High diversity of *Salmonella* serotypes found in an experiment with outdoor pigs

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Abstract

Little is known about the risk of *Salmonella* infection in outdoor pig production, but seroprevalence data have indicated a higher incidence of *Salmonella* in outdoor than in conventional indoor production systems. This higher incidence may be due to an increased exposure of the animals to the surrounding environment, including contact with wildlife. In a study on the transmission of *Salmonella* to outdoor pigs an unexpected high diversity of *Salmonella* serotypes that are not normally isolated from pigs, like for instance *S*. Uganda and *S*. Goldcoast, was detected in faecal and in soil and water samples. However, in a small-scale wildlife survey to elucidate the potential source of the different *Salmonella* serotypes, the bacterium was not detected in any of a total of 22 rats, mice and shrews nor in 22 birds (mainly crow-birds; Corvidae). The unidentified source of the *Salmonella* serotypes isolated implies inadequate control possibilities and may therefore pose a problem to outdoor pig production in terms of food safety.

Additional keywords: food safety, environmental persistence, rodents, birds

Introduction

Salmonella enterica bacteria are important causal agents of human enteritis throughout the world. *Salmonella* bacteria are divided into more than 2300 distinct serotypes, with *S*. Typhimurium and *S*. Enteritidis further divided into definitive phage types (Anderson *et al.*, 1977; Popoff & Le Minor, 1997). The possible differentiation between *Salmonella* bacteria by serotype, phage type and DNA fingerprinting methods like pulse field gel electrophoresis (PFGE), helps to trace the source when a specific *Salmonella* type is detected, e.g. in the case of a human infection (Wegener & Baggesen, 1996).

Few serotypes are strictly host specific. Nevertheless, some are more common among humans, pigs, cattle, poultry and in different types of food than other ones. Although all *Salmonella* types are potential pathogens, some serotypes are less likely to cause infection than other and the serotypes may also vary with respect to robustness in terms of survival outside a host.

Pork plays a role in the food-borne transmission of *Salmonella* to humans. Normally, the major source of introduction of *Salmonella* into a pig herd is due to trade with sub-clinically infected animals. In addition, a wide variety of *Salmonella* serotypes can be introduced into pig herds through contaminated feed (Anon., 2003a). Due to a comprehensive control programme in the Danish animal feed industry, this is expected to play a minor role in Denmark (Anon., 2003b). A Danish National Surveillance programme initiated in 1995 monitors the seroprevalence of *Salmonella* in pigs (meat-juice) entering the slaughterhouses, assigning herds to different levels depending on their risk of infection (Nielsen *et al.*, 2001). A comparison of the *Salmonella*-seroprevalences in Danish organic, free-range, conventional and breeding pig herds (Wingstrand *et al.*, 1999) showed that the risk of meat juice samples being seropositive was higher for organic and free-range than for conventional herds (Odds Ratio = 1.7). The difference with conventional herds was statistically significant for the free-range herds ($P < 0.001$) but due to a limited number of samples not significant for the organic ones. Similar results were obtained in a Dutch study where the *Salmonella*-seroprevalence was statistically higher in free-range (44.6%) than in intensively housed finishers (24.5%) (Wolf *et al.*, 2001).

An important difference between conventional and the various kinds of alternative pig production systems is their requirement of access to outdoor areas. Organic and free-range pigs have contact with the external environment, which may constitute a risk of infection either through direct contact with wildlife and other non-production animals potentially hosting *Salmonella* or through a non-animal environment contaminated with *Salmonella*. Furthermore, the normal intervention strategies for eradication of *Salmonella* on farms may be difficult to apply to outdoor production systems, perhaps enhancing the level of *Salmonella*-contamination and the risk of infection.

Several investigations have demonstrated that *Salmonella* infections on conventional pig farms are able to persist for several months or even years (Baggesen *et al.*, 2000; Sandvang *et al.*, 2000; Baloda *et* al., 2001; Davies & McLaren, 2001). Even though it is difficult to differentiate persistence in pigs caused by sub-clinically infected animals from infection by contaminated environment, isolations of *Salmonella* from soil, slurry, manure and equipment indicated that a contaminated environment might constitute a risk of infection. However, whether a *Salmonella*-contaminated environment will result in infection in outdoor pigs will depend on the actual contamination level, the time and the sensitivity of the pigs.

This paper presents data on the detection of various *Salmonella* serotypes in both pigs and the pasture environment and results of a small-scale wildlife survey that was performed in search of potential sources of the different serotypes. The results form part of a study on the transmission of *Salmonella* from artificially infected to non-infected outdoor organic pigs, which will be described in a separate paper.

Materials and methods

Pastures and pigs

On three occasions 56 organic pigs were obtained at the time of weaning (7 weeks old) from a Danish organic farmer. Their average weight was 16.9 ± 4.0 kg, 12.7 ± 2.4 kg and 20.6 ± 3.9 kg, respectively. On arrival at the University Research Farm (Taastrup, Denmark) rectal faecal and blood samples were collected (zero samples) from the pigs to determine their *Salmonella* status both by microbiological culturing methods and by ELISA serology as described in Baggesen *et al*. (1999). To avoid parasite contamination of the experimental pastures the pigs were treated with fenbendazole (10 g a.i. per 56 animals; 4% premix; Panacur, Intervet Danmark A.S., Skovlunde, Denmark) administered with the feed for two days.

From late April to the beginning of September 2003 three successive experiments were carried out, each lasting for 6 weeks. In each experiment the 56 *Salmonella*-free pigs were distributed over 6 pastures, 4 with 10 pigs each, and 2 with 8 pigs each. The pastures were rectangular and measured 50 m^2 per pig. The pastures were enclosed with electric fence and spaced 2 m apart to avoid direct contact between animals. In each pasture the pigs had free access to an insulated house (straw bedding) located at one end of the pasture, to water-drinking nipples, a wallowing area and a feed dispenser (FRH-3, Domino AS, Denmark). The vegetation in the pastures was a three-year-old mixture of grasses and red and white clover.

The pigs were fed *ad libitum* with pelleted organic feed (Natur Starter 80 fi/Natur Gris 80, DLG, Nørresøby, Denmark) and pea/barley silage as roughage.

Sampling methods

Faecal samples

Rectal faecal samples from each pig were collected once a week for 6 weeks during each of the three periods, with the first sample taken four days after the pigs had been allowed to the pasture.

Environmental samples

Environmental samples from the pasture were also collected weekly and included composite soil samples consisting of 5 small sub-samples of surface soil from each of 6 distinct locations per pasture, and water samples (50 ml) that were collected from the water cup. As a rule, to avoid cross-contamination between animals, materials and samples, only disposable or disinfected equipment was used for collecting samples along with good hygiene practices. The samples were transported at ambient temperature to the laboratory and stored at 4 °C until testing the next day.

Wildlife

Rodents were trapped between and around the experimental pastures for a period of two weeks in late August 2003. For rats a total of 31 single-capture wire-mesh traps (45 × 18 × 20 cm; Medana AS, Løsning, Denmark) and for mice and voles 48 multiple-

capture wire-mesh traps (25 × 7.8 × 6.5 cm; Ugglan special mouse trap and Ugglan special lemming trap; Grahnab, Hillerstorp, Sweden) were used. The traps were placed outside the pastures and at locations in the surrounding area with indications of rodent activity. The traps were baited with organically grown whole meat or with suet balls that had been irradiated before use in order to avoid introduction of bacteria. The traps were inspected once a day and animals caught were killed with CO_2 and stored at 4 °C until examination within two days.

Different crow-birds (Corvidae) frequently foraged inside the pastures and in the surrounding area. The birds were either shot or caught with nets. Birds were killed and stored at 4 °C until examination within three days.

Bacteriological examination

All samples were examined for *Salmonella,* using conventional bacteriological culturing methods. The rodents and the birds were dissected and the whole intestinal set was removed, weighed and homogenized in a laboratory blender (2× 30 sec). Buffered peptone water (BPW) supplemented with the antibiotic Novobiocin (BPW-N; 22 μg ml^{-1}) was added in a ratio of 1:9 to the wildlife intestines (variable weight), pig faeces (5 g) and homogenized soil (25 g) samples (Jensen *et al.,* 2003). Water samples (50 ml) were vacuum-filtered (0.45 mm filter; HAWG 047 S3, Millipore, Billerica, MA, USA) after which the filters were transferred to 9 ml of BPW-N. All samples were incubated at 37 °C for 16–20 hours (pre-enrichment culture). Following enrichment, 100 ml of each enriched culture was inoculated as three drops on selective modified semi-solid rappaport-vassiliadis (MSRV) agar plates and incubated for 18–24 hours at 41.5 °C. Growth of *Salmonella* was indicated by the formation of a swarm zone around the inoculated spot. Material from these swarm zones was streak-inoculated onto brilliant-green agar (BGA) plates and incubated for 18–24 hours at 37 °C for confirmation of *Salmonella* (Jensen *et al.,* 2003).

Typical *Salmonella* colonies were identified using serotyping by means of slide agglutination and polyclonal sera (Statens Serum Institut, Copenhagen, Denmark) according to the Kauffmann-White method (Popoff & Le Minor, 1997). All isolates of *S.* Typhimurium were phage-typed according to Anderson *et al.* (1977).

Results

A total of 14 different serotypes and 6 different *S.* Typhimurium definitive phage types (DT) including groups of non-typable (NT) strains and strains with unspecific phage types (RDNC) were found on 23 and 40 occasions in pigs and environment, respectively (Table 1). The *S.* Typhimurium DT 107 was detected in two different pigs for three and two successive weeks in periods 2 and 3, respectively, and *S.* Derby was detected in the same pig twice in two consecutive weeks in period 2. The other serotypes were only detected once in the same pig throughout the experimental period. Furthermore, there appeared to be only a small overlap between the serotype strains detected in the pigs and the environment, as 11 serotypes were found in one sample only. However, *S.* Newport, *S.* Livingstone, *S.* Typhimurium DT 41 and *S.* Typhimurium DT 107

Table 1. Number of times *Salmonella* serotypes were found in pigs and in pasture environments during 3 periods[1] (1, 2, 3) in an experiment with outdoor pigs.

Salmonella serotype[2]	Pig isolates			Environment isolates		
	1	2	3	1	2	3
S. Typhimurium DT 41		2			2	
S. Typhimurium DT 107		3	3		1	
S. Typhimurium DT 109			1		1	4
S. Typhimurium DT 170					6	
S. Typhimurium NT[3]						1
S. Typhimurium DT RDNC[4]			1			1
S. Typhimurium (DT not done)					3	1
S. Agona			1			
S. Anatum					3	
S. Derby		3				
S. Goldcoast	1	1				
S. Indiana			1			
S. Livingstone		1			1	1
S. Newport		1	1		1	7
S. Ohio						2
S. Reading					1	
S. Stanley					1	
S. Uganda					2	1
3.10:-:1.5			1			
4.12:d:-			2			

[1] Period 1 = 2 May – 10 June 2003; period 2 = 12 June – 21 July 2003; period 3 = 23 July – 1 September 2003. Sampling once a week for 6 weeks during each period.

[2] DT = definitive phage type.

[3] NT = non-typable strain.

[4] RDNC = routine dilution no conformity; non-specific phage types.

occurred in the pasture environment at the same time or in the weeks after pigs had been excreting these specific serotypes.

Around the pastures a total of 22 small mammals were caught, including two Norway rats (*Rattus norvegicus*), two house mice (*Mus musculus*), nine wood mice (*Apodemus sylvaticus*), eight field voles (*Microtus agrestis*) and one common shrew (*Sorex araneus*). Two great tits (*Parus major*) and one skylark (*Alauda arvensis*) were trapped unintentially. A total of 19 crow-birds were caught, consisting of 15 jackdaws (*Corvus monedula*), two hooded crows (*Corvus corone cornix*) and two magpies (*Pica pica*). None of these animals was found to host *Salmonella*.

Discussion

Salmonella diversity

An unexpected high number of 14 different *Salmonella* serotypes was detected in this outdoor pig experiment. This is in contrast to findings in infected conventional herds, which according to the national surveillance in Denmark rarely have more than one type of *Salmonella* (Baggesen *et al.,* 1999). Moreover, in previous Danish studies on the persistence of *Salmonella* in soil amended with slurry from *Salmonella*-infected conventional pig herds, only one serotype was found in the soil and this was the same serotype and even the same clone as in the pigs for up to 20 months after the first isolation (Sandvang *et al.,* 2000; Baloda *et al.,* 2001).

Serotypes may differ with respect to their ability to invade pigs, and some types are more common than other ones. For instance, the serotypes *S.* Typhimurium, *S.* Derby, *S.* Infantis, *S.* Livingstone and *S.* Stanley are the five most common types isolated from Danish conventional pigs (Baggesen *et al.,* 1999). *S.* Infantis was never detected in our outdoor experiment and *S.* Stanley was only found in the pasture environment. Instead, *S.* Newport appeared to be the second most frequent serotype after *S.* Typhimurium in both types of sample, and since this specific serotype is within the top ten of human isolate types (Anon., 2002a) its presence may be of special concern. However, although some of the serotypes detected, like *S.* Goldcoast and *S.* Ohio, are quite rare, they still hold a food safety aspect.

Generally, in our study there was only a small overlap between the serotypes found in the pigs and those in the pasture environment: only *S.* Typhimurium, *S.* Newport and *S.* Livingstone were detected in both types of sample. This is in contrast to an American study, where it appeared typical for any given serotype of *Salmonella* to be represented in multiple ecological compartments on a farm, which also indicated that *Salmonella* was readily transmitted (Barber *et al.,* 2002). However, excretion of bacteria at levels below the detection limit or intermittent excretion could have caused a *Salmonella*-negative result when cultured, even if the bacterium was present in the pig

The detection of some serotypes in the environment only, indicates the potential of extra-intestinal survival of *Salmonella,* which has been suggested to be an adaptation to ensure passage to the next host (Winfield & Groisman, 2003). Without a host, proliferation of the bacteria is unlikely, at least under Danish weather conditions, and their numbers will decline depending on UV-light, heat and drying. For example, the dissemination time (T_{90}) of *S.* Typhimurium on grass has been shown to vary between 24 days near the ground (0–8 cm) and 18 days in the top (> 16 cm) (Schlundt, 1982). Normally, *Salmonella* detection is based on culturing methods. Culture-independent methods, however, have shown the presence of stressed but active *Salmonella* cells that failed to grow. However, since the virulence of these bacteria also seemed to be affected, their presence may be of minor concern (Lesne *et al.,* 2000).

Sources of *Salmonella*

The source of the many different serotypes detected in this experiment was not clear. Since the establishment of a *Salmonella* infection in a herd is often due to introduction of sub-clinically infected or asymptomatic carrier animals, the organic pigs were screened for *Salmonella* by both bacteriological and serological methods before initiating the experiment. The results show that the pigs were *Salmonella*-free when the experiment started. In our study some of the pigs were artificially infected with *Salmonella*, but since the inoculum used was from a specific *S*. Typhimurium DT 12 rifampicin-resistant strain, this could not explain the occurrence of the other serotypes. Moreover, although *S*. Enteritidis was present in a poultry experiment carried out elsewhere on the research farm, this serotype was not found in our samples.

Since feed serves as a potential source for the introduction of *Salmonella* into the pasture environment, feed could be suspected to be a source of the serotypes found. However, in Denmark the occurrence of *Salmonella* in compound pig feed is rare (Anon., 2002b). Furthermore, in our experiment, environmental samples were collected at specific locations including the feeding area but nothing indicated a higher occurrence of *Salmonella* at this or any other location. The feed stock was controlled according to the official control programme and additional samples were taken twice for microbiological analysis, both of which yielded a *Salmonella*-negative result.

In order to further elucidate the potential source of the different serotypes, a small-scale wildlife survey was done in the surrounding environment. The results indicate that neither birds nor rodents were serving as a high-risk reservoir of the various *Salmonella*. Likewise, the results from a Danish project indicated that non-production animals seem to play a minor role in relation to the spread of *Salmonella*-infection to conventional pig herds (M.N. Skov, personal communication). Furthermore, in a Swedish study only one *Salmonella*-positive bird was found among 2377 migratory wild birds examined (Hernandez *et al.,* 2003). On the other hand, in Norway a study by Refsum *et al*. (2002) of *Salmonella* in avian wildlife indicated a high prevalence of the bacterium particularly in passerines, but except for one case these were all serotype Typhimurium. This did not point to birds as a potential source of the various serotypes found in our study. Though flies have been shown to be able to play a role in the transmission of *Salmonella* (Barber *et al.,* 2002) we did not examine insects as a possible source. So whether they serve as an actual source is not clear.

Conclusions

This study showed an unexpected high diversity of *Salmonella* serotypes in an experiment with outdoor pigs, compared with the low diversity known from Danish conventional herds (Baggesen *et al.,* 1999). The wildlife in the surrounding environment was shown not to be the source of these serotypes, but because of the small number of samples examined its possible role cannot be ruled out. The knowledge about *Salmonella* risk in relation to outdoor pig production is still limited, but the unidentified

sources of *Salmonella* indicated that the outdoor environment is harder to control with respect to the occurrence of pathogens. However, it is still not clear how their presence influences the introduction of *Salmonella* infection in outdoor organic pigs. Nevertheless, all *Salmonella* serotypes are potential pathogens especially in case of immunodepression, and their presence therefore plays a potential role in food safety.

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