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Original Article

Role of Wild Bird and Rodents in the Epidemiology of Subclinical Salmonellosis in Finishing Pigs

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Abstract

Wild birds and rodents may play an important role in the dynamics of subclinical pig salmonellosis, either as the introducers of the bacteria into the farm or as receptors of an infection already established in the farm. We tried to gain further insight into the epidemiology of this infection by studying the phenotypic (i.e., serotype and antimicrobial resistance patterns) and molecular characteristics of *Salmonella* strains isolated from samples collected from pigs and wildlife captured in the vicinity of pig farms. *Salmonella*-positive pig fecal samples were identified in 56.1% of the 41 farms investigated. Birds shedding *Salmonella* spp. were detected in 21.4% of the farms despite the low numbers of birds captured in many farms. Most *Salmonella* isolates from birds (74%) did not show any antimicrobial resistance (AR) pattern and belonged to phage types rarely seen in the pig population (U310, DT56, DT137, DT164), supporting the likely avian source of infection for most birds. The proportion of farms showing *Salmonella*-infected rodents was higher (46.2%), with *Salmonella* isolates showing a high homology with those likely originated from pigs. *Salmonella*-positive environmental samples were found in >50% of the farms, and the characteristics of these *Salmonella* strains supported the idea of pigs as a major source of *Salmonella* contamination of the farm environment. Dissemination of *Salmonella* in pig farms from areas of high *Salmonella* prevalence appeared to depend to some extent upon rodents and wild birds present in the farm, but the role of rodents in its maintenance seemed to be somewhat more relevant than that of birds. In conclusion, activities aimed at reducing the contact of these wild species with pigs will probably assist in the control of pig salmonellosis. Strict hygienic measures should be considered in areas of high prevalence of infection to lower the high load of environmental contamination.

Introduction

Pigs and pork products are major sources of human salmonellosis (Hauser *et al.,* 2010; Hopkins *et al.,* 2010; Gallati *et al.,* 2013), and could be responsible for more than 50% of cases in Europe (EFSA, 2012). Asymptomatically infected pigs are the primary source of contamination of pork products (Dickson *et al.,* 2002), but pig salmonellosis is not specifically addressed by the meat-inspection system at the abattoir (EFSA, 2011). Thus, the control of the infection at previous stages in the pork production chain (farm level and transport) is of utmost importance to decrease the load of *Salmonella* arriving at the abattoir (Bahnson *et al.,* 2006).

Salmonella infection at the farm level depends on many factors such as pen cleaning and disinfection, biosecurity, feed type, herd size, mixing of pig batches, proximity between swine herds, etc. (Fosse *et al.,* 2009; Rostagno *et al.,* 2012). The importance of each of these factors may differ depending upon the presence of other factors and their interactions. Thus, approaches to control pig salmonellosis should be tailored for each farm based on its own characteristics (De Busser *et al.,* 2013).

Specific biosecurity measures for preventing the introduction of the infection into the farm are proposed according to the different sources of contamination identified. Pests (i.e., rodents and wild birds) appear to play an important role

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as sources of *Salmonella* spp. for swine (Funk and Gebreyes, 2004; Tizard, 2004; Backhans *et al.,* 2013; Horton *et al.,* 2013). Pig farms without rodent-control programs are at higher risk of salmonellosis (Mejía *et al.*, 2006; Vico *et al.,* 2011), and the lack of bird-proof nets has been positively associated with *Salmonella* seropositivity in finishing pigs (Bahnson *et al.,* 2001; Creus *et al.,* 2004; Vico *et al.,* 2012). A reduction up to 10–20% of infection prevalence in slaughter pigs is anticipated if *Salmonella* infection from rodents and birds is prevented (EFSA, 2010).

However, the role that pests may be playing as reservoirs of *Salmonella* for pigs needs to be further characterized, as they may behave mostly as mere receptors of the infection from pigs instead of a major cause of introduction of the bacteria into the pig premises. Actually, *Salmonella* prevalence in wild birds is usually low, but birds near farm premises show higher probability of shedding *Salmonella* spp. than birds living far from livestock (Craven *et al.,* 2000; Andrés *et al.,* 2013).

We tried to gain further insight into the epidemiology of pig salmonellosis by looking at the relationship between *Salmonella* strains isolated from wild animals captured in the vicinity of pig farms, or their droppings, and strains from pig feces, through phenotypic and genotypic analyses.

Materials and Methods

Sample collection

Between March 2010 and September 2012, a convenience sample of 41 fattening-pig farms from farmers willing to collaborate were selected from an area (NE of Spain) presenting high prevalence of pig salmonellosis (Vico *et al.,* 2011). Average farm size was 2000 pigs (range: 500–6000). Four randomly selected pens from each individual fattening unit in the farm were sampled. A pool of five individual fresh fecal samples was collected from each pen. Collection of samples from wild birds, rodents, and the farm environment was also carried out during the same day.

Mist netting was the method used to trap wild birds. Four nets (2.5 × 9 m each) were set up in locations where birds were usually spotted, and these nets were used during the entire morning. Birds were captured either from inside the premises or within a 200-m radius from the farm, identified, and kept in sterilized dark cages until they defecated. Droppings were collected through sterile swabs for bacteriology, and birds were tagged and released. When many birds were captured simultaneously, they were grouped by species and pooled samples were considered.

Rodents were trapped using snap traps situated by pest control experts during the night previous to the sampling day on different farm locations. For each animal, the whole intestine, the liver, and the spleen were the samples taken for bacteriology.

Environmental samples consisted of a pool of bird and/or rodent droppings collected from locations pigs never have direct access to and could not contaminate directly (window ledges, top of pen walls, underneath outside feed silos), but they could have been contaminated with dust, insects, etc. The number of samples collected in each farm was variable, depending upon the observation of dropping accumulations in the farm.

Salmonella *spp. isolation and characterization*

Samples were processed and cultured following the procedure described by the ISO 6579:2002/Amd.1:2007 (ISO, 2007) with slight modifications (i.e., the culture on selective Modified Semisolid Rappaport Vassiliadis [MSRV] was performed by triplicate to increase the probability of *Salmonella* detection). When more than one MSRV plate was positive, then at least two were further cultured on selective media. Serotyping was performed following the White–Kauffman–Le Minor scheme (Guibourdenche *et al.,* 2010) at the National Reference Laboratory (NRL) for Animal Salmonellosis (Madrid, Spain) and phage typing of *Salmonella* Typhimurium and monophasic variant of *Salmonella* Typhimurium (*Salmonella* 4,[5],12:i:-) strains at the NRL for Human Salmonellosis (Madrid, Spain) (Anderson *et al.,* 1977).

Susceptibility to a panel of 10 antimicrobials (i.e., ampicillin, chloramphenicol, streptomycin, sulfisoxazole, trimethoprim, tetracycline, gentamicin, nalidixic acid, ciprofloxacin and cefotaxime) was determined by the Kirby-Bauer disc-diffusion method (Murray *et al.*, 2003). *Escherichia coli* ATCC 25922 and both *Salmonella* Typhimurium ATCC 14028 and DT104 reference strains were used as controls. *Salmonella* strains were classified as resistant, intermediate, or susceptible according to the Clinical and Laboratory Standard Institute recommendations (CLSI, 2012). In order to establish the antimicrobial resistance (AR) profiles, antimicrobials were grouped into the following classes: aminopenicillins (A), phenicols (C), aminoglycosides (S), sulphonamides and dihydrofolate reductase inhibitors (Su), tetracyclines (T), cephalosporins (Cf) and quinolones (Na).

Pulsed-field gel electrophoresis (PFGE) was used for the genotyping of all *Salmonella* isolates (Ribot *et al.,* 2006). Details on the PFGE procedure are described elsewhere (Andrés *et al.,* 2013).

Statistical analysis

A farm was classified positive for a given type of sample when *Salmonella* spp. was isolated from at least one of these samples. The Mann–Whitney *U* test was used to assess the association between the number of samples collected in the farm and the prevalence of *Salmonella* spp.

Results

Salmonella *isolation*

A summary of results is presented in Table 1. In 23 farms (56.1%), *Salmonella* was isolated from pig feces. Overall, 54 (13.6%) pig fecal samples were positive.

Wild birds were captured in all farms but in variable numbers (median: 23; range 1–192). They belonged to 47 different species. Birds shedding *Salmonella* spp. were detected in 9 (21.9%) farms. The median number of captured birds in the positive farms was larger than in the negative ones (58 vs. 17, respectively; $p < 0.005$). Out of 672 fecal samples from 1433 birds, 27 (4%) were positive.

Eighty-eight rodents (30 rats and 58 mice) were trapped in 13 farms (median: 4; range: 1–20) and 9 (10.2%) were infected. *Salmonella*-infected rodents were found in 46.2% of these farms. No significant differences regarding the number of captured rodents were observed between positive and negative farms ($p = 0.61$).

Table 1. Number of Sampled Farms According to Sample Type, Number, and Percentage of *Salmonella*-Positive Farms and Samples, and *Salmonella* Serotypes/Subspecies Identified

Type of sample	*No. farms*	*No. (%) of positive farms*	*No. samples*	*No. (%) of positive samples*	Salmonella *serotypes/subspecies (%)*
Pig feces	41	23 (56.1)	397	54 (13.6)	Typhimurium (37.5) 4,[5],12:i:- (35.7) Rissen (17.8) Anatum (5.4) Brandenburg (3.6)
Wild bird feces	41	9 (21.9)	672	27 (4.0)	Typhimurium (70.4) Anatum (11.1) subsp. *diarizonae* (11.1) Mikawasima (3.7) subsp. *arizonae* (3.7)
Farm rodents[a]	13	6 (46.2)	88	9 (10.2)	Typhimurium (55.5) Brandenburg (22.2) Havana (11.1) Derby (11.1)
Environmental samples	40	21 (52.5)	139	32 (23)	
Bird droppings	33	15 (45.4)	68	20 (29.4)	Typhimurium (30) 4,[5],12:i:- (20) Rissen (15) Bredeney (5) Reading (5) subs. *houtenae*(5) Anatum (5) Mikawasima (5) Havana (5) subs. *arizonae* (5)
Rodent droppings	26	7 (26.9)	39	8 (20.5)	4,[5],12:i:- (50) Brandenburg (12.5) Derby (12.5) Rissen (12.5) Choleraesuis (12.5)
Undetermined droppings from outside feed silos	21	3 (14.3)	32	4 (12.5)	Rissen (50) Anatum (25) Typhimurium (25)

[a]Intestines, spleens, and livers were analyzed.

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Environmental samples were collected in 40 farms (median: 3; range: 1–8), and 21 (52.5%) were *Salmonella* positive. A larger number of samples were collected in the positive farms (median three vs. two, respectively; $p = 0.03$). The proportion of environmental positive samples ranged from 12.5% (underneath the outside silos) to 29% (bird droppings).

Salmonella *characterization*

A total of 125 *Salmonella* strains were isolated from 122 *Salmonella*-positive samples (in 3 samples 2 different serotypes were identified) and 15 serotypes/subspecies were identified. *Salmonella* Typhimurium (42%), *Salmonella* 4,[5],12:i:- (22.4%), and *Salmonella* Rissen (12.8%) were the most prevalent. The distribution of *Salmonella* serotypes/subspecies is shown in Table 1. *Salmonella* 4,[5],12:i:- was found neither in wild birds nor in rodents. The environmental samples showed the greatest diversity of *Salmonella* serotypes.

Almost 80% of the *Salmonella* strains showed AR to at least 1 antimicrobial (Table 2), and 86.6% of the resistant isolates showed AR to ≥3 antimicrobial classes. The most common AR profiles were ASSuT (46.4%) and ACSSuTNa

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Table 2. Classification of *Salmonella* spp. Strains According to Serotype and Antimicrobial Resistance (AR) Characteristics

Serotypes	*No. (%) of strains isolated*[a]	*No. (%) of strains with AR*[b]
Typhimurium	52 (41.6)	31 (59.6)
4,[5],12:i:-	28 (22.4)	28 (100)
Rissen	16 (15.4)	16 (100)
Anatum	8 (6.4)	8 (100)
Brandenburg	5 (4.0)	5 (100)
Derby	2 (1.6)	2 (100)
Havana	2 (1.6)	1 (50)
Mikawasima	2 (1.6)	0 (0)
Other (7 serotypes)	10 (8.0)	6 (54.5)
Total: 15	125	97 (77.6)

[a]Percentage out of the total number of strains isolated.
[b]Percentage of strains with AR to at least one agent out of the total number of the strains for that serotype.

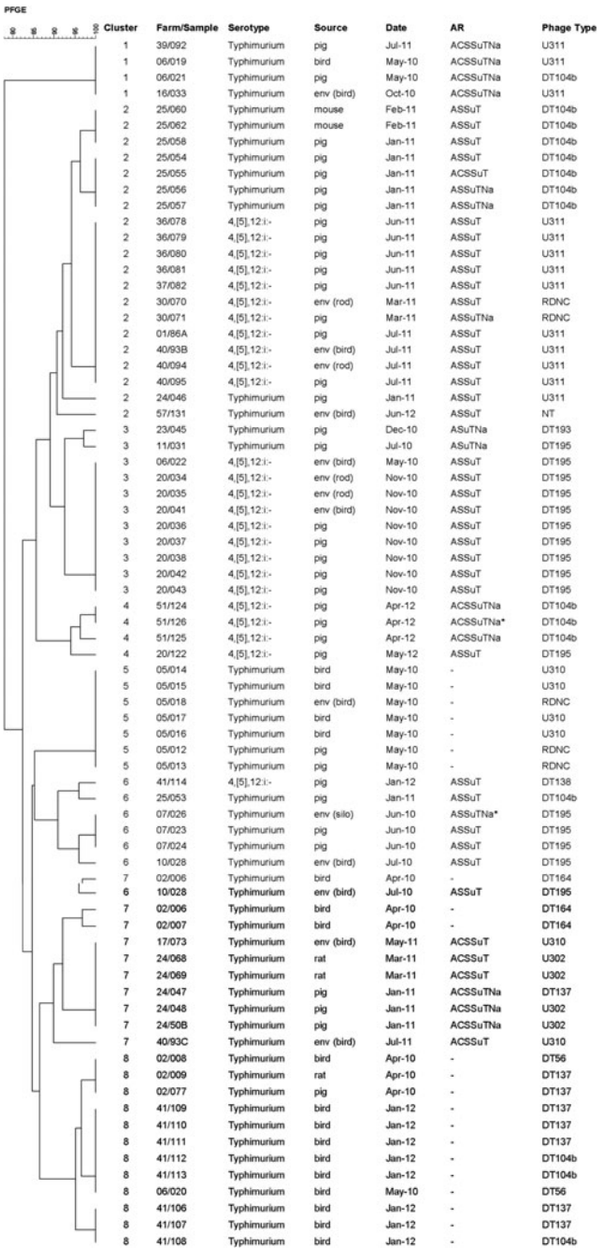

FIG. 1. Dendrogram showing the main *Xba*I patterns (>90% homology) for the *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- strains isolated along with their origin, source, date, antimicrobial resistance (AR) profile, and phage type.

F1 ▶ (14.4%) (Figs. 1 and 2). *Salmonella* strains from pig feces showed the highest AR prevalence (95%), followed by environmental and rodent samples (91% and 77.8%, respectively). By contrast, isolates from wild birds were mostly susceptible to all the antibiotics tested (74%), but the resistant strains showed AR patterns similar to that of the *Salmonella* strains from pig feces from the corresponding farm. Only two (8.7%) *Salmonella*-positive farms showed all their *Salmonella* isolates from pigs to be susceptible to all the antibiotics tested. Interestingly, all the *Salmonella* isolates from wild birds from these two farms were also susceptible to all antimicrobials. All *Salmonella* 4,[5],12:i:- and *Salmonella* Rissen strains showed AR (Table 2), with ASSuT being the most frequent profile (78.6% and 43.7%, respectively). For *Salmonella* Typhimurium, the AR prevalence was lower (59.6%) but with the same predominant AR pattern.

Twelve phage types were recognized among the 80 *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- strains. The most frequent were DT104b (23.1%) for *Salmonella* Typhimurium, and DT195 and U311 (35.7%) for *Salmonella* 4,[5],12:i:- (Fig. 1).

All *Salmonella* isolates except one (*Salmonella* Kapemba) were characterized by PFGE. Sixty *Xba*I patterns grouped into 31 clusters (90% similarity level) were identified. Most isolates (87%) were grouped into 15 clusters (Figs. 1 and 2).

Four clusters consisted exclusively of *Salmonella* Typhimurium strains; all but one originated from different farms and from a variety of sources. Another cluster included only strains of *Salmonella* 4,[5],12:i:- from two different farms. Three clusters consisted of a mix of *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:-.strains (Fig. 1).

Salmonella Rissen strains were isolated from pig feces and environmental bird samples from 8 farms, and were all

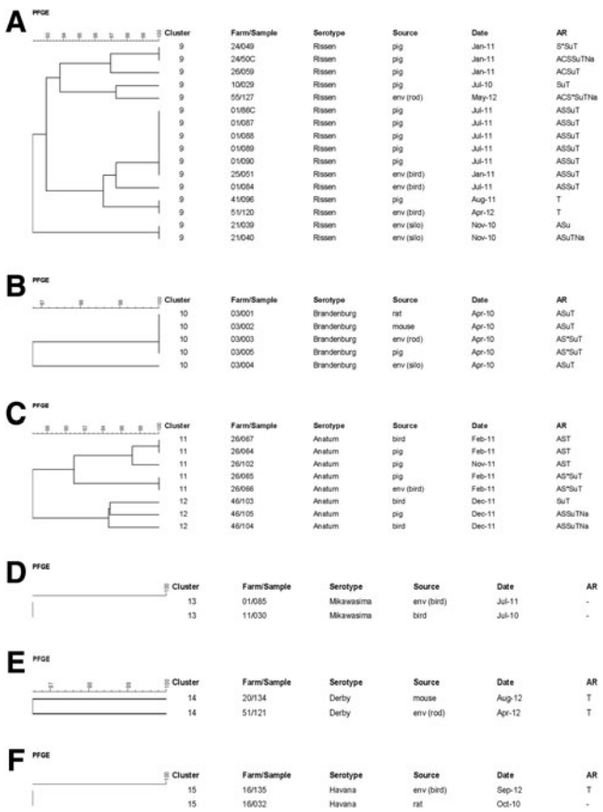

FIG. 2. Dendrogram showing the main *XbaI* patterns (>90% homology) for all the *Salmonella* Rissen **(A)**, *Salmonella* Brandenburg **(B)**, *Salmonella* Anatum **(C)**, *Salmonella* Mikawasima **(D)**, *Salmonella* Derby **(E)**, and *Salmonella* Havana **(F)** strains isolated along with their origin, source, date and antimicrobial resistance (AR) profile. ◀AU9

grouped within a single cluster (92% similarity) (Fig. 2A). Strains of *Salmonella* Brandenburg grouped within the same cluster (97% similarity) and shared similar AR patterns. They originated from two animal sources (rodents and pig feces) from the same farm (Fig. 2B). Eight isolates of *Salmonella* Anatum were grouped into 2 clusters (91% and 95% similarity), which matched with two different farms, each of them including isolates from different animal sources (pig feces and birds) (Fig. 2C). *Salmonella* Mikawasima, *Salmonella* Derby, and *Salmonella* Havana were grouped within its own cluster (100%, 96%, and 100% similarity, respectively) (Figs. 2D, E, and F). Overall, within a cluster it was common to observe different strains from different sources, locations, and isolated on different dates. Most *Salmonella*-positive bird and rodent samples were grouped within clusters containing pig samples (74% and 77.8%, respectively).

Discussion

More than 50% of the farms were *Salmonella* positive when pig feces were analyzed, which was in agreement with that expected for an area of high prevalence of infection. The predominant serotypes *Salmonella* Typhimurium, *Salmonella* 4,[5],12:i:-, and *Salmonella* Rissen, and the prevalence of AR were also in accordance with previous studies in the area (Vico *et al.,* 2011).

Birds shedding *Salmonella* were detected in more than 20% of the farms, indicating the significant circulation of the pathogen around farms. Unlike pig and rodent strains, most of the *Salmonella* Typhimurium isolates from wild birds (84.2%) did not show any AR pattern. They were mostly grouped into two separated PFGE clusters (nos. 5 and 8; Fig. 1), and most of them (76.4%) presented phage types (U310, DT56, DT137, DT164) rarely seen in the Spanish pig population (Instituto de Salud Carlos III, unpublished data), which suggested a likely avian source of infection of these birds. Indeed, the phage type DT56 has been commonly isolated from garden birds in England since 1995 (Hughes *et al.,* 2008; Lawson *et al.,* 2011; Pennycott *et al.,* 2006, 2010), and it appears to be a host-adapted *Salmonella* phage type that would not represent a large zoonotic risk (Hughes *et al.,* 2010). By contrast, bird strains showing AR (some *Salmonella* Typhimurium and all the *Salmonella* Anatum) displayed AR and PFGE patterns similar to those observed in *Salmonella* strains from pig feces from the corresponding farm, suggesting pigs as the main source of infection. These results supported a bidirectional pig–bird infection.

Considering the importance of the avian source of infection for most birds, and the fact that the number of captured birds would be a reflection of bird density, the significant positive relationship between the number of birds caught and the probability of detecting *Salmonella* in their feces may be explained by the greater risk of *Salmonella* transmission among birds in highly bird-populated areas. Therefore, activities aimed at preventing bird concentrations around the farm premises may help in the control of spillover infections from birds to pigs.

Rodents were trapped in few farms (13), which was explained by the presence of rodent-control methods in many farms. The proportion of farms showing *Salmonella*-infected rodents was high (46.2%) regardless of the number of rodents caught, indicating a high prevalence of salmonellosis in these animal species. A close relationship between *Salmonella* strains from farm rodents and pigs was evidenced by several findings. First, *Salmonella* Typhimurium was the most prevalent serotype in rodents, and it was always related to *Salmonella* Typhimurium from pig fecal samples (clusters nos. 2, 7, and 8; Fig. 1). In fact, *Salmonella* strains from rodents and pig samples from the same farm usually displayed a high percentage of PFGE homology. Second, two of the three phage types observed in farm rodents (DT104b and U302) are common in pigs (de la Torre *et al.,* 2003; Gebreyes *et al.,* 2004). In addition, rodent strains showed a high prevalence of AR, and AR patterns were similar to those observed in isolates from pigs. These findings supported the significant contribution of farm rodents to the maintenance of *Salmonella* infection in the pig farms.

Analyses of environmental samples showed that the proportion of positive farms was similar to that from pig fecal samples (>50%). The number collected was positively associated with the probability of isolation of *Salmonella*; thus, a more thorough farm sampling may have yielded higher farm prevalence. The prevalence of *Salmonella* and of AR was much higher in the environmental bird feces than when samples were collected directly from live birds (29% vs. 4%, and 90% vs. 26%, respectively). In addition, 78% of these strains showed PFGE patterns similar to those from *Salmonella* strains from pig feces. Since samples could be considered a composite of bird droppings and dust, these discrepancies may be explained by cross-contamination of the bird droppings due to the highly contaminated environment of the pig farms. Indeed, *Salmonella* spp. have been isolated from air and droplets from places very much cleaner than pig farms, such as abattoirs (Schmidt *et al.,* 2012), and insects, such as flies and cockroaches, are also considered potential carriers of the infection within the farm (Devi and Murray, 1991; Wang *et al.,* 2011). Another explanation may have to do with the fact that environmental samples usually included a large number of droppings, thus likely representing a large number of birds and therefore higher chances of detecting a positive individual. In any case, these results suggest that environmental *Salmonella* contamination was common in these pig farms, and emphasize the capacity of *Salmonella* spp. to survive in places remote from the pigs (Gotter *et al.,* 2011; Nathues *et al.,* 2013), and the need for significant improvements in the overall hygiene of these farms.

The incidence of *Salmonella* 4,[5],12:i:- in Spain has increased significantly since 1997 (de la Torre *et al.,* 2003). In this study, it was not detected either in wild birds or rodents, despite the fact that it was highly prevalent in pig and environmental samples. Interestingly, this serotype has been commonly isolated from dead Eurasian siskin (*Carduelis spinus*) in Germany (Hauser *et al.*, 2009), and from cattle egret (*Bubulcus ibis*) chicks in the United States (Phalen *et al.*, 2010). Both studies suggest that infection with *Salmonella* 4,[5],12:i:- may often be fatal, which could explain why it was not detected in this apparently healthy bird population. With regard to rodents, there are no published reports for this serotype, suggesting a very low prevalence in these species, which is supported by our results. The fact that *Salmonella* 4,[5],12:i:- was only isolated from pigs and environmental samples further supports the idea of pigs as a major source of *Salmonella* contamination of the farm environment.

As expected, some *Salmonella* 4,[5],12:i:- isolates clustered together with *Salmonella* Typhimurium (Fig. 1), evidencing that the former would be a mutation of the latter (de la Torre *et al.,* 2003; Zamperini *et al.*, 2007). In addition, the PFGE dendrogram showed that the majority of the clusters included isolates from different sources collected in distinct farms and on different dates, in most cases sharing the same phage type and even a similar AR profile (Figs. 1 and 2). These similarities indicated a clear dissemination and persistence of some strains throughout the region. A few PFGE clusters were composed exclusively by *Salmonella* isolates with very similar or even indistinguishable band patterns from the same farm (cluster nos. 5, 10, 11, and 12; Figs. 1 and 2) but isolated from different sources (pigs, birds, rodents, and the environment), showing a manifest circulation of these strains among different host species within a farm.

Conclusions

It appears that the dissemination of *Salmonella* in the pig farms from areas of high *Salmonella* prevalence may depend to some extent upon rodents and wild birds present in the farm. Thus, activities aimed at reducing their contact with pigs will probably assist in the control of pig salmonellosis. Given the high degree of homology between *Salmonella* isolates from rodents and pigs, and the apparent higher prevalence of salmonellosis in rodents compared to birds, the role of rodents in the maintenance of *Salmonella* within a pig farm seems to be somewhat more relevant than that of wild birds. However, the prevalence of *Salmonella* in birds may have been underestimated, since the number of captured birds was low in many farms, so the chances of observing similarities between bird and pig isolates were low. The infection appears to be bidirectional. Thus, a decrease of *Salmonella* prevalence in pigs would surely contribute to decrease the risk of infection in the wild bird and rodent populations (Backhans *et al.,* 2012). Strict hygienic measures should be implemented in high prevalence areas, given the considerable load of environmental contamination originating from pigs.

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Disclosure Statement

No competing financial interests exist.

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