Survival and Infectivity of Salmonella Choleraesuis in Swine Feces†

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ABSTRACT

Many serotypes of Salmonella survive well in the environment. Conversely, it is believed that Salmonella Choleraesuis, the host-adapted serotype of swine, does not survive well outside the host. We examined the survival capability of Salmonella Choleraesuis in swine feces. Six pigs were infected with Salmonella Choleraesuis and feces were collected and pooled on days 2, 4, 7, and 10 postinoculation (PI). Feces were stored in a wet and a dry form, and survival was measured over 13 months. Salmonella Choleraesuis was recovered from wet feces through 3 months of storage. In a desiccated (dry) form, Salmonella Choleraesuis was recovered from at least 13 months. Salmonella Choleraesuis shed from swine prior to 4 days PI did not survive as well as that shed 4 days PI or later. We also examined the infectivity of Salmonella Choleraesuis resident in dry feces. Six- or 13-week-old pigs were inoculated with dry feces that had been stored either 2 months or 4 months, respectively. Pigs were inoculated either intranasally or by mixing dry feces with the swine ration. Although clinical signs were mild, Salmonella Choleraesuis was widely disseminated among the tissues of all the pigs inoculated. This study demonstrates that Salmonella Choleraesuis remains viable and infective in the environment. Therefore, contaminated fecal matter can serve as a reservoir for Salmonella Choleraesuis as well as other Salmonella spp. Control measures must consider this environmental reservoir as a source of new infections.

Salmonella Choleraesuis is a host-adapted, facultative intracellular pathogen that causes swine paratyphoid (16, 25). It is the most frequent Salmonella serotype recovered from swine feces (6) and was isolated from >95% of swine salmonellosis outbreaks in Iowa in 1989. The National Animal Health Monitoring system estimated that porcine salmonellosis was responsible for annual production losses of 28 million dollars in Iowa and 100 million dollars in annual losses nationwide (19).

Although Salmonella Choleraesuis is the serotype most frequently isolated from swine, it is rarely isolated from swine feed or nonporcine Salmonella reservoirs such as the environment or other domestic and wild animal species (2, 10). The source of Salmonella Choleraesuis seems to be limited to carrier pigs and facilities previously contaminated with this serotype (11, 12, 25). Although several studies have demonstrated the ability of other Salmonella serotypes to survive in the environment, Salmonella Choleraesuis is believed to be inactivated rapidly. In soil, Salmonella Dublin has been shown to survive for up to 12 weeks (20) and Salmonella Typhimurium has been shown to survive for 18 weeks (21). Other studies have shown Salmonella spp. to survive for many years in cattle feces (15), in chicken fluff (14), and floor dust (18). A number of these studies examined Salmonella survival after deliberately infecting the environment with Salmonella grown in vitro. While many studies document long-term Salmonella survival, there are little or no data regarding the infectivity of Salmonella spp. after storage.

Salmonella Choleraesuis is rarely isolated during epidemiological studies of both swine and the swine environment. This creates a paradox between the ability of Salmonella Choleraesuis to survive in the environment and the relative frequency of Salmonella Choleraesuis infections. The purpose of this study was to evaluate the ability of Salmonella Choleraesuis to survive in the environment and remain infective after being shed from infected swine.

MATERIALS AND METHODS

Bacterial strains and fecal survival experiment. A group of six 8-week-old pigs were challenged with wild-type Salmonella Choleraesuis var. kunzendorf 3246pp (7) as previously described (8). The strain has a naturally acquired resistance to streptomycin (13). On days 2, 4, 7, and 10 postinoculation (PI), approximately 9 kg of feces shed over the previous 24 h from all of the infected swine were collected and mixed thoroughly. One-half of the feces was placed into a polypropylene bucket (30.5 cm deep, 25.4 cm diameter) to a depth of approximately 12.7 cm. A sterile, nylon mesh screen was placed over the top of the bucket, 500 ml of sterile water was added every 7 days, and this sample was designated as wet. The other half of the feces was spread evenly in a metal pan (6.35 cm deep, 25.4 cm wide, 43.2 length) to a depth of 5.0 cm. These samples were left open to the air and allowed to desiccate naturally. All samples were stored in an environmentally controlled, biologically secure, empty animal housing facility.

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throughout the experiment. Room temperature (26°C) was maintained throughout the period. Each of the wet and desiccated samples were cultured qualitatively and quantitatively for *Salmonella Choleraesuis* on days 0, 3, 14, and 28 and at 2, 3, 4, and 6 months using methods previously described (3). The dry samples were also cultured at 9 and 13 months.

**Infectivity experiments.** After farrowing, sows were screened as previously described (3) and *Salmonella* spp. was not recovered. At 2 weeks of age, pigs were weaned and transported to isolation facilities as previously described (3). Pigs were housed in a climate-controlled, fully enclosed building on concrete floors. Pigs were housed in isolation facilities and allowed to acclimate for 1 week. These pigs were also cultured and confirmed to be negative for *Salmonella* spp. as previously described (3). After removing feces manually, the pen was washed with water once daily throughout the experiment. Feces from the long-term survival experiment that had been stored dry for 2 months were either ground into a powder using a sterile mortar and pestle for intranasal inoculation or broken into small pieces for the feed inoculation. At 13 weeks of age (day 0) eight pigs were divided into two groups of four pigs. Four pigs were briefly anesthetized with a cocktail of xylazine, ketamine, and telazol and subsequently inoculated intranasally with 1 g (0.5 g in each nostril on inspiration, alternating nostrils) of *Salmonella Choleraesuis* 3246pp infected, dry feces. The concentration of *Salmonella Choleraesuis* in the ground feces was $2.3 \times 10^8$ CFU/g. The second group of four pigs was allowed to eat feed that had been mixed with the dry, infected feces. Each of these pigs received approximately 5 g of feces mixed with 227 g of raton resulting in an approximate challenge of $1 \times 10^9$ CFU/g.

Tonsil (T), nasal (N), and rectal (R) swabs were obtained on all pigs at 3 and 6 h PI and on days 1 through 7 PI. Individual fecal (FF) samples were collected (10- to 20-g samples) from pigs on days 1 through 7. Each swab and fecal sample was qualitatively cultured. Fecal pools for each group were obtained by combining approximately 2 g of feces from each pig per group. The *Salmonella Choleraesuis* environmental load was measured by combining all available feces, mixing well, and taking a 20- to 30-g sample. Fecal pools and environmental samples were qualitatively cultured as described previously (8).

All pigs were euthanized and necropsied on day 7 PI. Tissues were collected aseptically for bacteriologic examination (sterile gloves and instruments were used for each tissue). Tissues collected include the turbinates (1 g; approximate weight), tonsils (4 g), lungs (8 g), spleen (5 g), liver (5 g), middle ileum (11-mid; 4 g), ileocolic junction (ICJ; 6 g), ileocolic lymph node (ICLN; 3 g), cecum (4 g), colon (4 g), and colonic lymph node (CLN; 1 g); and cecal contents (cc cont, 25 g).

A second infectivity experiment was performed that had the following differences from the first trial: age of pigs (6 weeks for trial 1 versus 13 weeks for trial 1), length of time feces had been stored (4 months storage in trial 2, 2 months storage in trial 1), final challenge dose of *Salmonella Choleraesuis* in infected feces (trial 2 intranasal dose = $1.15 \times 10^6$ CFU and trial 2 oral feed dose = $5.6 \times 10^5$ CFU). Sampling was performed as described above, and in addition, quantitative bacteriology was performed on the ICLN and ICJ tissues from trial 2.

**Clinical signs.** Rectal temperatures and clinical signs were monitored twice daily until day 3 PI for pigs in all groups. Between days 4 and 7 PI, pigs were monitored once daily.

**Bacteriologic examinations.** Fecal samples, stored feces, and tissues were processed as previously described (9). All samples were incubated at 37°C in GN-Hajna (Difco, Detroit, Mich.) broth for 18 to 24 h then streaked on brilliant green agar with sulfadiazine (Microdiagnostics, Lombard, Ill.). Additionally, at 18 to 24 h, 100 μl of the GN-Hajna broth was transferred to Rappaport-Vassiliadis medium (23), incubated at 37°C for 18 h, then streaked on brilliant green agar with sulfadiazine. All brilliant green agar with sulfadiazine plates were incubated 24 h at 37°C. To facilitate recovery and identification of the experimental strain, streptomycin sulfadate (200 μg/ml; Sigma, St. Louis, Mo.) was added to the media for all specimens for trials 1 and 2.

Colonies having the typical appearance of *Salmonella* were picked and inoculated into triple sugar iron and lysine iron agar slants. Positive isolates were confirmed as serogroup C1 by agglutination with *Salmonella* antiserum group C1 (Difco). Representative isolates were serotyped at the National Veterinary Services Laboratory (Ames, Iowa). Samples of up to 10 g were used for qualitative bacteriology. Quantitative bacteriology was conducted using the five-tube most probable number method (27) with GN-Hajna, brilliant green agar with sulfadiazine, and Rappaport-Vassiliadis media as described previously (7), and results are reported as the mean of the quantitative levels for a respective group for each necropsy.

### RESULTS

*Salmonella Choleraesuis* survival in wet feces. The qualitative and quantitative bacteriologic results are presented for the wet feces in Table 1. Qualitative bacteriologic results indicated that *Salmonella Choleraesuis* survived in a culturable state in wet swine feces for at least 3 months after being shed from infected animals (Table 1). Quantitative bacteriology indicated that during the first 3 days of storage in the wet state the numbers of *Salmonella Choleraesuis* declined between 0.77 and 0.10 log$_{10}$ CFU/g. During the first 3 days of wet storage, the largest decline was noted in feces collected on day 2 PI, which equaled 0.77 log$_{10}$ CFU. For the same time period *Salmonella Choleraesuis* shed on days 4, 7 and 10, declined in concentration by 0.1, 0.15, and 0.49 log$_{10}$ CFU/g of feces, respectively. In wet feces *Salmonella Choleraesuis* was recovered for 3 months in feces shed on days 2 and 7 PI. Feces shed on day 10 PI retained quantifiable levels (1.2 log$_{10}$) of *Salmonella Choleraesuis* for 2 months.

**TABLE 1. Survival of *Salmonella Choleraesuis* in wet feces from infected swine over time**

<table>
<thead>
<tr>
<th>Time in storage</th>
<th>Log$_{10}$ CFU of <em>Salmonella Choleraesuis</em>/g of feces recovered PI</th>
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<tbody>
<tr>
<td>Day 2</td>
<td>2.46</td>
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<tr>
<td>Day 4</td>
<td>3.96</td>
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<tr>
<td>Day 7</td>
<td>2.45</td>
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<tr>
<td>Day 10</td>
<td>3.09</td>
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</tbody>
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$^a$ –, negative.

$^b$ +, positive by qualitative bacteriology only.
Survival and infectivity of Salmonella Choleraesuis

TABLE 2. Survival of Salmonella Choleraesuis in dry feces from infected swine over time

<table>
<thead>
<tr>
<th>Time in storage</th>
<th>Log$_{10}$ CFU of Salmonella Choleraesuis/g of feces recovered PI</th>
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</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>2.46</td>
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<td>3.69</td>
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<tr>
<td>Day 10</td>
<td>3.09</td>
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$^a$ +, positive by qualitative bacteriology only.
$^b$ -, negative.

Salmonella Choleraesuis survival in dry feces. The survival of Salmonella Choleraesuis in feces shed from infected swine and allowed to desiccate was more prolonged when compared to the wet feces (Table 2). Qualitative bacteriology indicated Salmonella Choleraesuis survived for at least 13 months in the dry fecal environment. As observed for the wet feces, a similar decline in numbers of Salmonella Choleraesuis occurred during the first 3 days of storage for each of the four collection time points. Conversely, quantitative bacteriology indicated an apparent increase in Salmonella Choleraesuis numbers from feces collected on day 4 and day 7. In reality, these numbers likely reflect a modest decrease in Salmonella Choleraesuis numbers and a loss of water weight in the desiccating feces. Gross appearance indicated the samples were completely dry after 2 weeks of storage. While a decline in numbers over time in storage was observed in wet and dry feces, Salmonella Choleraesuis survival appeared to be affected by time in host. Feces recovered at day 10 PI retained a larger number of viable bacteria than feces collected at any other time.

Clinical signs. No overt clinical signs were observed for pigs exposed to infected feces in either trial. A marked febrile response was recorded for the pigs exposed by the intranasal route in both trials 1 and 2. Peak rectal temperature was recorded on day 3 PI and was 41.2 and 40.8°C for trials 1 and 2, respectively.

Antemortem bacteriologic results. All source sows from which pigs were obtained were culture negative for Salmonella spp. All pigs were also culture negative for Salmonella sp. prior to challenge. After challenge, individual pigs were detected to be shedding Salmonella Choleraesuis with the following samples and time points PI: after intranasal inoculation 3 h (1R, 1T); 6 h (1T); D5 (1R); D6 (2T, 1N, 2R, 3IF); and after oral inoculation 6 h (1T). Qualitative fecal pool cultures after inoculation indicated on day 3 PI of trial 1 the pigs inoculated intranasally were shedding 0.41 log$_{10}$ CFU/g of feces. All other samples taken were negative for Salmonella.

Postmortem bacteriologic examination. The frequencies of Salmonella Choleraesuis recovery from tissue sample are presented in Table 3 for trials 1 and 2. Every animal that was challenged with the Salmonella Choleraesuis-infected feces has between 2 and 10 tissues positive for Salmonella Choleraesuis. Regardless of the route of inoculation or the age of the animal infected, the tissues that were most often positive for Salmonella Choleraesuis were the ICLN (15 of 16), cec cont (14 of 16), ICJ (12 of 16), and I1-mid (12 of 16). However, the intranasal route of exposure resulted in more extensive infection (73% for trial 1 and 50% for trial 2) when compared to the oral route (48% for trial 1 and 34% for trial 2).

Quantitative bacteriology on the ICI and ICLN obtained from pigs in trial 2 indicated mean populations ranging from 1.81 to 2.88 log$_{10}$ CFU/g of tissue and is presented in Table 4.

DISCUSSION

We evaluated the ability of Salmonella Choleraesuis that was shed from infected animals to survive after storage in a wet environment or a dry environment. These conditions may loosely replicate the fecal slurry that exits under and around many swine confinement facilities and the dry fecal matter that exists ubiquitously in and around livestock facilities.

The results of this study demonstrate that Salmonella...
Choleraesuis can survive for extended periods of time in swine feces in the environment and remains infective to naive animals in this state. Specifically, Salmonella Choleraesuis can survive for at least 3 months in a wet fecal slurry after being shed in feces from infected swine. If the same fecal material is allowed to desiccate, Salmonella Choleraesuis can survive for at least 13 months after being shed. Additionally, Salmonella Choleraesuis that has been desiccated in dry fecal material for either 2 or 4 months was found to be infectious for naive swine exposed to the fecal material either intranasally or per os.

The data collected indicate that Salmonella Choleraesuis survives relatively well in the wet environment. As would be expected in the wet fecal slurry, many mesophilic organisms were grossly evident after several weeks of storage. Conversely, the Salmonella Choleraesuis showed no evidence of replication in the slurry after being shed from the animal. For 3 days after excretion into the environment, Salmonella Choleraesuis numbers declined slowly followed by a more rapid decline until the 4th week of storage. After 4 weeks, a small population remained viable and culturable at roughly the same level for 3 months, after which no Salmonella Choleraesuis was detectable. Other Salmonella spp. have been shown to survive in a moist waste environment for short periods of time such as Salmonella Dublin survival in a cattle fecal slurry for 8 days to 12 weeks (20) or for 5 days in cattle urine (15). Alternatively, Salmonella spp. have been shown to survive in a culturable state for at least 5 weeks in aquatic environments (1) with increasing survival following removal of the competing microorganisms (17).

As noted with other Salmonella spp. (14, 15, 18, 20, 21), the survival of Salmonella Choleraesuis in a dry environment was found to be much greater. Under the environmental conditions used, the excreted fecal matter had dried to a state that could be ground into a powder after approximately 2 weeks. Due to water evaporation, comparisons of numbers of cells/g of feces prior to 2 weeks of storage and after 2 weeks of storage is difficult. The loss of water weight may have also contributed to relatively slight changes in Salmonella Choleraesuis numbers in the first 3 days of storage. However, in all but the day 10 sample large decreases of Salmonella Choleraesuis occurred between days 3 and 14 of storage. After day 14, a small population of Salmonella Choleraesuis existed in small pockets of surviving cells within the sample. This may indicate that the overall numbers of Salmonella Choleraesuis surviving in environmental feces may be low but it may survive in a relatively concentrated state in microcolonies.

Although numerous studies have documented survival of various Salmonella spp., very few have explored the likelihood of transmission of the organisms by testing their infectivity. In one study (20) a fecal slurry spiked with $10^6$ CFU/ml of Salmonella Dublin was spread on pasture ground and allowed to settle 18 h before cattle were allowed to graze. This resulted in fecal shedding, indicating that Salmonella Dublin remains infective for 18 h in the environment. However, long-term maintenance and infectivity of the pathogen in the environment remains inconclusive especially with serotypes such as Salmonella Choleraesuis. We found that feces, shed by animals infected with Salmonella Choleraesuis, remained infective after 2 or 4 months of storage in a dry state. The age of the swine exposed to the dry feces was not a factor as either 6- or 13-week-old pigs were equally susceptible. The numbers of organisms recovered were also approximately the same for both age groups, indicating no notable differences in colonization levels. Pigs exposed to the feces by the intranasal route were the same or slightly more likely to be positive for Salmonella Choleraesuis with similar levels of Salmonella Choleraesuis in the deep tissues cultured when compared to those exposed orally. This is consistent with other studies (4, 7) and may be due to the more rapid access to the lymphoid tissue of the upper respiratory tract shown to be important to Salmonella infections (4).

The dose that the infected swine were given varied by groups from $10^4$ to $10^6$ CFU. In controlled dose titration studies with organisms grown in vitro it has been shown that Salmonella Choleraesuis colonizes and persists for several weeks but results in only mild, transient clinical signs at a dose of $10^6$ CFU (9). At a dose of $10^3$ CFU little or no colonization with Salmonella Choleraesuis is observed. Conversely, studies on natural transmission have demonstrated colonization and clinical signs with Salmonella Choleraesuis (8) and Salmonella Typhimurium (5) in swine commingled with infected animals shedding as little as 2.61 log CFU/g of feces (5, 8). It is difficult to compare the actual dose received by each animal in the present experiment to other studies due to the inconsistent nature of intake associated with this type of exposure.

In the present experiment, relatively low doses of Salmonella Choleraesuis resulted in colonization after storage in a dry form for several months. The number of deep tissues positive for Salmonella Choleraesuis indicates that the invasive nature of this pathogen remains unchanged even after extended storage. In addition, the infection reaches the normal levels expected in the ICJ, a common site of colonization for Salmonella spp. infections (4, 7, 26). No overt clinical signs were observed in any of the exposed animals in this experiment, and while this is consistent with other studies, it does suggest that cofactors may be important for the development of clinical disease.

Many reports have been published regarding the ability of Salmonella (22) and other pathogens such as Vibrio (24) to enter into a viable but nonculturable state. Chmielewski and Frank (1) found that the entry of Salmonella into the viable but nonculturable state in the aquatic environment is enhanced by lower temperatures and/or lower phosphate buffer concentrations. It is possible that in this as well as other reports of Salmonella spp. long-term survival in the environment, that some of the reduction of cell numbers may be due to entry into the viable but nonculturable state. It is unclear under what circumstances these cells would remain infective in an animal host and could contribute to infection. In other studies, Turpin et al. (22) found the survival of Salmonella to be greater in sterile versus nonsterile soil due to the competition with other bacteria or microorganisms. It seems therefore, that Salmonella, which is
Aerosols generated during cleaning of premises have the potential to spread Choleraesuis remains infective for susceptible animals for several months after desiccation in feces. Therefore, the control of all Choleraesuis demonstrated here likely explains some of the new outbreaks that have been observed in apparently healthy, uninfected animals. In addition, we have provided direct evidence that Salmonella Choleraesuis remains infective for susceptible animals for several months after desiccation in feces. Therefore, the control of all Salmonella spp. in the environment must include removal of all organic matter followed by thorough disinfection.

Intranasal exposure of Salmonella spp. can be an important means of infection in swine (4, 7). The infections induced by this route can be severe (4, 7), and the organism can rapidly disseminate throughout the infected host (4). Aerosols generated during cleaning of premises have the potential to spread Salmonella spp. further to naive animals, therefore animal and human aerosol exposure should be avoided. Because of the long-term environmental survival and infectivity of Salmonella Choleraesuis, shedding of the organism by infected animals can result in long-term environmental contamination and continual reinfection of newly introduced animals.

REFERENCES