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A case-control study on the occurrence of *Salmonella* spp. in the environment of pigs

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SUMMARY

The objective of this study was to compare the occurrence of *Salmonella* spp. found in the animal environment in pig herds with different *Salmonella* risks (61 herds with low seroprevalence, 81 herds with high seroprevalence) on a broad scale. The environmental samples were divided into two types: direct ($n=1105$) and indirect ($n=1220$) environmental samples. All samples were tested for *Salmonella* spp. via real-time polymerase chain reaction. Most of the indirect environments were more often *Salmonella*-positive in the high-seroprevalence herds than in the low-seroprevalence herds; significantly higher were compartment aisles [odds ratio (OR) 3·45, 95% confidence interval (CI) 1·61–7·41], driving boards (OR 3·06, 95% CI 1·38–6·92) and the central aisle of the barn (OR 3·03, 95% CI 1·35–6·83). The overall results show that especially areas in the indirect environment are the major, but mostly underestimated causes of residual *Salmonella*.

Key words: *Salmonella enterica*, veterinary epidemiology, zoonoses.

INTRODUCTION

With the total reconstruction of the European Food Law beginning in 2001 and other corresponding laws following in 2003, German farmers and veterinarians alike had to consider, for the first time, the concepts of food safety on the farm itself. Help came from the German QS System, a company dedicated to creating a 'farm-to-fork' monitoring system for animal-derived as well as other agricultural products [1]. In this monitoring system, herds are divided into three categories:

Category I: less than 20% of all samples taken are *Salmonella* spp. antibody positive.
Category II: 20–40% of all samples taken are *Salmonella* spp. antibody positive.
Category III: more than 40% of all samples taken are *Salmonella* spp. antibody positive [i.e. >40% optical density (OD)].

As a rule, 60 slaughtered pigs per herd each year are sampled, and the sampling must be spread out over a 12-month period [1]. The samples for this serological monitoring are either meat juice samples taken at slaughter or blood serum samples taken not earlier than 14 days prior to slaughter. Either kind of sample

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is then analysed via enzyme-linked immunosorbent assay (ELISA). It is a requirement to find the cause of the *Salmonella* problem for herds in category III and implement measures against it [1]. This is not a requirement for herds in category II, but farmers are encouraged to do so [1, 2]. In 2007 the German government made participation in the described *Salmonella* monitoring system mandatory for all farmers supplying slaughter pigs.

Although many risk factors, such as more than three supplier herds [3], rodent infestation [4] and contaminated feed [5] have been discussed for a long time and many reports on studies into these risk factors have been frequently published [6], there is still a great deal of uncertainty in the field about how to overcome the problem. This is in marked contrast to the situation found in layer hens, were the critical points of residual *Salmonella* contamination are well known and the strategy of prevention is well defined [7–9].

In the pig industry, however, the veterinary practitioners and their clients often feel lost in a multitude of suggestions and options. Frustration runs high, when, as in many cases, herds are categorized into category III although many known measures against *Salmonella* infections, e.g. a strict all-in/all-out management [10, 11], acidifying of feed and/or coarsely ground feed [12] and correct external biosecurity measures [13], are already in place. Further complications arise when the farmer does not fully understand what the risk categorization of his herd signifies. 'My animals aren't ill, why is their meat a risk for human consumption?' is a question often encountered by veterinary practitioners. The frustration is regularly exacerbated by the fact that even the experts themselves rarely agree on which management or hygienic factors are key to a successful prevention of *Salmonella* infections in pigs [14].

Keeping this frustration of the veterinarians and farmers in mind, a hypothesis was postulated: the usually discussed critical points in the animal environment such as ventilation [4], floors and feeders [15] are apparently not the only points of *Salmonella* contamination. Several other points, for instance the driving boards, central aisles of the barn and the compartment aisles, are possibly major 'retreat' areas for residual *Salmonella* as well.

This hypothesis also exemplifies one of the unique points of our study, because the mentioned areas have not (to the best of our knowledge) been the focus of previous research in the pig industry.

Table 1. *Herd types by category and county*

	Category I		Category III	
County	F	F–F	F	F–F
Emsland	12	7	23	10
Cloppenburg	10	0	6	5
Diepholz	9	5	4	0
Grafschaft Bentheim	7	1	1	0
Vechta	6	1	12	5
Osnabrück	3	0	8	4
Oldenburg	0	0	0	1
Aurich	0	0	0	2
Total	47	14	54	27

F, Finisher herds; F–F, farrow-to-finisher herds.

Another unique point, from the German perspective, is that due to the new monitoring system, it was possible for the first time to differentiate between herds of high and low seroprevalence and compare them on a large scale.

The objectives for this study were therefore the following:

(1) To utilize the possibilities of the monitoring system by including a large number of herds in the study.
(2) To confirm or refute the hypothesis that there are critical points for residual *Salmonella* which have been previously underestimated.

MATERIAL AND METHODS

Study farms

The farms examined in the study are situated in the northwestern part of Germany with the highest pig density within Europe (spread over eight counties). All farms participated voluntarily and were chosen because of their serological classification and geographical distribution. In total 142 herds with 61 herds in category I and 81 herds in category III participated. The herds were either just finishing or farrow-to-finishing units. The names of the counties in which the herds were located are listed in Table 1. Beyond the clear risk categorization in one of the previously mentioned categories and the locations of the herds in Lower Saxony, no other requirements were made. The study period lasted from July 2007 to December 2009. During this period, each herd was visited at least once to obtain the samples. All herds were visited by the same examiner.

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Sampling

To determine the major 'retreat areas' of residual *Salmonella*, the animals' environment was divided into 'direct' and 'indirect' environments. Samples of the direct environment were defined as having been taken from objects to which the pigs were able to have continuous or repeated physical contact: pen floors, pen walls, walls, feeders and troughs, drinking nipples (only those outside the feeders), and toys. The walls were only sampled up to the level of the back height of the animals. Samples of the indirect environment were defined as having been taken from areas with which the pigs have no physical contact such as anterooms, ceilings, gas heaters, pipes, ventilation (fans), or very rare physical contact such as the compartment aisles, driving boards, the central aisle of the barn, boots/shoes of the farmer, animal scales, loading ramps, transporters and other miscellaneous objects.

The number of samples taken per herd was 14–20. If possible, samples were taken from three separate places:

(1) A cleaned and disinfected compartment.
(2) A compartment in which the youngest animals of the herd were housed (excluding suckling piglets).
(3) A compartment in which the oldest animals of the herd were housed (excluding sows).

Pig toys, central aisles, boots and shoes, anterooms, gas heaters, ventilation, ceilings, animal scales and transporters could not be sampled in every herd, either because the object was not present or it was out of reach of the examiner.

The environmental samples were taken via swabs. A swab is a 10×30 cm gauze tissue (tg® Size 7, Lohmann & Rauscher International GmbH Germany) drenched, i.e. thoroughly wet but not dripping, in buffered peptone water (BPW; Oxoid Ltd, UK) and sterilized in a sealed heat-resistant plastic envelope. These swabs were prepared by the examiner 2–3 days prior to the investigation of a farm. The swabs were stored at 7 °C until required.

All objects with a small surface (pig toys, drinking nipples, driving boards) were swabbed entirely. Other objects with areas too large to be completely swabbed (central aisles, anterooms, ceilings, compartment aisles, animal scales, loading ramps, transporters), were swabbed in 3–5 locations of ~ 1 m² depending on the overall size. From the feeders, pig toys and drinking nipples at least 50% of those present in the compartment were swabbed with one swab, since it was not important to find out which specific feeder or toy was contaminated, only the type of environment which was contaminated. On the day of sampling, all samples were transported in cooling boxes to the laboratory and were cultured in BPW at 37 °C for 18 ± 1 h.

Testing

Each sample was tested by a standardized real-time polymerase chain reaction (PCR) protocol. The PCR was performed with the TaqMan® Salmonella Detection kit (Applied Biosystems, USA) according to the manufacturer's instructions. Reactions were performed using a real-time PCR System 7500 and the results were analysed with SDS software package v. 1.4 (all Applied Biosystems, USA). The laboratory personnel conducting the analyses were blinded with respect to the category and the type of sample. A sample was categorized as positive if the crossing point value was less than 45 cycles. Due to the fact that the samples were incubated before analysis, the results in the tables are only given as positive or negative and not as a quantitative result.

Data handling

The odds ratios (OR) of the cumulative results were calculated with WinEpiscope 2.0® (CLIVE, UK); for small sample sizes Fisher's exact test was used, these values were calculated with SAS v. 9.0 (SAS Institute, USA). The confidence interval (CI) level was set at 95% and the cut-off of the P value was set at 0·05.

RESULTS

Because all herds participated voluntarily in the study, a matching of herds, although initially attempted, was not possible.

Although samples were, when possible, taken from three separate locations in the herd (a cleaned and disinfected compartment, a compartment with the youngest animals of the herd and a compartment of the oldest animals of the herd), the results were only calculated in two groups, i.e. before cleaning and disinfection (C + D) and after. This was because there was too much missing information regarding the age of the pigs on the day of sampling, so that satisfactory subgroups could not be established. Because of time constraints, it was not possible to sample an empty compartment prior to C + D and then sample it again afterwards.

Table 2. *Results of the direct environment samples*

Sample	No.	Category III	Category I	OR	95% CI
Pen floors	361	27·6%	9·3%	3·69	1·82–7·48
Feeders/ troughs	182	28·8%	21·8%	1·46	0·73–2·89
Pig toys	181	25·6%	15·4%	1·89	0·90–3·96
Pen walls	156	32·5%	20·5%	1·86	0·90–3·87
Drinking nipples	127	31·8%	18·0%	2·12	0·92–4·88
Walls	98	21·4%	10·7%	2·27	0·74–6·98
Total	1105	28·2%	15·7%	—	—

OR, Odds ratio; CI, confidence interval.

The percentage of positive samples from the total sample size before C+D ($n=2325$) was 22·97%. Differentiating between direct ($n=1105$) and indirect ($n=1220$) environments showed little difference between the groups (22·90% *vs.* 23·03% respectively). Of the samples taken after C+D ($n=222$) 17·57% were positive.

Table 2 shows the results of samples of the direct environment. Listed are the different kinds of samples with their respective total amounts, the percentage of positive samples within each category (as a percentage of the amount of samples in that particular category, not the total), as well as the OR and 95% CI. Of the direct environment, the only significant difference between the categories was found between the samples from the pen floor (OR 3·69, 95% CI 1·82–7·48).

Parts of the results from the indirect environment are illustrated in Table 3*a*. They are given in the same manner as the results in Table 2. In this case, three significant differences could be found in the samples: they were the compartment aisles (OR 3·45, 95% CI 1·61–7·41), driving boards (OR 3·06, 95% CI 1·34–6·92) and central aisles of the barn (OR 3·03, 95% CI 1·35–6·83).

As in Tables 2 and 3*a*, Table 3*b* shows the different kinds of samples from the direct and indirect environments with their respective total amounts, and the percentage of positive samples within each category (again as a percentage of the amount of samples in that particular category, not the total). However, due to very small sample sizes Fisher's exact test had to be used to calculate a two-sided P value (cut-off 0·05) for the ceilings, gas heaters, animal scales, loading ramps, transporters and other miscellaneous objects. The P value for the ceilings was 0·04, the others were not statistically significant.

Table 4 lists the results of the sampling after C+D; these samples are not included in the total of the samples in Table 2. Because of the small sample sizes, only an overall OR and CI between the categories was calculated (OR 2·94, 95% CI 1·38–6·25). No further calculations were made and the values given in the table are the total of the samples, the number of positives within each category and the percentage of positive samples with respect to the number of samples taken in that category.

DISCUSSION

Although we were aware of a recruiting bias, since voluntary participating herds were more likely to be aware of the nature of the problem and may have already implemented measures against it, it was impossible to exclude this bias, due to the nature of voluntary recruiting *per se*. In reference to the first objective, the utilization of the possibilities of the monitoring system, success was partial. While it was simple to assign the fairly large number of recruited herds ($n=142$) to one of the two study categories, we were unable to determine two further points of interest, as official data was unavailable:

(1) Is the distribution of the herds as recruited with respect to *category* representative of the true distribution in each county?
(2) Is the distribution of the herds as recruited with respect to *type of farm* representative of the true distribution in each county?

Knowledge of these points would considerably increase the value of the statistical results as well as the conclusions that could be drawn from them.

Sampling bias was excluded as far as possible by having a fixed pattern of where and how the sampling was executed. If possible a cleaned and disinfected compartment, a compartment with the youngest animals of the herd and a compartment of the oldest animals of the herd was sampled. Objects with a clearly defined small surface were swabbed entirely; the others were swabbed in 3–5 locations of $\sim 1\ m^2$ depending on the overall size. Naturally, even this approach has its faults: for example, a central aisle (20 m long × 5 m wide) was found to be 'negative', simply because the 'correct' (i.e. those with *Salmonella*) five locations were not swabbed – therefore it may be said that if a sample was negative, it was negative with respect to the area swabbed.

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Table 3*a*. *Results of the indirect environment samples* (*part 1*)

Sample	No.	Category III	Category I	OR	95% CI
Comparment aisles	195	35·8%	13·9%	3·45	1·61–7·41
Driving boards	181	28·8%	11·7%	3·06	1·38–6·92
Central aisle in barn	138	42·5%	19·6%	3·03	1·35–6·83
Pipes	143	22·4%	10·5%	2·45	0·97–6·22
Ventilation (fans)	111	22·2%	10·5%	2·43	0·84–7·02
Boots/shoes of farmer	84	35·0%	18·2%	2·42	0·89–6·62
Anterooms	92	27·7%	17·8%	1·77	0·65–4·79

OR, Odds ratio; CI, confidence interval.

Table 3*b*. *Results of the indirect environment samples of* (*part 2*)

Sample	No.	Category III	Category I	*P* value
Ceilings	52	23·5%	0·0%	0·04
Gas heaters	50	20·8%	19·2%	1·00
Animal scales	36	31·8%	14·3%	0·43
Loading ramps	38	10·5%	26·3%	0·41
Transporters	12	36·4%	0·0%	1·00
Other	88	22·7%	18·2%	0·77
Total	1220	29·5%	14·4%	—

Table 4. *Results of the sampling after cleaning and disinfection*

Sample	No.	Category III		Category I	
Feeders and troughs	40	6	28·5%	1	5·3%
Pig toys	25	3	25·0%	3	23·1%
Pen walls	25	2	14·3%	2	18·2%
Drinking nipples	20	3	30·0%	1	10·0%
Walls	15	1	16·7%	0	0·0%
Compartment aisles	17	4	40·0%	2	28·6%
Driving boards	10	2	28·6%	0	0·0%
Pipes	20	3	33·3%	1	9·1%
Other	50	4	16·7%	1	3·8%
Total	222	28	24·8%	11	10·1%

A possible bias of analysis was excluded by blinding the laboratory personnel to the category of the herd and type of sample submitted for examination at a given time.

One drawback of the utilized PCR is the fact that it was not clear whether the detected *Salmonella* were viable and therefore likely to infect a pig or not. Similarly, this particular PCR does not differentiate which serovar of *Salmonella* was detected. However, for the purposes of this study it was assumed that even if the detected *Salmonella* were not viable, they must have been so at one point and therefore every positive sample represented a risk of infection. Moreover, since pigs in Germany are most frequently infected with *Salmonella* Typhimurium [16], a differentiation of isolates via culture was deemed unnecessary. The PCR was therefore a suitable time- and labour-saving alternative to the culture method usually employed in such studies.

Regarding the second objective, it was possible to confirm the hypothesis that there are indeed previously underestimated critical points of residual *Salmonella* infection.

As the results show, category III herds have a higher risk of residual *Salmonella* in the environment compared to herds in category I.

This finding once more demonstrates the usefulness of serological monitoring as a means for estimating the risk that herds pose for carrying *Salmonella* into the slaughterhouse, which has been previously established by several authors [17–19]. This is of great importance, since occasionally farmers are sceptical about the validity of the assumption that many animals with antibodies against *Salmonella* in a herd really means a higher risk of the occurrence of *Salmonella* in slaughter pigs – especially farmers with an apparently high level of hygiene.

Statistically significant differences between category I and category III samples from individual sampling sites were found in the pen floor, compartment aisles, central aisle of the barn as well as the driving boards. As these areas are likely to be contaminated with faeces, it supports the general knowledge that the faecal–oral route is the most important form of transmission, which is also consistent with previous studies [20, 21]. Three of the sampling sites with significant differences (compartment aisle, central aisle, driving board) are part of the indirect environment of the pigs as defined in the Materials and Methods section.

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The fact that *Salmonella* can be isolated from samples taken after C + D has also been observed by other authors [15, 22]. However, the objective of such studies was to evaluate C + D measures in general [8, 15, 22–24], but areas outside the compartment in which the pigs are held were not the focus of such studies, i.e. no differentiation of areas with a different intensity of the animal contacts was undertaken. In view of the fact that the current C + D protocols had been developed for controlling mostly pig-associated pathogens, it is understandable that routine C + D measures, even in very well-managed herds, focus mainly on the animal contact areas and spaces (pens and compartments), but not so much on areas out of reach of the animals. Herein lays a possible explanation for the validity of the presented hypothesis: the areas not included in the traditional C + D routine are the areas of previously underestimated critical points. A similar assumption can be made with respect to the result of the sampling of the ceilings. Even though located inside the compartment, the ceilings may not be as thoroughly cleaned and disinfected as the rest, because of two reasons:

(1) Especially in old buildings, it may not be possible to clean the ceiling with water via a high-pressure cleaner because the ceiling is made of weak materials (thin wood, straw).
(2) The farmers assume that since the pigs never reach the ceiling, nothing there could be harmful to the animals and therefore the ceiling is not cleaned at all.

The fact, however, that dust is a well-known 'bearer' of *Salmonella* [4, 23, 25], implies that dust from the ceiling is as much a cause for residual *Salmonella* that are able to infect *Salmonella*-free piglets after their introduction into a herd.

The overwhelming importance of C + D is emphasized in this study by the fact that of samples taken after C + D, category III herds had significantly more positive samples after C + D than category I herds (OR 2·94 CI 1·38–6·25). There are three possible explanations for this phenomenon:

(1) The management of hygiene in category III herds is not as elaborate as in category I herds and/or lacks a standardized protocol. A literature review and an exploratory study have both identified this as an aspect of deficient biosecurity and a main risk factor for *Salmonella* infections [26, 27].
(2) A protocol for correct C + D does exist in these herds, but its execution is deficient. This particular explanation has also been suggested previously by other authors [23].
(3) In contrast to category III herds, there are other management and working procedures in category I herds that are obviously capable of permanently minimizing the dissemination of *Salmonella* spp. throughout the herd.

Which of these three explanations is the most important needs further research.

To summarize with respect to the hypothesis of the study: the indirect environmental areas of pigs in any pig farm are a thus far underestimated cause of residual *Salmonella*. This is probably due to the fact that they are not included in routine C + D measures.

A comprehensive strategy against residual *Salmonella* contaminations must therefore begin with the implementation and correct execution of biosecurity measures from the threshold of the barn door.

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