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Role of wild birds and environmental contamination in the epidemiology of *Salmonella* infection in an outdoor pig farm ~~in the United Kingdom~~

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ABSTRACT

Foodborne outbreaks caused by *Salmonella* are often attributed to the pork consumption. *Salmonella* contamination of retail pork is directly linked to the *Salmonella* prevalence on farm. In UK, approximately 40% of breeding pigs are kept outdoors. Aim of this study was to investigate the role of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm. Three sampling visits were carried out at monthly intervals to an outdoor farm consisting of two fields, one left empty of pigs for more than 2 years (field A) while the second (field B) was occupied by pigs during the first visit only. Faeces from wild bird droppings, environmental samples and pig faeces were tested for ~~the presence of~~ *Salmonella*. *Salmonella* spp. was isolated from environmental samples ~~even~~ also in field A~~B~~ that had not been occupied by pigs more than 2 years. Interestingly, the wild bird population accessing the fields increased considerably once the pigs had left the farm and the proportion of *Salmonella* positive wild bird droppings increased over time with 7.4%, 15.8% and 44.3% at the first, second and third visit, respectively. The levels of *Salmonella* identified in some of the wild bird droppings were unusually high (10^5 - 10^6 CFU/g) suggesting that *Salmonella* was actively replicating in the gastrointestinal tract of these birds. Monophasic *Salmonella* Typhimurium DT193 was the predominant serotype isolated in pigs as well as ~~,~~ in wild bird droppings and the environment, suggesting~~pporting the hypotheses~~ that the pigs were the original source of infection, as this serovar is typically associated with pigs.

Highlights

- *Salmonella* was isolated from a field left empty by pigs for more than 2 years.
- Wild birds and pigs had the same *Salmonella* serotypes and phage types.
- The close relatedness of a selection of monophasic *S.* Typhimurium and *S.* Rissen isolated from wild birds and pigs was confirmed using whole genome sequencing.
- Pigs are the likely to be the original source of *Salmonella* infection in wild birds.~~Pigs are the likely source of *Salmonella* infection in wild birds~~.

- Wild birds are likely recycling and contributing to the persistence of *Salmonella.*

Keywords: *Salmonella*, Outdoor pig farm, Wild birds, Environment

INTRODUCTION

In the European Union, among the top-5 combinations related with the highest number of cases of illness and hospitalisations in foodborne outbreaks in people, *Salmonella* is always included as causative agent. F~~Evidence f~~oodborne outbreaks caused by *Salmonella* are~~were~~ often attributable to the consumption of contaminated eggs, pig meat, products thereof and other foods ~~In the European Union, among the top-5 combinations related with the highest number of cases of illness and hospitalisations in outbreaks in people, *Salmonella* is always included as causative agent, in combination with eggs, pig meat, products thereof and other foods~~ (EFSA, 2016).

Salmonella infection can be introduced into a pig herd by many routes, for example through the purchase of *Salmonella*-infected pigs, contaminated feed or other animals. Movements of pigs between premises at different life stages represent a risk because during transport pigs are subjected to stress. Stress makes pigs more susceptible to infection and increases the shedding rate of infected pigs (Verbrugghe et al., 2011). Furthermore, especially for outdoor and organic farms, wild fauna, synanthropic and domestic animals living on the farm can constitute a source of introduction and transmission of *Salmonella* through direct contact with pigs or indirectly through faecal contamination of feed, water troughs or farm equipment (Zheng et al., 2007).

The herd prevalence of *Salmonella* infection in pig production holdings in the United Kingdom (UK) was reported to be 44.4% in 2008 by EFSA (EFSA, 2009).

Andres and Davies (2015) suggested that there is a correlation between *Salmonella* prevalence on farm and contamination of retail pork. Biosecurity measures applied at the farm ~~therefore~~ play an important role in the reduction of contamination at retail, even if some of the risk of contamination can be reduced at slaughter (Martelli et al., 2017). Biosecurity measures are also important to prevent further spread within the pig industry, to other food animal sectors and potential zoonotic infections due to contact with infected pigs and manure (Andres and Davies, 2015).

Several studies have been conducted to understand the role of wild birds in the cycle of *Salmonella* infection in pigs (Andres-Barranco et al., 2014; Andres and Davies, 2015; Tizard, 2004; Zheng et al., 2007). Various phage types of *S*. Typhimurium have been associated with wild birds in the UK. *S*. Typhimurium definitive phage types (DT) 56, 40, 41, 195 were isolated from finches, waterfowl, house sparrows, rooks, greenfinches, gulls. *S*. Typhimurium DT2 and DT99 are associated with pigeons, and DT8 and DT30 with game birds (Pennycott et al., 2006). According to the data published by APHA on the isolation of *Salmonella* from pig livestock in Great Britain between the 2011 and 2015, only 0.3% of *Salmonella* isolates from pigs were *Salmonella* serotypes and phage types commonly associated with wild birds ~~strains of *Salmonella*~~ suggesting birds do not present a major risk of infection for pigs (APHA, 2017). Andres et al., (2013), reported that wild birds could be a reservoir of farm-resident strains and that birds can recycle the infection, but are less likely to be the source of introduction. The presence of wild birds, rats and mice is of particular importance in outdoor pig units where they can represent a risk factor for *Salmonella* seropositivity and where measures of control are more challenging (Andres and Davies, 2015). In the UK, around 40% of the pig breeding stock is kept outdoors, whilst most grower and finisher pigs are reared in indoor units (Houston, 2013).~~, while in the~~ In other European countries the number of pigs bred in organic or outdoor farms has increased in recent years (European Commission, 2016).

The aims of the study were to investigate the role of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm and asses *Salmonella* prevalence in the environment when the farm was stocked with pigs and after depopulation.

MATERIALS AND METHODS

Sampling

Between the 8th of September and the 15th November 2015, three sampling visits were carried out in one outdoor pig farm at times determined by the depopulation schedule, during one production~~stocking~~ cycle.

The farm sampled in~~of~~ this study was a fattening farm housing pigs from weaning to finishing. The first visit was carried out when the pigs were still present, the second visit one month later and one week after depopulation, and the third visit one month later. The farm consisted of two adjacent fields: field A has been left empty of pigs for more than 2 years while the field B was occupied by weaners and growers pigs during the first visit only.

All pigs were housed in pens adjacent to each other and located in a portion of the field B. The soil of the fields was sandy and partially covered by weeds and wild shrubbery. The sizes of the fields were 10.4 ha and 8.2 ha for field A and B respectively. Adjacent to the farm, there was a water course, populated by a large number of aquatic wild birds.

In both~~all two~~ fields, swab samples of bird droppings, and environmental samples (soil, water puddles and farm equipment) were collected from the areas unoccupied by pigs and all faecal samples appeared to be fresh at the time of collection. The swab samples were either collected in sterile plastic pots or directly placed into 225 ml of Buffered Peptone Water (BPW) using a hand held gauze.

The sample size was calculated to estimate *Salmonella* prevalence considering an expected prevalence of 50%, an acceptable error of 5~~6~~% and 95% confidence level.

During the first farm visit, intensive sampling (211 of 242 samples collected) was performed in field B, which was occupied by pigs. In addition to what described above, pooled faeces samples were taken from the weaners' and growers' pens at this visit.

A description of the samples taken at each visit is available in Tables 1 and Table 2.

Bacteriological analyses

Individual samples and pooled samples were suspended in Buffered Peptone Water (BPW) enrichment media (1:10 w:v). All samples in BPW were incubated for 18 ± 2 hours at 37 ± 1˚C and

after incubation, 100 µl of each sample was pipetted onto a semi-solid isolation medium; Modified Semi-solid Rappaport Vassiliadis (MSRV) agar containing 0.01% novobiocin (MSRV; Difco 218681) and incubated at 41.5°C for 24 ± 3 hours. After incubation, Rambach agar was inoculated from the MSRV by using a 1 µl loop from the edge of opaque growth on the MSRV (consistent with *Salmonella* growth on MSRV). The Rambach agar plates were incubated at 37°C for 24h ± 3h. The MSRV plates in which bacterial growth consistent with *Salmonella* was observed and ~~were~~ negative for *Salmonella* on the Rambach agar plates were sub-cultured again onto Rambach agar after 48h incubation. Suspect *Salmonella* colonies were identified by complete serotyping according to the Kaufmann–White-Le Minor Scheme (Grimont and Weill, 2007). A random selection of the *S.* Typhimurium and monophasic *S.* Typhimurium (mST) strains were also phage typed (Anderson et al., 1977).

Quantitative analysis was performed on a random selection of positive individual faecal samples from each age class (weaners and growers) and on a random selection of environmental samples collected without enrichment media. Decimal dilutions and subsequent cultures of each dilution, as above, were carried out to semi-quantitatively estimate the level of *Salmonella* per gram of sample (Wales et al., 2006).

Sequencing and sequence data analyses

To further study the relatedness among wild bird and pig *Salmonella* isolates, a total of 6 isolates were whole genome sequenced (WGS). These included 2 *S.* Rissen isolates, one isolate S05753-15 from a wild bird and one from pig (isolate S06138-15), and 4 mST isolates, 3 from wild birds (isolates S05620-15, S05798-15 and S06144-15) and one from pigs (isolate S05634-15).

The assembled draft genome of the *S.* Rissen wild bird isolate S05753-15 was used as reference to map the pig *S.* Rissen genome S06138-15 and extract the SNPs within the whole genome sequence. The assembled draft genome S05620-15 was used as reference for the mST isolates.Genomic DNA and was extracted from 6 *Salmonella* isolates using a commercial kit MagMAX™ CORE Nucleic

Acid Purification Kit together with a KingFisher Duo Prime magnetic particle processor (both from Thermofisher Scientific, Waltham, USA) following manufacturer's instructions. The extracted genomic DNA was fragmented, tagged for multiplexing with the Nextsera XT DNA Sample Preparation Kit (Illumina, Inc. San Diego, USA) and sequenced at the APHA on the Illumina NextSeq platform (Illumina, San Diego, USA) to generate 150 base pair paired-end reads with minimum coverage of 50 x. The quality of the short reads was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The short reads were quality trimmed with Trimmomatic (Bolger et al., 2014) and mapped to the 2 de novo assembled draft genomes S05753-15 and S05620-15 using Snippy (Kwong et al., 2015). The genomes were de novo assembled using SPAdes (Bankevich et al., 2012) and assemblies corrected using Shovill (https://github.com/tseemann/shovill). The ~~allignments~~alignments were further parsed to extract only single nucleotide polymorphism (SNP) with the minimum number of 10 reads~~ covering the variant position and the minimum proportion of those reads which must differ from the reference being 0.9~~. For phylogenetic analyses, a maximum-likelihood phylogenetic tree was constructed from the SNP alignments after Gubbins was run to remove regions of recombination in the pseudofasta files from SNP calling (Croucher et al., 2014). The recombination regions were removed using SNP-sites (Page et al., 2016). The phylogenetic analysis was performed on the generated SNP alignment file to infer core SNP phylogeny using the maximum likelihood method at 100 bootstraps by RAxML and visualised using the tree of life (iTol) (Letunic and Bork, 2016). The SNP distance tables were obtained using ~~a program called~~ snp-dist ~~[snp-dist -~~ (https://github.com/tseemann/snp-dists). The raw fastq files of the six isolates were also passed through the *Salmonella* pipeline which consists of several programs including three serotyping programs, MOST, SeqSero and SISTR to identify the *Salmonella* serotypes based on WGS (Tewolde et al., 2016; Yoshida et al., 2016; Zhang et al., 2015).

Statistical analyses

Salmonella prevalence in environmental and wild bird droppings samples collected during the 3 farm visits was investigated. Results from field A (empty at all visits) were compared to field B (occupied by pigs at visit 1).

The change in *Salmonella* prevalence during the three visits was also studied in environmental and wild bird droppings samples in each of the two fields using Chi-squared test ~~for trend~~. Chi-Square test was used to compare all the data and the significance limit was set at $P < 0.05$. Confidence intervals were calculated by binomial (Clopper-Pearson) "exact" method based on the β distribution. Finally, Odds ratio (OR) was calculated for the risk of *Salmonella* contamination. All statistical analyses were performed using the software SPSS 23.0 (IBM SPSS Statistics, NY, US).

RESULTS

During the three farm visits a total 661 samples were collected from the two areas investigated, field A (empty at all visits) and field B (occupied by pigs at the firs visit). Of these, 324~~182~~ were environmental samples (254 ~~individual~~ swabs collected without enrichment media and 70 swabs collected in enrichment media), 182 were bird dropping (167 swabs collected without enrichment media ~~individual~~ and 15 swabs collected in enrichment media~~swabs~~), 155 were swine feces (120 ~~swabs collected without enrichment media~~individual faecal samples~~individual~~ and 35 ~~swabs~~ pooled faeces collected in enrichment media).

The ~~individual~~ non-enriched environmental and wild bird dropping samples collected from field B were more likely to be *Salmonella* positive (P=0.001) than those collected from field A (Table 5). Overall, the odds of a sample being *Salmonella*-positive was 20.2 times higher in field B compared to field A (P=0.001). For environmental samples and wild bird dropping, the OR values were 27.8 (95% CI: 12.5-62.2) and 12.6 (95% CI: 5.4-29.6), respectively (P=0.001) (Table 5).

At the first visit, 120 individual pig faeces samples were collected from field B. *Salmonella* was isolated from 70.0% (42) and 91.7% (55) respectively of 60 samples from weaners' pens and 60

samples from growers' pens (Table 3). *Salmonella* was also isolated from 58.3% (35 of 60) of environmental samples and from 7.4% (2 of 27) of ~~individual~~ wild bird faeces samples.

At the second visit, the 26.9% (36 of 134) of environmental samples and 15.8% (12 of 76) of wild bird droppings were found to be *Salmonella* positive.

During the final third visit, *Salmonella* was isolated from 27.7% (36 of 130) of environmental samples and from 44.3% (35 of 79) of wild bird samples.

~~Overall, a significant difference (P=0.001) (P < 0.05) was observed in *Salmonella* prevalence over time in the field B.~~ Wild bird dropping samples collected in field B at the third visit were significantly more contaminated with *Salmonella* than the samples collected in empty fields (field A) (P=0.001)~~(P < 0.05)~~.

Although *Salmonella* prevalence in environmental samples and wild bird samples collected from field A did not vary significantly during the three visits (respectively: P=0.82 and P=0.84), significant differences were observed in samples collected from field B over time. In particular, the proportion of *Salmonella*-positive wild bird faeces increased significantly over time (P=0.001)~~P < 0.015)~~ while no difference was observed for the environmental samples (P=0.07).~~.~~

Enriched ~~S~~swab samples

At the first visit *Salmonella* was isolated from 83.3% (15 of 18) and 100% (17 of 17) respectively of the weaners'and growers' pens. Furthermore, *Salmonella* was isolated from all the 5 environmental samples collected during the first farm visit and from the 23.1% (3 of 13) and 41.7% (5 of 12) of environmental samples collected respectively during the second and third visit (Table 4).

From wild bird droppings, *Salmonella* was detected in 71.4% (5 of 7) and 100% (8 of 8) swabs collected respectively during the second and third visit.

Semi-quantitative culture results

High levels of *Salmonella* were found in individual pig faecal samples (Table 3). In the growers' faeces, 7 samples had a *Salmonella* concentration of 10^2-10^3 CFU/g, 3 samples of 10^3-10^4 CFU/g and 2 samples of 10^5-10^6 CFU/g. In the weaners' faeces the maximum *Salmonella* load was 10^3-10^4 CFU/g (3 samples) followed by 8 samples with 10^2-10^3 CFU/g while a lower *Salmonella* level was found in the remaining 8 samples (10-10^2 CFU/g and 1-10 CFU/g).

In the environmental samples (Table 2) the levels of *Salmonella* were found to be low (1-10 CFU/g or 10-10^2 CFU/g) for the majority of the samples. Only the environmental samples collected from the field in which pigs had been housed had higher CFU/g (two samples had 10^2-10^3 CFU/g). Unusually high *Salmonella* levels were found in some of the wild bird droppings: 10^5-10^6 CFU/g in geese droppings collected during the second and third visit both of the two fields sampled (Table 2).

Serovars and phage types

In total 151 *Salmonella* strains were serotyped, most *Salmonella* isolates were *S. enterica* serovar 4,5,12:i:- (mST) (121, 80.1%), followed by *S.* Rissen (22, 14.6%), *S.* Senftenberg (3, 2%), *S.* Typhimurium (2, 1.3%), *S.* Panama (2, 1.3%) and *S.* Derby (1, 0.7%) (Tables 1, 3 and 4).

Among individual faeces and swab samples mST was detected from 85.1% (40 of 47) of weaners' and growers' samples, from 78.4% (29 of 37) of wild bird droppings samples (the majority of them were from geese droppings) and 77.6% (52 of 67) of environmental samples (Tables 3 and 4).

S. Typhimurium was isolated from only two wild bird droppings samples collected during the first and second visits.

Thirty four *S.* Typhimurium and mST isolate were phage typed. Different sample types, collected during all the visit, such as bird droppings (12), environmental (18) and pig fecal samples (4) were selected. Only two phage types were identified among the 34 *S.* Typhimurium and mST strains tested. All were DT193 except for one isolate of *S.* Typhimurium, isolated from wild bird droppings, which was phage type DT41.

262 **Phylogenetic clustering of wild bird and pig *Salmonella* isolates**

263 All 6 isolates sequenced in this study were highly related with only 1 SNP difference between the 2
264 *S.* Rissen isolates and a maximum observed difference of 9 SNPs between the 4 mST isolates. The
265 SNP difference of the monophasic *S.* Typhimurium isolates from wild birds was between 4 and 6.
266 Maximum likelihood core genome SNP phylogeny of *S.* Rissen and mST isolates and SNP
267 differences are presented in Figure 1 and Figure 2, respectively.

268

269 **DISCUSSION**

270

271 On farms rodents, birds, insects, are common inhabitants that can all be carrier vectors and can
272 mechanically transmit pathogens (Backhans et al., 2013). Bait traps or chemical pesticides can aid in
273 the management of rodent problems as well the removal of waste and feed spills can be helpful to
274 limit the attraction of birds and rodents (Andres and Davies, 2015).
275 The role of wild birds is a controversial matter in relation to potential hazards to livestock and to for
276 human health. Several studies support the hypothesis that wild birds play an important role in
277 *Salmonella* epidemiology in both humans and animals (Andres et al., 2013; Phalen et al., 2010; Vico
278 and Mainar Jaime, 2011). In contrast, other studies suggest that they do not represent a major public
279 health hazard, considering the low numbers of organisms shed and the short duration of *Salmonella*
280 carriage shedding (Hughes et al., 2008; Jensen et al., 2004; Marin et al., 2014). It is well recognized
281 that *Salmonella* is an ubiquitous agent that can colonize asymptomatically the gut of birds and
282 consequently can be shed in their faeces (Andres et al., 2013). *Salmonella* prevalence studies are
283 usually associated with wild birds and focussed on host-adapted strains in some bird species, but
284 some studies report that birds near pig farms have higher probability of shedding *Salmonella* than
285 birds living far from pig premises (Andres et al., 2013).

This study was carried out on one outdoor pig farm occupied by *Salmonella* infected pigs at the first visit only. Individual samples of pig faeces, environmental samples and wild bird droppings were collected and a quantitative analysis of *Salmonella* was performed on positive samples.

Pools of faecal samples were also collected as they are regarded as more effective for isolating *Salmonella*~~salmonellae~~, than the sampling of a large number of individual samples (Cook et al., 2005). A significantly higher prevalence~~ (P < 0.001)~~, as well~~ the~~ as the higher odds of *Salmonella*-positive samples detected in samples collected from the field occupied by pigs, suggest that pigs are the likely source of *Salmonella* in the pig farm environment. At the first visit >50% environmental samples were found to be *Salmonella*-positive, and one and two months after the pigs had left the farm, 27% of environmental samples were still *Salmonella*- positive. *Salmonella* was also found in environmental samples in a field that had been empty for 2 years. It is likely that *Salmonella* can survive outside the host for a significant length of time as reported by several authors (Funk and Gebreyes, 2004; Jensen et al., 2004; Sandvang et al., 2000), and therefore the environment itself can become a potential source of infection for subsequent batches of pigs and wildlife. However, it is also possible that wild birds contributed to re-contaminate the soil, considering that *Salmonella* was isolated from wild bird droppings (7.4%~~; 2 of 27)~~ of samples collected during the first farm visit, 15.8 % ~~(12 of 76)~~ and 44.3% ~~(35 of 79)~~ collected during the second and third farm visit, respectively). It is apparent that once the pigs had left the farm, the proportion of *Salmonella*-positive wild bird faeces increased significantly~~ (P < 0.01)~~. This could be linked to the fact that the wild bird population accessing the fields increased considerably once the pigs left the farm (as observed by the sampling team). The increase may be due to the presence of leftover pig feed and worm populations being nearer the surface of the soil (Andres et al., 2013; Andres and Davies, 2015). Furthermore, the increase in wild birds density over time, may have caused an increase in the transmission rate of this infection among birds (Andres et al., 2013). It was not possible to collect samples from a field that had never been occupied by pigs on this farm, as all fields had been occupied by pigs on a rotational

basis in the last decade. It was therefore not possible to assess the levels of contamination exclusively related to wild bird droppings.

Livestock farms can act as areas where wild birds congregate for the availability of food and shelter (Andres et al., 2013). At the same time farm environment with high levels of *Salmonella* contamination, as well other pathogens may be an important potential source of infection and potential biodiversity threat for those avian species of wild birds susceptible to the infection (Andres-Barranco et al., 2014; Andres et al., 2013). It has been suggested that ~~S~~salmonellosis ~~has been suggested as can~~can be ~~be~~ one of the causes of the decline of the house sparrow population (Pennycott et al., 2006). *Salmonella* enterica serovar Typhimurium for passerine can result in a sever disease with significant mortality (Tizard, 2004). S~~In this context, s~~everal authors reported that the feeding and migration behaviour as well as the seasonality may influence the prevalence of salmonellosis in free-ranging birds (Andres-Barranco et al., 2014; Andres et al., 2013).

Salmonella prevalence was significantly higher in wild bird droppings collected from the field occupied by pigs at the first visit (field B).

~~The majority of wild birds present in the farm were gulls or geese, both those bird species feeding on the ground resulting in higher chances of getting infected than birds feeding from hanging feeders. Moreover the significant positive relationship between the of detecting *Salmonella* in wild bird droppings collected in field B. This that pigs represent an important risk factor as source of *Salmonella* for wild birds.~~

Moreover in field B the odd of *Salmonella* positive samples was ~~12.6 times~~ higher compared with those samples collected from field A. These results together suggested that pigs represent an important risk factor as source of *Salmonella* for wild birds.

In the proximity of the farm sampled in this study, there was also a river, which attracted a large number of wild birds especially aquatic birds, such as Canada geese and seagulls.

Geese droppings had higher ~~(10^5-10^6)~~ *Salmonella* CFU/g, ~~whilst~~ compared with *Salmonella* levels ~~were lower~~ in environmental samples ~~(10-10^2 and 10^2-10^3 CFU/g)~~. A study by Pennycott et al., (2006)

concluded that *Salmonella* strains in Great Britain originating from wild birds do not represent a major primary source of infection, considering the low percentage of wild bird associated phage types isolated from livestock. However, the high levels of *Salmonella* in geese faeces suggest that geese can represent an important source of infection, able to maintain *Salmonella* in areas ~~with~~ where geese ~~bird populations~~are present.

The *Salmonella* serotypes found in pigs, mST and *S*. Rissen, were the same as those found in wild bird droppings. We confirmed the close relatedness of the mST and *S*. Rissen isolated from wild birds and pigs using whole genome sequencing as ~~the~~an highly discriminative method for studying population heterogeneity in bacteria. We found a single SNP difference between the *S*.~~,~~ Risen isolates and maximum of 9 SNPs among the mST isolates. In recent year WGS has been used successfully in investigating a number of *Salmonella* related outbreak and trace back investigations and is becoming a method of choice in linking different sources of infection (Ashton et al., 2015; Inns et al., 2017; Inns et al., 2015)~~(Ashton at al., 2015, Inns et al., 2015, Inns et al., 2017)~~. Within the *S*. Typhimurium serovar a cluster of isolates that are grouped together in time and space and sharing 0-10 SNPs are considered as common source of infection (Ford et al., 2018). *S*. Senftenberg, and *S*. Typhimurium were found in wild bird droppings, and these are also serotypes typically commonly found in housed breeding pigs in the UK (Hughes et al., 2008). *S*. Typhimurium is reported to be the most common serotype identified in wild bird droppings (Andres et al., 2013; Horton et al., 2013; Hughes et al., 2008; Lawson et al., 2011; Palmgren et al., 2006; Vico and Mainar Jaime, 2011). In contrast, the majority of *Salmonella* isolates from wild bird droppings during this study were mST DT193. One of the isolated *S*. Typhimurium serovars was phage type DT 41. *S*. Typhimurium DT 41 has been reported previously in wild birds from the UK and is particularly associated with waterfowl (Barua et al., 2013; Hughes et al., 2008; Pennycott et al., 2006).

Pigs have been recognized as the main reservoir of mST DT193 (Crayford et al., 2014), supporting the hypothesis that pigs can act as a source of wild bird salmonellosis.

Interestingly, the 25 of 37 mST-positive samples, were from geese droppings and three of them collected during the second and third visit presented an unusually high level of *Salmonella*-shedding ~~(10^5-10^6 CFU/g)~~. Therefore, it is reasonable to postulate that mST infection in wild geese does not cause clinical symptoms in birds. However, further studies are required to better understand the role of geese and their role in the cycle of *Salmonella* infection in outdoor pig farms.

This study suggests a possible cyclical dissemination of *Salmonella* between pigs and wild birds, and that wild birds are capable of contributing to the persistence of *Salmonella* between batches of pigs. Adequate management practices to minimize the contact between pigs and wild birds (e.g. cover feed and water sources, use of nets) should be implemented in outdoor pig units.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Highlights

- *Salmonella* was isolated from a field left empty by pigs for more than 2 years.
- Wild birds and pigs had the same *Salmonella* serotypes and phage types.
- The close relatedness of a selection of monophasic *S*. Typhimurium and *S.* Rissen isolated from wild birds and pigs was confirmed using whole genome sequencing.
- Pigs are the likely to be the original source of *Salmonella* infection in wild birds.
- Wild birds are likely recycling and contributing to the persistence of *Salmonella.*

Role of wild birds and environmental contamination in the epidemiology of *Salmonella* infection in an outdoor pig farm

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ABSTRACT

Foodborne outbreaks caused by *Salmonella* are often attributed to the pork consumption. *Salmonella* contamination of retail pork is directly linked to the *Salmonella* prevalence on farm. In UK, approximately 40% of breeding pigs are kept outdoors. Aim of this study was to investigate the role of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm. Three sampling visits were carried out at monthly intervals to an outdoor farm consisting of two fields, one left empty of pigs for more than 2 years (field A) while the second (field B) was occupied by pigs during the first visit only. Faeces from wild bird droppings, environmental samples and pig faeces were tested for *Salmonella*. *Salmonella* spp. was isolated from environmental samples also in field A that had not been occupied by pigs more than 2 years. Interestingly, the wild bird population accessing the fields increased considerably once the pigs had left the farm and the proportion of *Salmonella* positive wild bird droppings increased over time with 7.4%, 15.8% and 44.3% at the first, second and third visit, respectively. The levels of *Salmonella* identified in some of the wild bird droppings were unusually high (10^5 - 10^6 CFU/g) suggesting that *Salmonella* was actively replicating in the gastrointestinal tract of these birds. Monophasic *Salmonella* Typhimurium DT193 was the predominant serotype isolated in pigs as well as in wild bird droppings and the environment, suggesting that the pigs were the original source of infection, as this serovar is typically associated with pigs.

Highlights

- *Salmonella* was isolated from a field left empty by pigs for more than 2 years.
- Wild birds and pigs had the same *Salmonella* serotypes and phage types.
- The close relatedness of a selection of monophasic *S.* Typhimurium and *S.* Rissen isolated from wild birds and pigs was confirmed using whole genome sequencing.
- Pigs are the likely to be the original source of *Salmonella* infection in wild birds..
- Wild birds are likely recycling and contributing to the persistence of *Salmonella.*

Keywords: *Salmonella*, Outdoor pig farm, Wild birds, Environment

INTRODUCTION

In the European Union, among the top-5 combinations related with the highest number of cases of illness and hospitalisations in foodborne outbreaks in people, *Salmonella* is always included as causative agent. Foodborne outbreaks caused by *Salmonella* are often attributable to the consumption of contaminated eggs, pig meat, products thereof and other foods (EFSA, 2016).

Salmonella infection can be introduced into a pig herd by many routes, for example through the purchase of *Salmonella*-infected pigs, contaminated feed or other animals. Movements of pigs between premises at different life stages represent a risk because during transport pigs are subjected to stress. Stress makes pigs more susceptible to infection and increases the shedding rate of infected pigs (Verbrugghe et al., 2011). Furthermore, especially for outdoor and organic farms, wild fauna, synanthropic and domestic animals living on the farm can constitute a source of introduction and transmission of *Salmonella* through direct contact with pigs or indirectly through faecal contamination of feed, water troughs or farm equipment (Zheng et al., 2007).

The herd prevalence of *Salmonella* infection in pig production holdings in the United Kingdom (UK) was reported to be 44.4% in 2008 by EFSA (EFSA, 2009).

Andres and Davies (2015) suggested that there is a correlation between *Salmonella* prevalence on farm and contamination of retail pork. Biosecurity measures applied at the farm play an important role in the reduction of contamination at retail, even if some of the risk of contamination can be reduced at slaughter (Martelli et al., 2017). Biosecurity measures are also important to prevent further spread within the pig industry, to other food animal sectors and potential zoonotic infections due to contact with infected pigs and manure (Andres and Davies, 2015).

Several studies have been conducted to understand the role of wild birds in the cycle of *Salmonella* infection in pigs (Andres-Barranco et al., 2014; Andres and Davies, 2015; Tizard, 2004; Zheng et al., 2007). Various phage types of *S.* Typhimurium have been associated with wild birds in the UK. *S.* Typhimurium definitive phage types (DT) 56, 40, 41, 195 were isolated from finches, waterfowl,

house sparrows, rooks, greenfinches, gulls. *S.* Typhimurium DT2 and DT99 are associated with pigeons, and DT8 and DT30 with game birds (Pennycott et al., 2006). According to the data published by APHA on the isolation of *Salmonella* from pig livestock in Great Britain between the 2011 and 2015, only 0.3% of *Salmonella* isolates from pigs were *Salmonella* serotypes and phage types commonly associated with wild birds suggesting birds do not present a major risk of infection for pigs (APHA, 2017). Andres et al., (2013), reported that wild birds could be a reservoir of farm-resident strains and that birds can recycle the infection, but are less likely to be the source of introduction. The presence of wild birds, rats and mice is of particular importance in outdoor pig units where they can represent a risk factor for *Salmonella* seropositivity and where measures of control are more challenging (Andres and Davies, 2015). In the UK, around 40% of the pig breeding stock is kept outdoors, whilst most grower and finisher pigs are reared in indoor units (Houston, 2013). In other European countries the number of pigs bred in organic or outdoor farms has increased in recent years (European Commission, 2016).

The aims of the study were to investigate the role of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm and asses *Salmonella* prevalence in the environment when the farm was stocked with pigs and after depopulation.

MATERIALS AND METHODS

Sampling

Between the 8th of September and the 15th November 2015, three sampling visits were carried out in one outdoor pig farm at times determined by the depopulation schedule, during one production cycle. The farm sampled in this study was a fattening farm housing pigs from weaning to finishing. The first visit was carried out when the pigs were still present, the second visit one month later and one week after depopulation, and the third visit one month later. The farm consisted of two adjacent fields: field

A has been left empty of pigs for more than 2 years while the field B was occupied by weaners and growers pigs during the first visit only.

All pigs were housed in pens adjacent to each other and located in a portion of the field B. The soil of the fields was sandy and partially covered by weeds and wild shrubbery. The sizes of the fields were 10.4 ha and 8.2 ha for field A and B respectively. Adjacent to the farm, there was a water course, populated by a large number of aquatic wild birds.

In both fields, swab samples of bird droppings, and environmental samples (soil, water puddles and farm equipment) were collected from the areas unoccupied by pigs and all faecal samples appeared to be fresh at the time of collection. The swab samples were either collected in sterile plastic pots or directly placed into 225 ml of Buffered Peptone Water (BPW) using a hand held gauze.

The sample size was calculated to estimate *Salmonella* prevalence considering an expected prevalence of 50%, an acceptable error of 5% and 95% confidence level.

During the first farm visit, intensive sampling (211 of 242 samples collected) was performed in field B, which was occupied by pigs. In addition to what described above, pooled faeces samples were taken from the weaners' and growers' pens at this visit.

A description of the samples taken at each visit is available in Tables 1 and Table 2.

Bacteriological analyses

Individual samples and pooled samples were suspended in Buffered Peptone Water (BPW) enrichment media (1:10 w:v). All samples in BPW were incubated for 18 ± 2 hours at 37 ± 1˚C and after incubation, 100 µl of each sample was pipetted onto a semi-solid isolation medium; Modified Semi-solid Rappaport Vassiliadis (MSRV) agar containing 0.01% novobiocin (MSRV; Difco 218681) and incubated at 41.5°C for 24 ± 3 hours. After incubation, Rambach agar was inoculated from the MSRV by using a 1 µl loop from the edge of opaque growth on the MSRV (consistent with *Salmonella* growth on MSRV). The Rambach agar plates were incubated at 37°C for 24h ± 3h. The MSRV plates in which bacterial growth consistent with *Salmonella* was observed and negative for

Salmonella on the Rambach agar plates were sub-cultured again onto Rambach agar after 48h incubation. Suspect *Salmonella* colonies were identified by complete serotyping according to the Kaufmann–White-Le Minor Scheme (Grimont and Weill, 2007). A random selection of the *S.* Typhimurium and monophasic *S.* Typhimurium (mST) strains were also phage typed (Anderson et al., 1977).

Quantitative analysis was performed on a random selection of positive individual faecal samples from each age class (weaners and growers) and on a random selection of environmental samples collected without enrichment media. Decimal dilutions and subsequent cultures of each dilution, as above, were carried out to semi-quantitatively estimate the level of *Salmonella* per gram of sample (Wales et al., 2006).

Sequencing and sequence data analyses

To further study the relatedness among wild bird and pig *Salmonella* isolates, a total of 6 isolates were whole genome sequenced (WGS). These included 2 *S.* Rissen isolates, one isolate S05753-15 from a wild bird and one from pig (isolate S06138-15), and 4 mST isolates, 3 from wild birds (isolates S05620-15, S05798-15 and S06144-15) and one from pigs (isolate S05634-15).

The assembled draft genome of the *S.* Rissen wild bird isolate S05753-15 was used as reference to map the pig *S.* Rissen genome S06138-15 and extract the SNPs within the whole genome sequence. The assembled draft genome S05620-15 was used as reference for the mST isolates.Genomic DNA and was extracted from 6 *Salmonella* isolates using a commercial kit MagMAX™ CORE Nucleic Acid Purification Kit together with a KingFisher Duo Prime magnetic particle processor (both from Thermofisher Scientific, Waltham, USA) following manufacturer's instructions. The extracted genomic DNA was fragmented, tagged for multiplexing with the Nextera XT DNA Sample Preparation Kit (Illumina, Inc. San Diego, USA) and sequenced at the APHA on the Illumina NextSeq platform (Illumina, San Diego, USA) to generate 150 base pair paired-end reads with minimum coverage of 50 x. The quality of the short reads was evaluated with FastQC

(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The short reads were quality trimmed with Trimmomatic (Bolger et al., 2014) and mapped to the 2 de novo assembled draft genomes S05753-15 and S05620-15 using Snippy (Kwong et al., 2015). The genomes were de novo assembled using SPAdes (Bankevich et al., 2012) and assemblies corrected using Shovill (https://github.com/tseemann/shovill). The alignments were further parsed to extract only single nucleotide polymorphism (SNP) with the minimum number of 10 reads. For phylogenetic analyses, a maximum-likelihood phylogenetic tree was constructed from the SNP alignments after Gubbins was run to remove regions of recombination in the pseudofasta files from SNP calling (Croucher et al., 2014). The recombination regions were removed using SNP-sites (Page et al., 2016). The phylogenetic analysis was performed on the generated SNP alignment file to infer core SNP phylogeny using the maximum likelihood method at 100 bootstraps by RAxML and visualised using the tree of life (iTol) (Letunic and Bork, 2016). The SNP distance tables were obtained using snp-dist (https://github.com/tseemann/snp-dists). The raw fastq files of the six isolates were also passed through the *Salmonella* pipeline which consists of several programs including three serotyping programs, MOST, SeqSero and SISTR to identify the *Salmonella* serotypes based on WGS (Tewolde et al., 2016; Yoshida et al., 2016; Zhang et al., 2015).

Statistical analyses

Salmonella prevalence in environmental and wild bird droppings samples collected during the 3 farm visits was investigated. Results from field A (empty at all visits) were compared to field B (occupied by pigs at visit 1).

The change in *Salmonella* prevalence during the three visits was also studied in environmental and wild bird droppings samples in each of the two fields using Chi-squared test. Chi-Square test was used to compare all the data and the significance limit was set at $P < 0.05$. Confidence intervals were calculated by binomial (Clopper-Pearson) “exact” method based on the β distribution. Finally, Odds

ratio (OR) was calculated for the risk of *Salmonella* contamination. All statistical analyses were performed using the software SPSS 23.0 (IBM SPSS Statistics, NY, US).

RESULTS

During the three farm visits a total 661 samples were collected from the two areas investigated, field A (empty at all visits) and field B (occupied by pigs at the firs visit). Of these, 324 were environmental samples (254 swabs collected without enrichment media and 70 swabs collected in enrichment media), 182 were bird dropping (167 swabs collected without enrichment media and 15 swabs collected in enrichment media), 155 were swine feces (120 individual faecal samples and 35 pooled faeces collected in enrichment media).

The non-enriched environmental and wild bird dropping samples collected from field B were more likely to be *Salmonella* positive (P=0.001) than those collected from field A (Table 5). Overall, the odds of a sample being *Salmonella*-positive was 20.2 times higher in field B compared to field A (P=0.001). For environmental samples and wild bird dropping, the OR values were 27.8 (95% CI: 12.5-62.2) and 12.6 (95% CI: 5.4-29.6), respectively (P=0.001) (Table 5).

At the first visit, 120 individual pig faeces samples were collected from field B. *Salmonella* was isolated from 70.0% (42) and 91.7% (55) respectively of 60 samples from weaners' pens and 60 samples from growers' pens (Table 3). *Salmonella* was also isolated from 58.3% (35 of 60) of environmental samples and from 7.4% (2 of 27) of wild bird faeces samples.

At the second visit, the 26.9% (36 of 134) of environmental samples and 15.8% (12 of 76) of wild bird droppings were found to be *Salmonella* positive.

During the final third visit, *Salmonella* was isolated from 27.7% (36 of 130) of environmental samples and from 44.3% (35 of 79) of wild bird samples.

Wild bird dropping samples collected in field B at the third visit were significantly more contaminated with *Salmonella* than the samples collected in empty fields (field A) (P=0.001).

Although *Salmonella* prevalence in environmental samples and wild bird samples collected from field A did not vary significantly during the three visits (respectively: P=0.82 and P=0.84), significant differences were observed in samples collected from field B over time. In particular, the proportion of *Salmonella*-positive wild bird faeces increased significantly over time (P=0.001) while no difference was observed for the environmental samples (P=0.07).

Enriched swab samples

At the first visit *Salmonella* was isolated from 83.3% (15 of 18) and 100% (17 of 17) respectively of the weaners'and growers' pens. Furthermore, *Salmonella* was isolated from all the 5 environmental samples collected during the first farm visit and from the 23.1% (3 of 13) and 41.7% (5 of 12) of environmental samples collected respectively during the second and third visit (Table 4).

From wild bird droppings, *Salmonella* was detected in 71.4% (5 of 7) and 100% (8 of 8) swabs collected respectively during the second and third visit.

Semi-quantitative culture results

High levels of *Salmonella* were found in individual pig faecal samples (Table 3). In the growers' faeces, 7 samples had a *Salmonella* concentration of 10^2-10^3 CFU/g, 3 samples of 10^3-10^4 CFU/g and 2 samples of 10^5-10^6 CFU/g. In the weaners' faeces the maximum *Salmonella* load was 10^3-10^4 CFU/g (3 samples) followed by 8 samples with 10^2-10^3 CFU/g while a lower *Salmonella* level was found in the remaining 8 samples (10-10^2 CFU/g and 1-10 CFU/g).

In the environmental samples (Table 2) the levels of *Salmonella* were found to be low (1-10 CFU/g or 10-10^2 CFU/g) for the majority of the samples. Only the environmental samples collected from the field in which pigs had been housed had higher CFU/g (two samples had 10^2-10^3 CFU/g). Unusually high *Salmonella* levels were found in some of the wild bird droppings: 10^5-10^6 CFU/g in geese droppings collected during the second and third visit both of the two fields sampled (Table 2).

Serovars and phage types

In total 151 *Salmonella* strains were serotyped, most *Salmonella* isolates were *S. enterica* serovar 4,5,12:i:- (mST) (121, 80.1%), followed by *S.* Rissen (22, 14.6%), *S.* Senftenberg (3, 2%), *S.* Typhimurium (2, 1.3%), *S.* Panama (2, 1.3%) and *S.* Derby (1, 0.7%) (Tables 1, 3 and 4).

Among individual faeces and swab samples mST was detected from 85.1% (40 of 47) of weaners' and growers' samples, from 78.4% (29 of 37) of wild bird droppings samples (the majority of them were from geese droppings) and 77.6% (52 of 67) of environmental samples (Tables 3 and 4).

S. Typhimurium was isolated from only two wild bird droppings samples collected during the first and second visits.

Thirty four *S.* Typhimurium and mST isolate were phage typed. Different sample types, collected during all the visit, such as bird droppings (12), environmental (18) and pig fecal samples (4) were selected. Only two phage types were identified among the 34 *S.* Typhimurium and mST strains tested. All were DT193 except for one isolate of *S.* Typhimurium, isolated from wild bird droppings, which was phage type DT41.

Phylogenetic clustering of wild bird and pig *Salmonella* isolates

All 6 isolates sequenced in this study were highly related with only 1 SNP difference between the 2 *S.* Rissen isolates and a maximum observed difference of 9 SNPs between the 4 mST isolates. The SNP difference of the monophasic *S.* Typhimurium isolates from wild birds was between 4 and 6. Maximum likelihood core genome SNP phylogeny of *S.* Rissen and mST isolates and SNP differences are presented in Figure 1 and Figure 2, respectively.

DISCUSSION

On farms rodents, birds, insects, are common inhabitants that can all be carrier vectors and can mechanically transmit pathogens (Backhans et al., 2013). Bait traps or chemical pesticides can aid in

the management of rodent problems as well the removal of waste and feed spills can be helpful to limit the attraction of birds and rodents (Andres and Davies, 2015).

The role of wild birds is a controversial matter in relation to potential hazards to livestock and to for human health. Several studies support the hypothesis that wild birds play an important role in *Salmonella* epidemiology in both humans and animals (Andres et al., 2013; Phalen et al., 2010; Vico and Mainar Jaime, 2011). In contrast, other studies suggest that they do not represent a major public health hazard, considering the low numbers of organisms shed and the short duration of *Salmonella* carriage shedding (Hughes et al., 2008; Jensen et al., 2004; Marin et al., 2014). It is well recognized that *Salmonella* is an ubiquitous agent that can colonize asymptomatically the gut of birds and consequently can be shed in their faeces (Andres et al., 2013). *Salmonella* prevalence studies are usually associated with wild birds and focussed on host-adapted strains in some bird species, but some studies report that birds near pig farms have higher probability of shedding *Salmonella* than birds living far from pig premises (Andres et al., 2013).

This study was carried out on one outdoor pig farm occupied by *Salmonella* infected pigs at the first visit only. Individual samples of pig faeces, environmental samples and wild bird droppings were collected and a quantitative analysis of *Salmonella* was performed on positive samples.

Pools of faecal samples were also collected as they are regarded as more effective for isolating *Salmonella*, than the sampling of a large number of individual samples (Cook et al., 2005). A significantly higher prevalence, as well as the higher odds of *Salmonella*-positive samples detected in samples collected from the field occupied by pigs, suggest that pigs are the likely source of *Salmonella* in the pig farm environment. At the first visit >50% environmental samples were found to be *Salmonella*-positive, and one and two months after the pigs had left the farm, 27% of environmental samples were still *Salmonella*- positive. *Salmonella* was also found in environmental samples in a field that had been empty for 2 years. It is likely that *Salmonella* can survive outside the host for a significant length of time as reported by several authors (Funk and Gebreyes, 2004; Jensen et al., 2004; Sandvang et al., 2000), and therefore the environment itself can become a potential source

of infection for subsequent batches of pigs and wildlife. However, it is also possible that wild birds contributed to re-contaminate the soil, considering that *Salmonella* was isolated from wild bird droppings (7.4% of samples collected during the first farm visit, 15.8 % and 44.3% collected during the second and third farm visit, respectively). It is apparent that once the pigs had left the farm, the proportion of *Salmonella*-positive wild bird faeces increased significantly. This could be linked to the fact that the wild bird population accessing the fields increased considerably once the pigs left the farm (as observed by the sampling team). The increase may be due to the presence of leftover pig feed and worm populations being nearer the surface of the soil (Andres et al., 2013; Andres and Davies, 2015). Furthermore, the increase in wild birds density over time, may have caused an increase in the transmission rate of this infection among birds (Andres et al., 2013). It was not possible to collect samples from a field that had never been occupied by pigs on this farm, as all fields had been occupied by pigs on a rotational basis in the last decade. It was therefore not possible to assess the levels of contamination exclusively related to wild bird droppings.

Livestock farms can act as areas where wild birds congregate for the availability of food and shelter (Andres et al., 2013). At the same time farm environment with high levels of *Salmonella* contamination, as well other pathogens may be an important potential source of infection and potential biodiversity threat for those avian species of wild birds susceptible to the infection (Andres-Barranco et al., 2014; Andres et al., 2013). It has been suggested that salmonellosis can be one of the causes of the decline of the house sparrow population (Pennycott et al., 2006). *Salmonella* enterica serovar Typhimurium for passerine can result in a sever disease with significant mortality (Tizard, 2004). Several authors reported that the feeding and migration behaviour as well as the seasonality may influence the prevalence of salmonellosis in free-ranging birds (Andres-Barranco et al., 2014; Andres et al., 2013).

Salmonella prevalence was significantly higher in wild bird droppings collected from the field occupied by pigs at the first visit (field B). Moreover in field B the odd of *Salmonella* positive samples

was higher compared with those samples collected from field A. These results together suggested that pigs represent an important risk factor as source of *Salmonella* for wild birds.

In the proximity of the farm sampled in this study, there was also a river, which attracted a large number of wild birds especially aquatic birds, such as Canada geese and seagulls.

Geese droppings had higher *Salmonella* CFU/g, compared with *Salmonella* levels in environmental samples. A study by Pennycott et al., (2006) concluded that *Salmonella* strains in Great Britain originating from wild birds do not represent a major primary source of infection, considering the low percentage of wild bird associated phage types isolated from livestock. However, the high levels of *Salmonella* in geese faeces suggest that geese can represent an important source of infection, able to maintain *Salmonella* in areas where geese are present. The *Salmonella* serotypes found in pigs, mST and *S*. Rissen, were the same as those found in wild bird droppings. We confirmed the close relatedness of the mST and *S.* Rissen isolated from wild birds and pigs using whole genome sequencing as an highly discriminative method for studying population heterogeneity in bacteria. We found a single SNP difference between the *S*. Risen isolates and maximum of 9 SNPs among the mST isolates. In recent year WGS has been used successfully in investigating a number of *Salmonella* related outbreak and trace back investigations and is becoming a method of choice in linking different sources of infection (Ashton et al., 2015; Inns et al., 2017; Inns et al., 2015). Within the *S.* Typhimurium serovar a cluster of isolates that are grouped together in time and space and sharing 0-10 SNPs are considered as common source of infection (Ford et al., 2018). *S.* Senftenberg, and *S.* Typhimurium were found in wild bird droppings, and these are also serotypes typically commonly found in housed breeding pigs in the UK (Hughes et al., 2008). *S.* Typhimurium is reported to be the most common serotype identified in wild bird droppings (Andres et al., 2013; Horton et al., 2013; Hughes et al., 2008; Lawson et al., 2011; Palmgren et al., 2006; Vico and Mainar Jaime, 2011). In contrast, the majority of *Salmonella* isolates from wild bird droppings during this study were mST DT193. One of the isolated *S*. Typhimurium serovars was phage type DT 41. *S.* Typhimurium DT 41

has been reported previously in wild birds from the UK and is particularly associated with waterfowl (Barua et al., 2013; Hughes et al., 2008; Pennycott et al., 2006).

Pigs have been recognized as the main reservoir of mST DT193 (Crayford et al., 2014), supporting the hypothesis that pigs can act as a source of wild bird salmonellosis.

Interestingly, the 25 of 37 mST-positive samples, were from geese droppings and three of them collected during the second and third visit presented an unusually high level of *Salmonella*-shedding. Therefore, it is reasonable to postulate that mST infection in wild geese does not cause clinical symptoms in birds. However, further studies are required to better understand the role of geese and their role in the cycle of *Salmonella* infection in outdoor pig farms.

This study suggests a possible cyclical dissemination of *Salmonella* between pigs and wild birds, and that wild birds are capable of contributing to the persistence of *Salmonella* between batches of pigs. Adequate management practices to minimize the contact between pigs and wild birds (e.g. cover feed and water sources, use of nets) should be implemented in outdoor pig units.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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1 **Table 1**. *Salmonella* isolated from environmental and wild bird dropping samples (not collected in enrichment media) from the two fields sampled at

2 the 3 sampling visits. Number of *Salmonella* positive samples/number tested, serotyping results are also reported. The number of positives for each

3 category is shown in brackets.

Field	Samples	Farm visit	*Salmonella* positives/tested	Prevalence (%)	95% CI	Serotype
A	Bird dropping	1	1/19	5.3	0.1-26.0	Typhimurium (1)
		2	7/57	12.3	5.1-23.7	4,5,12:i:- (3), Senftenberg (3),Typhimurium (1)
		3	3/46	6.5	1.4-17.9	4,5,12:i:- (1), Rissen (2)
		Total	11/122	9.0	4.6-15.6	
	Environment[a]	1	2/12	16.7	2.1-48.4	4,5,12:i:- (2)
		2	3/54	5.6	1.2-15.4	4,5,12:i:- (3)
		3	3/63	4.8	1.0-13.3	4,5,12:i:- (3)
		Total	8/129	6.2	2.7-11.9	
B	Bird dropping	1	1/8	12.5	0.3-52.7	
		2	0/12	0.0	0.0-26.5	
		3	24/25	96.0	79.7-99.9	4,5,12:i:- (23)
		Total	25/45	55.6	40.0-70.4	
	Environment[a]	1	28/43	65.1	49.1-79.0	4,5,12:i:- (16), Rissen (4), Panama (1)
		2	25/47	53.2	38.1-67.9	4,5,12:i:- (2), Rissen (3)
		3	28/35	80.0	63.1-91.6	4,5,12:i:- (23), Rissen (3), Derby (1)
		Total	81/125	64.8	55.8-73.1	
Total			125/421	29.7	25.4-34.3	

4 [a] soil and water puddle samples

Table 2. *Salmonella* isolated from environmental and wild bird dropping samples (not collected in enrichment media) from the two fields sampled at the 3 sampling visits. Enumeration and serotyping results are also reported. Number of isolates that were serotyped is shown in brackets.

Field	Samples	Farm visit	Count (CFU/g)	Serotype
A	Bird dropping	1	1-10	Typhimurium (1)
		2	1-10	Senftenberg (2), Typhimurium (1)
			10-10^2	Senftenberg (1)
			10^3-10^4	4,5,12:i:- (1)
			10^4-10^5	4,5,12:i:- (1)
			10^5-10^6	4,5,12:i:- (1)
		3	1-10	Rissen (1)
			10-10^2	Rissen (1)
			10^3-10^4	4,5,12:i:- (1)
	Environmental*	1	1-10	4,5,12:i:- (2)
		2	1-10	4,5,12:i:- (3)
		3	1-10	4,5,12:i:- (1)
B	Bird dropping	3	1-10	4,5,12:i:- (3)
			10-10^2	4,5,12:i:- (6)
			10^2-10^3	4,5,12:i:- (8)
			10^3-10^4	4,5,12:i:- (4)
			10^5-10^6	4,5,12:i:- (2)
	Environmental*	1	1-10	4,5,12:i:- (14), Rissen (3), Panama (1)
			10-10^2	4,5,12:i:- (2), Rissen (1)
		2	1-10	4,5,12:i:- (2), Rissen (3)
		3	1-10	4,5,12:i:- (17)
			10-10^2	4,5,12:i:- (5), Rissen (3)
			10^2-10^3	4,5,12:i:- (1), Derby (1)

* Environmental= soil and water puddle samples

12 **Table 3**. Prevalence of *Salmonella*-positive pig individual faecal samples from pigs at visit 1.

13 Number of *Salmonella* positive samples/number tested, enumeration and serotyping are also

14 reported. Number of isolates that were serotyped is shown in brackets.

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Samples	*Salmonella* positives/tested	Prevalence %	95% CI for prevalence	Count (CFU/g)	Serotype
Weaners	42/60	70.0	56.8-81.2	1-10	4,5,12:i:- (5) Rissen (1)
				$10-10^2$	4,5,12:i:- (2)
				10^2-10^3	4,5,12:i:- (8)
				10^3-10^4	4,5,12:i:- (3)
Growers	55/60	91.7	81.7-97.2	1-10	4,5,12:i:- (1)
				$10-10^2$	4,5,12:i:- (6)
				10^2-10^3	4,5,12:i:- (7)
				10^3-10^4	4,5,12:i:- (1), Rissen (2)
				10^5-10^6	4,5,12:i:- (2)
total	97/120	80.8	72.6-87.4		

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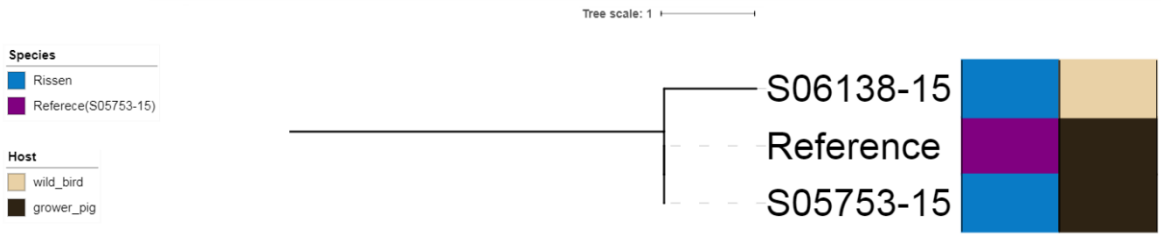
Table 4. *Salmonella* isolated in swab samples collected in enrichment media from the two fields sampled at the 3 sampling visits. Number of *Salmonella* positive samples/number tested, and serotyping are also reported. Number of isolates that were serotyped is shown in brackets.

Field	Samples	Farm visit	no. positives/ no. tested	Serotype
A	Environmental	2	3/13	Rissen (3)
		3	5/12	
B	Environmental	1	5/5	4,5,12:i:- (3)
		2	5/20	
		3	0/20	
	Bird dropping	2	5/7	4,5,12:i:- (2)
		3	8/8	Rissen (1)
	Weaner	1	15/18	4,5,12:i:- (3), Panama (1)
	Grower	1	17/17	4,5,12:i:- (2), Rissen (3)

* Environmental= farm equipment samples

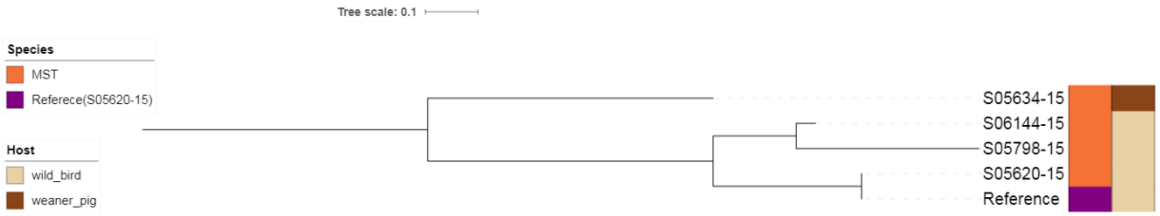
Table 5. Odds Ratio (OR) for environmental and wild bird dropping (not collected in enrichment media) from field A and B at the 3 sampling visits.

Sample	Field	Positive	Negative	Total	P	OR	95%CI for OR
	B	25	20	45	< 0.001	12.6	5.4-29.6
Bird dropping	A	11	111	122			
	Total	36	131	167			
	B	81	44	125	< 0.001	27.8	12.5-62.2
Environment	A	8	121	129			
	Total	89	165	254			
	B	106	64	170	< 0.001	20.2	11.5-35.4
Total	A	19	232	251			
	Total	125	296	421			



	Reference	S05753-15	S06138-15
Reference	0	0	1
S05753-15	0	0	1
S06138-15	1	1	0

Figure 1. Maximum likelihood core genome SNP phylogeny of 2 *Salmonella* Rissen isolates S06138-15 and S05735-15 with assembled draft genome of S05735-15 used as reference. Figure created with Interactive Tree of Life (iTOL) (https://itol.embl.de). SNP, single-nucleotide polymorphism. Table: Number of SNPs in each isolate compared to the reference genome.



	Reference	S05620-15	S05634-15	S05798-15	S06144-15
Reference (bird)	0	0	9	6	4
S05620-15 (bird)	0	0	9	6	4
S05634-15 (pig)	9	9	0	9	9
S05798-15(bird)	6	6	9	0	4
S06144-15(bird)	4	4	9	4	0

Figure 2. Maximum likelihood core genome SNP phylogeny of 4 monophasic *Salmonella* Typhimurium isolates S05620-15, S05634-15, S05798-15 and S06144-15 with assembled draft genome S05620-15 used as reference. Figure created with Interactive Tree of Life (iTOL) (https://itol.embl.de). SNP, single-nucleotide polymorphism. Table: Number of SNPs in each isolate compared to the reference genome.