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Epidemiology of subclinical salmonellosis in wild birds from an area of high prevalence of pig salmonellosis: phenotypic and genetic profiles of *Salmonella* isolates

S Andrés[1], JP Vico[1], V Garrido[2], MJ Grilló[2], S Samper[3], P Gavín[3], S Herrera-León[4], RC Mainar-Jaime[1]*

[(1)]Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA). Avda. Montañana, 930. 50059 Zaragoza, Spain;

[(2)]Instituto de Agrobiotecnología (CSIC-UPNA-Gobierno de Navarra). 31006. Pamplona, Spain;

[(3)]IIS Aragón, Laboratorio Investigación Molecular, Hospital Universitario Miguel Servet. 50009 Zaragoza, Spain;

[(4)]Sección de Enterobacterias, Servicio de Bacteriología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid, Spain.

*Corresponding author: rcmainar@aragon.es

Place where most of the work was carried out: Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA). Avda. Montañana, 930. 50059 Zaragoza, Spain

Summary

The epidemiology of subclinical salmonellosis in wild birds in a region of high *Salmonella* prevalence in pigs was studied. Three hundred and seventy nine fecal samples from 921 birds trapped in 31 locations nearby pig premises and 431 samples from 581 birds of 10 natural settings far from pig farms were analyzed for the presence of *Salmonella* spp. Positive samples were serotyped and analyzed for antimicrobial resistance (AR). Phage typing and PFGE on *S.* Typhimurium isolates were also carried out. The overall proportion of *Salmonella* positive samples was 1.85% (95%CI=0.93-2.77). *Salmonella* isolation was positively associated with samples collected from birds in the proximity of a pig operation (OR= 16.5; 95%CI=5.17, 52.65), and from non-migratory (or short distance migration) birds (OR=7.6; 95%CI=1.20, 48.04), and negatively related to mostly granivorous birds (OR=0.4; 95%CI=0.15, 1.13). *Salmonella* Typhimurium was the most prevalent serotype and 4 different *XbaI* PFGE patterns were observed that matched the 4 phage types identified (U310, U311, DT164, DT56). Only 20% of the strains showed multi-AR. In 3 farms a high degree of homogeneity among isolates from different birds was observed. These findings suggested that pig farms may act as amplifiers of this infection among wild birds, and the degree of bird density may have much to do on this transmission. Some of the *Salmonella* serotypes isolated from bird feces were of potential zoonotic transmission and associated with AR. Monitoring salmonellosis in wild bird is advised.

KEY WORDS: Salmonellosis; wild birds; prevalence; antimicrobial resistance; bacteriology; Spain.

Impacts

- In areas where pig salmonellosis is highly prevalent pig farms may act as amplifiers of salmonellosis among wild birds, regardless the origin (pig or bird) of the *Salmonella* strains infecting the birds.
- Although prevalence of *Salmonella* spp. among wild birds is low, birds can carry *Salmonella* serotypes of potential zoonotic transmission and sometimes associated with antimicrobial resistance, thus monitoring of wild bird salmonellosis in these areas is advised.
- Long-distance migration birds were less likely to carry *Salmonella* spp., although dispersion of this pathogen through this type of birds cannot be discarded.

Introduction

Wild birds are considered as potential sources for zoonoses as they are natural hosts for enteropathogens such as *Salmonella* or *Campylobacter* spp., leading zoonotic pathogens in the developed world (Chomel et al., 2007). Birds can acquire these pathogens from contaminated environments and spread it directly to humans or indirectly by contaminating commercial livestock operations (Alley et al., 2002, Daniels et al., 2003). They could also acquire drug-resistant microorganisms from livestock farms and disseminate these strains into the human population, hence contributing to the global spread of emerging infectious diseases (Guenther et al., 2011, Reed et al., 2003).

Salmonella is considered a ubiquitous agent that usually colonizes asymptomatically the guts of birds and can be further excreted through their feces (Connolly et al., 2006). It is

also relatively common to associate avian salmonellosis with die offs of back-yard passerine birds (Alley et al., 2002, Refsum et al., 2003) or with sick birds arriving to wildlife rehabilitation centers (Molina-Lopez et al., 2011, Reche et al., 2003). Reports on unapparent *Salmonella* carriers are less common, although from a zoonotic point of view, these birds would be the most problematic animals for people and livestock due to the potential risk they pose. Wild birds have been implicated as source of human infection and contamination of feed (Hoelzer et al., 2011), and of outbreaks of clinical salmonellosis in livestock (Luque et al., 2009).

The prevalence of *Salmonella* infection among wild birds is variable but appears to be low (Kobayashi et al., 2007, Kirk et al., 2002, Fallacara et al., 2001, Gaukler et al., 2009, Brittingham et al., 1988, Cizek et al., 1994). Factors such as season, feeding behavior or migration patterns, may influence on the prevalence of salmonellosis in free-ranging birds (Skov et al., 2008). For instance, clinical salmonellosis has been associated with winter months (Refsum et al., 2002). Raptors that usually prey on sick or dead animals may be infected with *Salmonella* spp. at higher proportions than non-predators birds (Millan et al., 2004, Molina-Lopez et al., 2011, Reche et al., 2003). Likewise, birds feeding on the ground may have higher chances of getting infected than those feeding from hanging feeders (Refsum et al., 2003). Long-distance migrations may also enhance susceptibility to certain diseases (Reed et al., 2003). In addition, environments with high levels of *Salmonella* contamination (urban settings, livestock facilities, etc.) may be a potential source of infection for those species of wild birds more adapted to these places (Cizek et al., 1994, Gaukler et al., 2009, Skov et al., 2008).

Thus, some aspects on the *Salmonella* infection in apparently healthy wild birds, i.e. its relationship with migration patterns or other potential risk factors, the relatedness among *Salmonella* strains isolated from different birds, or their levels of antimicrobial resistance, are of utmost interest in order to gain further insight into the epidemiology of subclinical salmonellosis in wild birds. In addition, this knowledge may help further in identifying potential epidemic *Salmonella* strains (Brouwer et al., 2011), and in the ensuing design and implementation of control measures against this infection both in human and production animals.

Materials and methods

Sample collection

Birds were trapped between September 2009 and October 2011 in an area from the Northeast of Spain (provinces of Zaragoza and Huesca) that had shown a high prevalence of pig salmonellosis (Vico et al., 2011). Mist netting was the method used to trap birds in 31 locations nearby pig premises (birds were trapped either from inside the premises or within 200 m radius), hereafter "near pig premises site" (NPPS), and in 10 natural settings far (> 2km) from pig farms and mostly related to bank rivers and forests (far from pig premises site -FPPS-).

Once birds were identified they were kept in sterilized cages under a dark environment to reduce stress until they defecate. Bird droppings were collected through sterile swabs for bacteriological processing. Afterwards, birds were released after being measured and tagged by a licensed bander. When many birds were captured simultaneously, they were

grouped by species and kept together in the same cage. Thus, pooled samples of a variable number of birds were obtained instead of individual samples.

Salmonella spp. isolation

Fecal samples were processed within the same day of collection. All samples were cultured following the procedure described by the ISO 6579:2002/DAM 2005 (Anonymous, 2005a) after slight modifications. Briefly, approximately 0.1 or 1 grams of, respectively, individual or pooled fecal samples were homogenized in, respectively, 0.9 or 9 ml (around a 1:10 dilution) of buffered peptone water (BPW) (Panreac Química SAU, Castellar del Vallés, Spain) for 18±2 hours at 37±1 ºC. To try to increase the sensitivity of the ISO 6579:2002 method, 100 µl of the incubated BPW interface were inoculated by triplicate onto Modified Semi-solid Rappaport Vassiliadis (MSRV) (Oxoid Ltd., Hants, England) medium plates (3 plates containing 100 µl/plate distributed in 3 drops of around 33.3 µl/drop) and plates were incubated for 24±3 h at 41.5±1 ºC. If typical halo was observed on any of the plates at 24 or 48 hours, a 1 µl loop of the growth area was plating on the surface of two selective media (Xylose Lysine Desoxycolate -XLD- and Brilliant Green -BG-) (Laboratorios MICROKIT, Valdemorillo, Spain). Suspected colonies were confirmed biochemically (Triple sugar iron -TSI- agar, urea agar, L-Lysine decarboxylation broth, and indol reaction) (Panreac Quimica SAU), and one representative colony was sent to the *Centro Nacional de Salmonelosis Animales* (Madrid, Spain), for serotyping according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). Bacteriophage typing of all *Salmonella* Typhimurium isolates was performed at *Instituto de Salud Carlos III*

Salmonellosis Reference Centre (Madrid, Spain) according to the methods previously described (Anderson et al., 1977).

Antimicrobial resistance (AR)

Salmonella isolates were tested against a panel of 10 antimicrobials (i.e. nalidixic acid, ciprofloxacin, cefotaxime, ampicillin, chloramphenicol, streptomycin, gentamicin, sulfisoxazole, trimethoprim, and tetracycline) using the Kirby-Bauer disk diffusion method (Murray *et al.*, 2003), and following the antimicrobial concentrations recommended by the European Committee on Antimicrobial Susceptibility Testing (Anonymous, 2007), and the Clinical and Laboratory Standards Institute (CLSI) (Anonymous, 2005b). *Salmonella* strains were classified as resistant (R), intermediate (I) or susceptible (S), according to the CLSI guidelines.

Genotyping

Salmonella isolates were genotyped by pulsed-field gel electrophoresis (PFGE) according to the Pulse-Net protocol (Ribot et al., 2006). Briefly, genomic DNA was prepared by embedding cells of *Salmonella* isolates in agarose plugs (Lonza, Rockland, ME, USA) and lysing the cells using sarcosyl (Sigma-Aldrich Co., St. Louis, MO, USA) and proteinase K (Ambion Inc., Austin, TX, USA). *Salmonella* Braenderup H9812 (Culture Collection, University of Göteborg, Sweden) was used as molecular size marker. After digestion of genomic DNA with the restriction enzyme *XbaI* (Roche Diagnostics, Mannheim, Germany), the electrophoresis to separate fragments by size was carried out using the CHEF-DR III system (BioRad, U.S.A.). The PFGE pulsing

and running conditions were an initial 2.2 sec to a final 64 sec for 17 hr and at 6 V/cm at 14ºC. BioNumerics software (version 6, Applied Maths, Belgium) was used to compare the PFGE patterns by cluster analysis using Dice coefficient and unweighted-pair group method with arithmetic averages (UPGMA dendrogram type) with a position tolerance of 1.5% and optimization of 2.0%.

Statistical analyses

Since fecal samples were collected either from individual birds or from birds in groups (pooled samples), estimates of individual prevalence of *Salmonella* in birds were not possible. Thus, only rough estimates (i.e. minimum and maximum possible values) of *Salmonella* prevalence in birds were calculated. The overall proportion of *Salmonella*-positive samples and their 95% confidence intervals was also estimated.

Unweighted chi-squared analyses were used to compare the proportion of *Salmonella*-positive samples by factors such as location (NPPS *vs*. FPPS), season, type of feeding (mostly granivorous *vs*. mostly insectivorous) and migration patterns (long *vs*. short distances/no migration). Multivariable logistic regression was used further to determine major factors associated to prevalence of subclinical salmonellosis. Since the number of pooled samples and the number of animals contributing to a fecal pool may differ among factor categories, a weight variable was included in the model. This weight variable was computed as the inverse of the number of birds contributing to the sample. Since few variables were considered and all of them could be potential confounders regardless their univariable statistical significance, they all were included in the multivariable model to reduce the likelihood of confounding. As birds captured in the

same site were expected to be more alike regarding probability of *Salmonella* infection compared to birds coming from different capture sites, observations were clustered by site of capture and robust estimates of the standard errors of the coefficients obtained. The software Intercooler Stata 12.0 (StataCorp LP, College Station, Texas) was used for all statistical analyses.

Results

Birds from 50 different species were captured during the two-year period of the study. Most of them belonged to the order of passeriforms and a few to the columbiforms. The number and species captured depended on factors such as the season of the year and the bird habits (i.e. migration patterns, diet, etc.). For instance, blackcaps were trapped mostly at the beginning of the autumn, when they crossed the areas sampled in their way to southern locations for wintering. Around 50% of the birds trapped were considered mostly granivorous. The variety of bird species captured in both locations was quite similar (39 from FPPS *vs*. 42 from NPPS).

Investigation of the presence of *Salmonella* spp. was performed on a total of 810 fecal samples corresponding to 1.502 birds. Three hundred and seventy nine samples (921 birds) were from NPPS and 431 (581 birds) from FPPS. On average each pooled sample represented 3.7 (95% CI= 3.3 to 4.1) individual birds. *Salmonella* spp. was isolated in 15 (1.85%; 95%CI=0.93-2.77) of the fecal samples collected. The overall *Salmonella* prevalence in the captured birds ranged between 1% (from a minimum of 15 *Salmonella*-positive birds out of 1.502) and 4.4% (from a maximum of 66 out of 1.502).

The proportion of *Salmonella* positive samples was significantly higher ($P<0.001$) when collected from birds captured in NPPS (3.46%) than from birds in FPPS (0.46%) (Table 1). It was also significantly higher in samples collected in spring (4.44%) than in samples from birds captured during the other seasons (average of 0.8%) (Table 1). However, no significant differences were observed in the proportion of *Salmonella*-positive samples regarding feeding diets (Table 1). In addition, samples from migratory (long distance) birds presented lower proportion of *Salmonella* positive samples (0.6%) than those from non-migratory or short distance migratory birds (2.17%), but this difference was not significant in the univariable analysis (Table 1). Ranges of estimated *Salmonella* prevalence in birds for the different factors considered in this study are presented in Table 1.

In the multivariable analysis the proximity of the capture site to a pig operation remained as the main significant factors associated with *Salmonella* positive samples, followed by migration patterns (Table 2). Salmonellosis was much more prevalent in samples from birds captured in the vicinity of pig premises (Odds Ratio (OR) = 16.5) or when the birds were considered non-migratory (or travelled mostly short distances) (OR= 7.6). Seed-feeder birds presented a lower probability of finding positive samples compared to birds feeding mostly on insects or invertebrates (OR= 0.4; P=0.087). Regarding season, despite that samples from birds captured during the spring time appeared to have a higher proportion of *Salmonella* positivity (OR= 3.4), this variable was not statistically significant (Tables 1 and 2). A model with possible two-way interactions between significant factors could not be assessed as model convergence could not be reached due to the low number of positive samples.

The characterization of all the *Salmonella* isolates is shown in Table 3. Out of the 13 positive fecal samples from NPPS birds, most came from house sparrows (30.8%), European starlings (23.1%) and rock pigeons (15.4%). The two *Salmonella*-positive fecal samples from FPPS originated both from house sparrows.

Among the isolates collected from NPPS birds *Salmonella* Typhimurium was the most prevalent serotype (69.23%), followed by 4 other serotypes, 3 of which are seldom observed in pigs (*S. enterica* subsp. *arizonae* –IIIa-, *S. enterica* subespecie *diarizonae* - IIIb- and Mikawasima). The last positive sample in this group corresponded to *S.* Anatum, a serotype very common in pigs. Interestingly, one of the two *Salmonella* isolates from the FPPS was the emergent monophasic variant of the Typhimurium serotype (1,4,[5],12:i:-) which showed a pattern of multi-AR to ampicillin, streptomycin, sulfisoxazole, and tetracycline (ASSuT) (Table 3) considered of potential zoonotic transmission.

Overall, the levels of AR were low, with only 3 isolates (20%) presenting multidrug resistance. They belonged to two bird species well adapted to human environments, namely, house sparrow and European starling. Out of these 3 only 1 (33%) come from a FPPS bird (a house sparrow) and corresponded to the monophasic variant of Typhimurium. The other two were serotypes frequently isolated from pigs and presenting AR patterns commonly observed in this animal species (Table 3).

Salmonella Typhimurium isolates were further characterized by phage typing and PFGE. Four clear different *XbaI* PFGE patterns (>90% genetic homology) were observed among the 9 strains of Typhimurium isolated (Figure 1). Four isolates were

100% identical and belonged to samples from European starlings, barn swallows and house sparrows captured around the same pig farm (farm C). Another two isolates (96.8% homology) belonged to two rock pigeons also trapped within the same pig farm (Farm B). Two more isolates (96.6% homology) came from a house sparrow and a blackcap captured at different pig farms (B and D) located around 60 km each other. The last genetic profile belonged to a single isolate from a European starling (farm D).

Four phage types were identified among the Typhimurium isolates, which matched perfectly with the four PFGE profiles observed. The four isolates 100% identical from one of the farms belonged to phage type U310. The phage types from the two pigeons were DT164, and the last two PFGE-related isolated were DT56. The single phage type corresponding to the starling from farm D was U311.

Discussion

The overall proportion of *Salmonella* positive samples from wild birds captured in this area was low (1.85%). Likewise, the values for the expected *Salmonella* bird prevalence ranged between 1% and 4.3%. These figures agreed with results from many other surveys carried out in different countries on apparently healthy birds that show an overall low *Salmonella* prevalence (Brittingham et al., 1988, Gaukler et al., 2009, Kobayashi et al., 2007). In general, when higher prevalences have been observed, they were usually related to contaminated places (Cizek et al., 1994, Kirk et al., 2002), mortality outbreaks (Alley et al., 2002, Refsum et al., 2003), or birds held at rehabilitation centers (Millan et al., 2004, Molina-Lopez et al., 2011, Reche et al.,

2003). In the surveyed area, no reports of bird die-offs had been noticed during the last years.

As it happens is in other countries (Hudson et al., 2000, Kobayashi et al., 2007, Lawson et al., 2011, Palmgren et al., 2006), *Salmonella* Typhimurium was the most prevalent serotype in the bird samples. Interestingly, the monophasic variant of *S.* Typhimurium (1,4,5,12:i:-) was also detected in one sample from sparrows. The monophasic variant of *S.* Typhimurium was rarely identified before the mid-1990s and is now considered an emerging serotype around the world (Soyer et al., 2009). Monophasic *S.* Typhimurium strains have been shown to have similar virulence and AR characteristics to other strains of *S.* Typhimurium. Recent studies worldwide confirm the rapid emergence and dissemination of monophasic strains in animals and humans. The public health risk posed by these emerging monophasic strains is therefore considered comparable to that of other epidemic *S.* Typhimurium strains (Anonymous, 2010). Currently it is one of the most common serotypes associated with human and swine infections in Spain (Echeíta-Sarrionandia *et al.*, 2011, Vico et al., 2011), but there are no reports of this serotype in passerines. Interestingly, the AR pattern showed by this serotype (ASSuT) matches the one observed for a European clonal line first detected in Italy in the year 2000 and later in Denmark and United Kingdom, which seems to be spreading to other European countries (Lucarelli et al., 2010). This AR pattern is indeed one of the most prevalent in *S.* Typhimurium and its monophasic variant strains isolated from pigs in the surveyed area (Vico et al., 2011). The fact that this serotype has been now isolated from healthy sparrows captured in an area where is prevalent in pigs strongly suggest a pig-to-bird transmission.

The type of specimen collected (feces) and the diagnostic method used may have influenced somewhat on the sample prevalence observed. Shedding *Salmonella* is usually intermittent and infected non-shedders birds may have been overlooked. In addition, the MSRV medium is designed to detect motile *Salmonella* spp. and some serotypes that may affect birds (i.e. *S*. Gallinarum and *S*. Pullorum) are non-motile. However these latter serotypes have not been detected either in previous surveys of wild passerines where other less selective culture protocols were used (Kirk et al., 2002, Kobayashi et al., 2007, Pennycott et al., 2010, Tizard, 2004). An additional drawback was the expected limited sensitivity of bacteriology to detect *Salmonella* on feces (Hurd et al., 2004, Mainar-Jaime et al., 2008). With the aim of reducing this detection bias, samples were cultured by triplicate on MSRV (i.e. 3 plates of MSRV containing 100 µl/plate of BPW distributed in 3 drops of 33.3 µl/drop). All positive samples but one (93%) yielded a positive result (i.e. the characteristic growth halo) on the three plates (results not shown), suggesting that this approach did not have a significant impact on prevalence results.

The weighted multivariable analysis showed that sample positivity appeared to be related to some biological factors, mostly to the location where the birds were captured and their migratory habits (Table 2). When birds were trapped in areas in the vicinity of swine operations the proportion of *Salmonella* positive samples increased significantly, up to 3.46%, from a mere 0.46% observed in samples from birds trapped in environments apart from pig premises. After adjusting for other factors, the odds of being *Salmonella*-positive for a sample from birds captured in a pig farm was more than 16 times higher than that for a sample from birds from areas far from pig operations (Table 2). It is well recognized that livestock farms act as good providers of feed and

shelter for wild birds, and congregations of certain bird species such as house sparrows or European starlings around them is common, provoking damages associated with feed contamination and consumption (Carlson et al., 2011). A relationship between contamination of the environment with enterobacteria and the incidence of this type of infections in wild birds has been reported elsewhere (Cizek et al., 1994, Gaukler et al., 2009). In the region where the birds were trapped almost 95% of the pig farms were positive to *Salmonella* and 30% of the finishing pigs were estimated to be infected (Vico et al., 2011). The magnitude of the relationship between the proportion of *Salmonella* positive fecal samples and the proximity to pig premises suggested the importance that contaminated environments along with bird congregations may have on increasing the likelihood of infection in birds.

Migratory birds have the potential to carry certain pathogenic microorganisms over long distances (Hubalek, 2004). However, in this study non-migratory (sedentary) passerines presented a higher proportion of *Salmonella* positive samples than migratory ones (OR= 7.6; 95%CI: 1.2, 48) (Table 2), suggesting that the risk of transmission of *Salmonella* infection would be higher for non-migrant birds or birds travelling short distances. In a previous study in Denmark long-distance migrant birds were at some lower risk of contracting *Salmonella* infections than nonmigrating (resident) birds (Skov et al., 2008), supporting our findings.

While sedentary birds were repeatedly observed in the surroundings of the pig operations, most of migratory birds trapped in the vicinity of the pig farms were in their way to migration sites, likely spending less time around the pig premises and therefore being less prone to become infected. Migratory passerines might thus play a minor role

in the long-distance transmission of *Salmonella* infection. Bearing in mind that stressors can exert a suppressive effect on immunity, increasing infection virulence and the likelihood of become sick (Holt, 2000), stress associated to migration may lead to disease and the subsequent death of the sick migrating bird, therefore stopping the potential transmission of the infection over long distances. The fact that *Salmonella* was identified in a pool of feces from barn swallows may disagree with this hypothesis. However, these migratory birds were in very close contact with the farm environment for an extended period of time as they were nesting inside a pig fattening unit.

Although in the univariable analysis bird diet was not related to sample prevalence, after adjusting by other variables it turned out close to significant (P=0.08) (Table 2), showing the need for taking into account as many variables as possible when working with wildlife data to avoid confounding effects from many unknown factors. Similar to what was previously reported in Denmark (Skov et al., 2008), seed-feeder birds appeared to have less chances of *Salmonella* infection (OR=0.4; 95%CI: 0.15, 1.1) compared to mostly-insectivorous birds. Five out of the 7 (71.4%) bird species with *Salmonella*-positive samples were considered mostly insectivorous. Some studies have shown that flies and beetles, either as larval stages or adults, are carriers of *Salmonella* spp. (Barber et al., 2002, Liebana et al., 2003, Wales et al., 2010), and this pathogen has been isolated from insects from hen and pig farms (Holt et al., 2007, Olsen and Hammack, 2000, Wang et al., 2011). Pig farms allow for high concentrations of insects which would make *Salmonella* readily available for this type of birds, increasing significantly their odds of getting infected.

Nevertheless, classifying birds according to their diet is difficult. Many bird species change their diet following the availability of their main source of food according to seasonal changes. Thus, insectivorous birds may feed on small seeds and fruits during winter (i.e. European starling) which, in turn, will modify the intestinal flora and then possibly its susceptibility to some infections such as those by *E. coli* (Gaukler et al., 2009). Our classification as "insectivorous/mostly-insectivorous" and "granivorous/mostly-granivorous" was a simplistic categorization of the real nature of the bird diets. Thus these results should be further confirmed.

Evidences that pig farms may act as amplifiers of the *Salmonella* infection among surrounding birds were further brought about by the *XbaI* PFGE patterns and the phage types identified, and the AR profiles observed. For instance, in farm C, where barn swallows were nesting inside a fattening unit, the *Salmonella* strain isolated from them presented the same serotype (Typhimurium), the same phage type (U310) and 100% pulse type homology than those from house sparrows and European starlings captured in the same location (Table 3 and Figure 1). In addition, all isolates were susceptible to all drugs tested. Similar results were observed for the two *Salmonella* strains isolated from fecal samples from two rock pigeons from farm B (phage type DT164).

Interestingly, in the area surveyed AR to at least one drug was detected in 73% of the swine *Salmonella* strains analyzed, and ≥1 resistant strains were recovered in 93% of the pig herds analyzed. In addition, AR was significantly more frequent among the most prevalent serotypes, i.e. Typhimurium (Vico et al., 2011). The fact that 89% (8 out of 9) of the *S.* Typhimurium isolates from bird samples were susceptible to all the drugs tested suggested that most bird infections would have not been acquired from pigs.

However, pig farms may have favored the transmission of these strains among birds living in the surroundings of these farms.

It has been postulated that is more likely that pathogens from wildlife acquire AR through horizontal transfer of resistance genes from clinical isolates or the intake of already resistant bacteria from human waste, sewage and domesticated animal manure than through new parallel mutations in the respective genes (Martinez, 2009). The multi-AR patterns showed by the three of the *Salmonella* strains isolated here (Table 3) matched those more commonly observed in the pig population (Vico et al., 2011), supporting also a possible pig-to-bird pathway transmission.

Regarding the phage types identified, the U310 has been observed in retail pork and the environment of meat cutting rooms, being able to persist for long time (Prendergast et al., 2009). In Spain, this phage type has been isolated on a regular basis from clinical human samples the last 6 years (*Instituto de Salud Carlos III*, data not published). The phage type U311 was also one of the most commonly found from human isolates in Europe in 2009 (Anonymous, 2011). Its prevalence in human samples in Spain has shown a significant increase in the last two years, reaching up to 200 cases in 2009 and 150 in 2010 (*Instituto de Salud Carlos III*, data not published). It is worth noting that the U311 was the only Typhimurium strain among all isolates that showed multi-AR in this study (Table 3). On the contrary, the DT164 is an infrequent phage type that has not been detected neither in humans or domestic animals in Spain in the last years. It may represent a bird-adapted subtype of Typhimurium of limited risk to humans or livestock (Hoelzer et al., 2011, Tizard, 2004).

The fourth phage type, the DT56, along with its variant DT56v are reported as the most commonly *S.* Typhimurium phage types isolated from dead garden birds in England since 1995 (Hughes et al., 2008, Lawson et al., 2011, Pennycott et al., 2010, Pennycott *et al.*, 2006). It has been suggested that DT56 and its variant would be host-adapted *Salmonella* phage types maintained within the British wild bird population (Hughes et al., 2008, Hughes et al., 2010, Lawson et al., 2011). They lack the *sop*E gene associated with some *S.* Typhimurium disease outbreaks in humans and livestock and therefore they would not represent a large zoonotic risk in England (Hughes et al., 2010). However, this phage type has been isolated from human clinical samples in Spain in the last years although at very low frequency (*Instituto de Salud Carlos III*, data not published), and thus the chances of a direct or indirect (through livestock) spill over effect from wild birds to human beings, although low, would be plausible.

To these author's knowledge there are no reports of the phage type DT56 from birds outside of England, thus this may likely be the first time this phage type is detected in passerines from other country. Interestingly, one of the *S.* Typhimurium DT56 was isolated from a migratory blackcap. It has been reported that, since the 1960s, and favored by warmer climate and increasing food supply provided by humans in the United Kingdom, blackcaps established a new northwestern migration route between the breeding areas of southern Germany/Austria and the UK, besides the traditional southwestern route between central Europe and Spain/north Africa (Berthold et al, 1992). This new route may have facilitated the arrival of this phage type to Spain and, therefore, the passerines analyzed, although may not be considered common long-distance carriers of *Salmonella*, should not be fully discarded as such.

Despite the difficulties associated with the isolation of *Salmonella* from wild birds, i.e. low number of birds captured, low prevalence, limited culture sensitivity, etc., and the fact that only one colony was serotyped from each positive sample, these findings suggest that pig farms would act as potential amplifiers of this infection among wild birds surrounding the farms, as it has been observed for other infections such as influenza (Saenz et al., 2006). The degree of bird density (i.e. congregation) may have much to do on the transmission of this infection among birds as phenotypic and genotypic relatedness among isolates from different birds were observed only in farms where abundant birds were seen. Some of the *Salmonella* serotypes isolated from bird feces were of potential zoonotic transmission and associated with AR, therefore the monitoring of wild birds salmonellosis is advised in order to have a good understanding on the epidemiology of this infection in birds and their potential as transmitters of infection either directly or indirectly to humans.

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1 Table 1. Prevalence of *Salmonella*-positive wild-bird fecal samples according to different factors
2 in Northeast Spain.
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4 Table 2. Variables associated by weighted multivariable logistic regression* with *Salmonella*
5 prevalence in wild-bird fecal samples from Northeast Spain.
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7 Table 3. Characterization of the 15 *Salmonella* strains isolated from wild-bird fecal samples in
8 Northeast Spain.
9
10 Figure 1. Dendrogram showing the 4 *XbaI* patterns (>90% homology) of the 9 *S.* Typhimurium
11 strains identified from wild-bird fecal samples.
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