeks and 46% of the total number of fecal samples ($n = 56$) were *Salmonella* positive (Fig. 1b). Two of five pigs (F) with an excretion level of >100 cells g^{-1} were killed after week 4 because of poor health, and postmortem examinations confirmed symptoms of salmonellosis. An additional seven pigs from the other paddocks were excluded before the end of the second period, and one of these was found to be *Salmonella* positive.

The high infection rate of the tracer pigs introduced into paddock F was in accordance with a high level of contamination in the environment, since the inoculum strain, *Salmonella* serovar Typhimurium DT12 rifampin^{res} was detected in 88.1% of the environmental samples (Fig. 4b). In the other high-dose paddock (E), *Salmonella* was found in 45.2% of the samples, but no *Salmonella* was isolated from the pigs. Interestingly, the lower environmental contamination level in paddock D (11.9%) resulted in two *Salmonella*-positive tracer pigs (Fig. 1b and 4b). However, despite the rare isolation of *Salmonella*, 56% of the tracer pigs in paddocks C to E had seroconverted in the last week (Fig. 3).

When new tracer pigs were introduced into paddock F in the third period, only two of eight pigs excreted *Salmonella* on three occasions, while three pigs seroconverted, although 61.9% of the environmental samples ($n = 42$) contained *Salmonella*.

DISCUSSION

This study aimed to assess the *Salmonella* infection dynamics in organic outdoor pigs and to determine the level of *Salmonella* contamination in the paddock environment. *Salmonella*-negative tracer pigs were grouped with pigs artificially infected with *Salmonella* or exposed to a *Salmonella*-contaminated paddock environment to allow natural acquisition of *Salmonella* infections in the tracer pigs. The artificially infected pigs were

inoculated with two different doses of *Salmonella* in an attempt to resemble low and high infection risk scenarios for the tracer pigs. The study showed that *Salmonella* infections can spread among 8- to 13-week-old organic pigs reared at low densities in outdoor facilities. Furthermore, *Salmonella* persisted in the paddock environment, and these contaminated pastures were able to cause infections in introduced tracer pigs.

Imitation of *Salmonella* infection by artificial inoculation of conventionally reared pigs with moderate doses was previously shown to resemble natural infection with respect to excretion level and antibody response (8). Nevertheless, the *Salmonella* excretion level in our pigs was low (<100 CFU g^{-1}) in 72% of the *Salmonella*-positive animals 3 days after inoculation, compared to the higher excretion levels, 2 to 10 $\log_{10}$ CFU g^{-1}, found by Baggesen et al. (8). It is unclear whether this difference was related to an effect of the organic rearing of the pigs. Another possible explanation is a difference in inoculation methods. Baggesen et al. (8) administered the bacteria in small portions of feed, while we gave a *Salmonella* solution directly to each pig with a gastric tube to control the dose given to individual animals, which may have caused higher killing of *Salmonella* due to the gastric acid barrier. Furthermore, the additional selection for nalidixic acid resistance might have influenced the infectivity of the test strain, as mutations for resistance are likely to reduce the growth rate and virulence of the bacteria. However, Björkman et al. (13) found that *Salmonella* serovar Typhimurium resistant to rifampin and nalidixic acid made compensatory mutations that restored fitness without loss of resistance.

The 26 tracer pigs (56%) found to be *Salmonella* culture positive when grouped with artificially infected pigs were unequally distributed among the seven groups, as the proportion of *Salmonella*-shedding tracer pigs ranged from 0.14 to 1.0 (Table 2). This is similar to the known within-herd prevalence for Danish conventionally reared pigs, which ranges from 0.1 to 1 with a median of 0.2 (7, 16). When nine conventionally reared pigs were grouped with inoculated pigs shedding 2.7 $\log_{10}$ CFU *Salmonella* g^{-1} feces, individual rectal swabs were *Salmonella* negative 2 days after exposure, while pooled fecal and subsequent necropsy samples were *Salmonella* positive (21). However, these results were obtained with indoor pigs and therefore are not comparable to the current study. Overall, 29 tracer pigs (63%) seroconverted within the 6-week study period; when serology data were used to determine the proportion of *Salmonella*-positive tracer pigs, there were significant differences in infection frequencies in the low- and high-risk infection scenarios with different inoculation doses. This is in contrast to the proportion of *Salmonella*-shedding tracer pigs, which seemed independent of the inoculation dose. However, the inoculation dose seemed to influence the length of time before the pigs tested *Salmonella* positive in feces (2.1 versus 5.9 weeks), and this indicates the importance of maintaining low exposure to *Salmonella* in order to limit and control infections.

The observed higher seroprevalence, compared to bacteriological *Salmonella* prevalence, can be explained partly by the low *Salmonella* excretion level, intermittent excretion (57, 58), and the low sensitivity of the culture method, previously reported to be 0.5 to 0.6 (6). In this study, a majority of the positive tracer animals (93.7%) excreted low levels of *Salmonella* (<100 CFU g^{-1}), while an intermittent excretion pattern was observed in 30% of the positive tracer pigs. Another explanation is that serology captures the history of exposure to *Salmonella* antigens, as pigs may remain seropositive for 10 weeks or more after seroconversion, which has previously been shown to occur within 6 to 37 days after infection (23, 34, 40). This also stresses the importance of obtaining pigs for infection studies with no prior exposure to infections; however, organic rearing of pigs was prioritized in this study, which makes it more difficult to ensure a *Salmonella*-free status. The delay in seroconversion was seen particularly in the second period, in which the seroprevalence of the tracer pigs continued to increase for 3 weeks after the bacteriological peak (week 3) (Fig. 3), whereas the inoculated pigs exposed directly to high levels of *Salmonella* started to serorespond within the first week (Fig. 2). The initial delay in seroconversion was probably the major reason why eight *Salmonella*-shedding tracer pigs (total, 94 tracer pigs) remained seronegative. This concurs with findings showing that the ELISA method is valuable for stating the *Salmonella* infection risk at the herd level, whereas its capacity for detection of *Salmonella* in individual animals may be limited (36, 40, 48). The reported number of *Salmonella* culture-positive pigs was based on isolations of the test strain only, but within the current study there was an unexpectedly high level of detection of *Salmonella* types other than the test strain, e.g., *Salmonella* serovars Newport, Livingstone, and Typhimurium DT41 and DT107 (Table 3), which has been reported previously (29). These *Salmonella* strains may have contributed to the seroresponse; however, *Salmonella* serovars other than Typhimurium tend to give a moderate serological response in the mix-ELISA (3, 40, 52).

The detection of different *Salmonella* serovars in both pigs and the environment, including the control paddocks (29), probably reflects the widespread occurrence of *Salmonella* in nature and the fact that outdoor pigs will be exposed thereto, but it is not clear how their presence influences the introduction of *Salmonella* in outdoor pigs. The non-test strains were detected in all paddocks except F, in which the infection rate (test strain) was high. A mixed infection was detected on one occasion in paddock C.

The frequent isolation of the *Salmonella* test strain in the environment (46%; $n = 296$) indicated the ability of *Salmonella* to survive outside the host, which has been suggested to be an adaptation to ensure passage to the next host (53). Heavy *Salmonella* serovar Typhimurium DT120 contamination of soil has also been reported for holding paddocks used to retain sheep prior to slaughter, where *Salmonella* persisted for 6 months in the soil with no *Salmonella*-reducing effect of liming, whereas plowing appeared to reduce the level of *Salmonella* contamination, presumably due to better mixture with other competitive soil bacteria (45).

The *Salmonella* organisms isolated from the environmental samples must have survived either in the external environment the entire time or via repeated passages through the pigs. *Salmonella* was detected in soil no longer than 5 weeks after the paddocks were vacated, except in some huts that remained contaminated for all 7 weeks. This underscores the need for good production hygiene to avoid persistence of *Salmonella*. *Salmonella* was detected for longer in this study than the reported survival rate of 7 to 14 days in soil amended with

naturally contaminated slurry from infected pig herds (9, 15). This further indicates a higher contamination potential when infected pigs in outdoor production systems are allowed to shed directly onto soil, compared to contamination from the spread of slurry. It also suggests that assessment of the *Salmonella* infection risk associated with contaminated slurry may not apply to outdoor pig production (12). A generally high fecal contamination level was also evident from detection of a high level of *E. coli* organisms (3 to 7 $\log_{10}$ CFU g^{-1}) in the paddock compared to the levels after normal application of pig slurry (3 $\log_{10}$ CFU g^{-1} soil) (15). Other studies have reported recovery of *Salmonella* from soil several months after inoculation, most likely due to different experimental conditions and higher inoculation doses, e.g., 5 log CFU g^{-1} (22, 28, 38, 39).

E. coli has been reported to a have a survival rate similar to *Salmonella*, thus providing a good indicator of decimation of *Salmonella* (38, 41). In contrast, Winfield and Groisman (53) found that *Salmonella* has better adaptation to nonhost survival than does *E. coli.* In the current study, the decimation time, T_{90}, of presumptive *E. coli* after vacation of the paddocks was estimated to be 3.4 weeks. A level of 1 CFU g^{-1} was estimated to be reached after 19 weeks, on average, for the seven locations and after 48 weeks for the huts. Assuming that *Salmonella* organisms are equivalent to *E. coli* with respect to survival, this indicates the possible persistence time of *Salmonella* in the paddock environment.

The physical and chemical properties of the external environment strongly influence the survival of *Salmonella*, and it can therefore be difficult to predict the duration of persistence and the associated infection risk for livestock. Alternating temperatures, variable rainfall, and other uncontrollable factors under natural conditions hamper examination of a possible seasonal effect. There have been reports showing no seasonal difference (26), longer survival in winter (41), more rapid decline of *Salmonella* after exposure to summer temperatures (47), and high summer temperatures increasing the contamination of vegetables fertilized with *Salmonella*-containing manure (38).

A potential *Salmonella* infection risk from contaminated soils could perhaps be overcome by soil treatments such as, e.g., plowing. A reduced survival of *Salmonella* in soil has been reported at depths of 5 to 50 cm, compared to subsurface (42), and after plowing of pastures contaminated by infected sheep (45) or slurry-amended fields (15). However, in other studies, plowing of slurry-amended fields did not prevent detection of *Salmonella* after 2 weeks (46), and incorporation of contaminated waste to a depth of 10 to 15 cm slowed the decline compared to surface application (26). A higher rate of elimination of bacterial pathogens in surface soil may be due to a more instable environment exposed to extreme temperatures (35, 12), desiccation (45, 59) and UV light (47). Thus, the persistence of *Salmonella* in the soil environment can be affected by the degree of infiltration into the soil column depending on, e.g., soil texture, water movements, and management (49), and soils with higher clay (38, 42) and organic matter (17) content may protect *Salmonella*. In the current study, the different contamination levels obtained in the paddocks did not permit a comparable examination of the pathogen-reducing effect of plowing.

The *Salmonella* infection risk associated with contaminated pastures was assessed by introduction of new tracer pigs in the second study period, where the first pig to contract infection could be assigned solely to the contamination carried over from the first period. A total of 12 pigs (30%) shed *Salmonella* at least once, while 24 (60%) pigs seroconverted. The presence of *Salmonella* was not necessarily predictive of the occurrence of *Salmonella* infection in pigs, as very low *Salmonella* levels gave rise to *Salmonella*-shedding pigs (paddock D; Fig. 1b and 4b) and rather high *Salmonella* levels did not (paddock E; Fig. 1b and 4b). Exposure to low levels of *Salmonella* (10^3 CFU g^{-1} material) in a preslaughter environment has previously been shown to promote *Salmonella* infection in pigs (24). Again, serology proved better for detection of the low-level infections with the test strain, as a minimum of 40% of the pigs in each group seroconverted, indicating that the pigs' immune systems were stimulated even at low contamination levels. The use of a 10 OD% cutoff in the mix-ELISA should avoid false-positive reactions, as evaluated in Danish pigs (38). However, this did not apply to Swedish pigs in a region of non-*Salmonella* endemicity (56). A dose-dependent protection against rechallenge with *Salmonella* has been found in conventionally reared pigs (55), although another study showed that pigs were receptive to reinfection when reexposed to *Salmonella* (31).

To our knowledge, the infection risk associated with *Salmonella*-contaminated pastures for pigs has not been assessed, but the infectivity of *Salmonella*-contaminated pastures has previously been assessed for calves grazing pastures spread with *Salmonella* serovar Dublin-containing slurry, leading to isolation of *Salmonella* in 1 of 12 calves (50). The different infection rates of *Salmonella* among the groups of organic pigs in this study probably reflects a variable susceptibility to *Salmonella* infections caused by a number of factors, including the general health status of the pigs and previous exposure. These variations in *Salmonella* infection rates are similar to those seen in infections of conventionally reared pigs (31). Furthermore, *Salmonella* organisms recovered from the paddock environment may have lost or attenuated virulence factors important for the ability to infect pigs (33, 37).

The results of our study showed that profound contamination of the environment with *Salmonella*, as in paddock F (88% positive environmental samples), e.g., due to pigs with symptoms of salmonellosis, tended to increase the risk of a high infection rate. However, even a low-grade *Salmonella* contamination of the environment confers a risk of infection in pigs, as was seen in paddock D in the second period. Organically reared pigs presumably benefit from a long suckling period, which is approximately 3 weeks longer than in conventional pig production. This allows the establishment of a more robust intestinal flora that renders the pig less susceptible to infection during the changes in the gut ecosystem following the shift from milk to solid feed at weaning (14). However, the organic rearing conditions and the low stocking density in the outdoor facilities do not prevent infections, probably because there is still close contact between the pigs, e.g., in the hut, and their rooting behavior is likely to pose a high risk of ingestion of *Salmonella* from the contaminated environment (20, 44). The high infection rate in paddock F points to the importance of removing pigs with clinical symptoms of salmonellosis as soon as possible, because these animals contribute significantly to the persistence of *Salmonella* and may in addition serve as vehicles for spread to other animals and the environment,

though no *Salmonella* was detected when a total of 22 rodents and 22 birds were examined for the occurrence of *Salmonella* in a small wildlife study performed in connection with this study, as described elsewhere (29). Finding of the test strain in the control paddocks and pigs on a few occasions indicated that dissemination occurs despite the preventive measures taken to avoid cross-contamination between paddocks and samples. Such reservoirs of *Salmonella* should be limited, to reduce long-term persistence (10, 46) and potential reentry into an active shedding status triggered by some favorable condition or stress, e.g., from transport to the slaughterhouse (25, 27). An active *Salmonella*-shedding status should be avoided because in particular it challenges food safety at slaughter due to fecal contamination of the carcasses (11).

It cannot be concluded from this study that organic rearing conditions serve as protection against *Salmonella* infections, as a high infection rate did occur under some circumstances. In addition, *Salmonella* organisms were able to survive in the paddock environment for several weeks, and even an estimated low level of *Salmonella* contamination was able to pose an infection risk to newly introduced animals. However, the longer-term persistence/infection risk was not assessed.

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