

SHORT COMMUNICATIONS

J. Raptor Res. 49(3):337–341

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MULTIRESISTANT *SALMONELLA* SEROVAR TYPHIMURIUM MONOPHASIC IN WINTERING RED KITES (*MILVUS MILVUS*) IN SEGOVIA, CENTRAL SPAIN

GUILLERMO BLANCO

Department of Evolutionary Ecology, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain

KEY WORDS: *Red Kite*; *Milvus milvus*; *avian scavengers*; *carcass dump*; *emergent pathogen*; *Salmonella*; *swine*.

Carcass dumps and urban refuse dumps are sources of superabundant and predictable food for avian scavengers, making them important to the conservation of these species' populations (Blanco 1997, Bertellotti et al. 2001, Donazar et al. 2009, Cortés-Avizanda et al. 2010, López-López et al. 2014). Birds frequenting dumps have been considered vectors in the dissemination of pathogens associated with rotting meat and waste, as well as livestock pathogens, and this has implications for human health (Hatch 1996, Fallacara et al. 2001, Tsiodras et al. 2008). Wild birds are also susceptible to many pathogens infecting domestic animals and humans (Friend et al. 2001, Thomas et al. 2008). However, much less effort has been devoted to assessing the effect of feeding on livestock carrion at carcass dumps on the acquisition of disease agents that may affect the health of avian scavengers.

My objective in this study was to evaluate the presence of *Salmonella* serotypes and their resistance to antimicrobials in Red Kites (*Milvus milvus*) feeding on a mix of wild prey and livestock carrion during the winter in central Spain. *Salmonella* is a zoonotic bacteria causing gastroenteritis and systemic disease in humans, domestic animals, and wildlife through ingestion of contaminated food and water (Wray and Wray 2000, Hilbert et al. 2012). Wildlife can act as long-term asymptomatic carriers of this bacterium, which can eventually lead to negative effects on health and increased mortality, depending on species and serotypes and also on environmental stress and host characteristics such as population density and diet (Wray and Wray 2000, Friend et al. 2001, Benskin et al. 2009).

METHODS

Study Area. During February 2013, we studied Red Kites in a poplar (*Populus × cultivar*) plantation of about 0.8 ha in Segovia Province, central Spain (40°53'N, 4°15'W). At this site, about 95 communally roosting kites were recorded at dusk following Blanco (1994). This area is part

of one of the main strongholds for wintering Red Kites from central and northern Europe, especially Germany, although population numbers and density have suffered a dramatic decline in recent decades that continues today (Blanco and Montoya 2004, Blanco 2014a). Wintering Red Kites primarily exploit common voles (*Microtus arvalis*) and livestock carcasses (especially swine [*Sus scrofa domestica*]) dumped at sites near rearing farms (Blanco 2014a). This province has one of the highest concentrations and numbers of farms devoted to fattening swine reared under intensive conditions in Spain (Blanco 2014a).

Sample Collection. Fresh excreta ($n = 36$) were sampled with sterile microbiological swabs that were subsequently inserted into tubes containing Amies transport medium. Sampling was conducted early in the morning, after the kites had left the roost, to reduce contamination or desiccation of the fresh excreta produced at dawn. Fresh excreta was not confused with excreta of other species because the sampling site was exclusively used by communally roosting Red Kites. The kites typically distributed themselves throughout the poplar plantation to roost, which allowed me to collect excreta samples that were somewhat separated, in order to avoid multiple samples from the same individual. Samples were transported in a container on ice to the laboratory (Laboratorio Regional de Sanidad Animal, Consejería de Medio Ambiente y Ordenación del Territorio, Comunidad de Madrid, Colmenar Viejo, Madrid, Spain) on the same day of collection and processed within 1–2 hr following their arrival (Blanco 2014b).

Culture, Serotyping, and Antimicrobial Resistance. Samples were cultured according to ISO 6579:2002/Amd. 1:2007. Pre-enrichment was carried out in buffered peptone water (Oxoid, Basingstoke, U.K.) for 16 hr at 37°C, and then 1 ml was cultured in Rappaport-Vassiliadis soya peptone (RVS) and 100 µl in modified semisolid Rappaport-Vassiliadis (MSRV) mediums (Bio Merieux, Marcy l'Etoile, France) for 24 hr at 41.5°C and subcultured onto solid media SM ID agar and XLD agar for 24 hr at 37°C. Suspect colonies were then subcultured onto nutrient agar and confirmation of *Salmonella* species was performed using oxidase, API 20E, and polyvalent antisera. Serotyping was carried out according to the Kauffman-White scheme at the Spanish

¹ Email address: gblanco@mncn.csic.es

National *Salmonella* Reference Laboratory (Laboratorio Central de Veterinaria, MAGRAMA, Algete, Madrid, Spain).

Antibiotic susceptibility of *Salmonella* to selected antimicrobials commonly used in livestock farming and human medicine was tested using the Kirby-Bauer disk diffusion method, which was performed and interpreted as described by the Clinical and Laboratory Standards Institute (2008) protocols. Commercial antimicrobial disks were used for a total of 24 tested antimicrobials, including aminoglycosides, β -lactams, polypeptides, quinolones, sulphenamides, tetracyclines, and macrolids. Each bacterial isolate was classified as susceptible, intermediate, or resistant, depending on the growth inhibition diameter.

RESULTS AND DISCUSSION

Salmonella was isolated from three of 36 excreta samples (8.3%) of wintering Red Kites (Blanco 2014b), a prevalence that can be considered intermediate compared to values recorded in surveys of apparently healthy wild birds (Benskin et al. 2009). This prevalence value may represent a minimum estimate, due to the limited sensitivity of microbiological culture-dependent methods for excreta samples and because infected individuals intermittently shed this bacterium in excreta (Tizard 2004). These results should be considered preliminary because of the relatively small sample size and the spatiotemporal limitation of the study. More research is required to evaluate the infection rate and the health effects of the identified *Salmonella* serovar and other pathogens on the population viability of Red Kites and other scavengers.

Salmonella is a major cause of zoonotic infections worldwide, with variable effects on multiple wildlife hosts, including high mortality, prominent and persistent gastrointestinal disease, and infertility in survivors, as well as embryonic and neonatal mortality (Battisti et al. 1998, Tizard 2004, Benskin et al. 2009). The potential adverse effects of *Salmonella* on wildlife have been reported repeatedly, and variability of effects depends primarily on the avian species or *Salmonella* species and serovars, and on the immunological state of the host (Wray and Wray 2000, Tizard 2004, Benskin et al. 2009). Contrary to most studies on wild birds in which *Salmonella* Typhimurium was the most prevalent serovar (e.g. Kobayashi et al. 2007, Lawson et al. 2011, Hernández et al. 2012), I isolated only *Salmonella enterica* subspecies *enterica*, serovar Typhimurium monophasic variant 4,5,12:i:- from wintering Red Kites (sample references 1301051: 117, 120, and 121 from the Spanish National Reference Laboratory). This serovar was initially characterized from pig samples in Spain, and began to emerge in the mid-1990s and is currently one of the most commonly found serovars in human clinical cases and food sources (mainly pigs and pork products) worldwide (e.g., Echeita et al. 1999, Hauser et al. 2010, Hopkins et al. 2010, Laorden et al. 2010). Most isolates of this serovar involved in human and livestock infections causing disease and mortality were resistant to multiple antimicrobials, especially ampicillin, streptomycin, sulphonamides,

and tetracyclines (Hopkins et al. 2010, García et al. 2014). There are few reports of this serovar in wild birds (e.g., Dobbin et al. 2005, Phalen et al. 2010, Andrés et al. 2012), although it has been recently found at high prevalence in Griffon Vultures (*Gyps fulvus*), in Spain (Marin et al. 2014). The potential adverse effects on this serovar on wildlife health remain generally unknown (but see Phalen et al. 2010), although it has similar virulence and antibiotic resistance features to those of related serovars associated with several outbreaks in passerine birds (Andrés et al. 2012, Hernández et al. 2012).

Carrión from swine and other stabled livestock such as poultry and domestic rabbits are typically only available to scavengers in carcass dumps, where unsatisfactory sanitation can increase the presence of *Salmonella* and other potentially pathogenic bacteria (Friend and Franson 1999, Benskin et al. 2009). The antibiotic resistance pattern of the *Salmonella* isolates collected from three Red Kites in this study was consistent with a multiresistant phenotype, as these isolates were resistant to 13 (54.2%), 11 (45.8%), and 12 (50.0%) of 24 tested antimicrobials (Table 1). All isolates ($n = 3$) were resistant to streptomycin, penicillin G, amoxicillin, ampicillin, piperacillin, and all tested tetracyclines and macrolids, but susceptible to gentamicin, kanamycin, ceftazidime, imipenem and all tested quinolones. The remaining antimicrobials yielded variable results, including intermediate inhibition, depending on the isolate sample (Table 1). Thus, these isolates showed the specific profile of resistance to antimicrobials of this serovar in cases of food-borne illnesses associated with the consumption of contaminated pork and poultry (Echeita et al. 1999, Astorga et al. 2007, Hopkins et al. 2010, Laorden et al. 2010, García et al. 2014). Therefore, both the emergence and comparatively frequent isolation of this *Salmonella* serovar in pigs and pork products, as well as the typical antimicrobial profile, suggest that carrión from swine could be a source of infection with this multiresistant serovar in Red Kites and other avian scavengers (Marin et al. 2014) foraging at carcass dumps, although other sources of infection such as contaminated water or wild prey cannot be dismissed. Carrión from stabled livestock operations, especially pigs, in different states of decomposition is habitually disposed in multiple carcass dumps used by Red Kites during the wintering period in Segovia (see Blanco 2014a for details on the distribution of carcass dumps in relation to the location of the sampled communal roost). In addition, this source of infection, as opposed to other potential sources such as wild prey, was further supported by the fact that *Salmonella* was not isolated from excreta ($n = 48$) of Red Kites wintering in northeastern Madrid Province, central Spain (Blanco 2014b), which lacks carcass dumps and where kites mostly feed on wild prey, primarily lagomorphs (Blanco 2014a).

A previous study in Spain has suggested that pig farms may act as amplifiers of salmonellosis among wild birds, and bird density may be a major factor in transmission, as

Table 1. Susceptibility test results of 24 antimicrobials against three isolates (a, b, c) of *Salmonella enterica* subspecies *enterica*, serotype Typhimurium monophasic variant 4,5,12:i:- from excreta samples of wintering Red Kites in Segovia, central Spain. Each bacterial isolate was classified as susceptible (S), intermediate (I) or resistant (R), depending on the growth inhibition diameter, in accordance with criteria set by the CLSI standard protocol for bacteria isolated from animals. Cut-off values represent the maximum inhibition zone diameter for considering an isolate as resistant.

ANTIMICROBIAL AGENT	DISK CONCENTRATION (µg)	CUT-OFF VALUE (mm)	SUSCEPTIBILITY TEST		
			a	b	c
Aminoglycosides					
Gentamicin	10	12	S	S	S
Kanamycin	30	13	S	S	S
Streptomycin	10	11	R	R	R
Neomycin	30	12	S	I	I
β-lactams					
Penicillin g	10	28	R	R	R
Amoxicillin	25	13	R	R	R
Amoxicillin+clavulanic acid	20+10	13	S	I	I
Ampicillin	10	13	R	R	R
Piperacillin	100	17	R	R	R
Cephalothin	30	14	R	I	I
Cephalexin	30	13	R	S	S
Ceftazidime	30	14	S	S	S
Imipenem	10	13	S	S	S
Polypeptides					
Colistin	10	8	S	I	I
Quinolones					
Norfloxacin	10	12	S	S	S
Enrofloxacin	5	16	S	S	S
Ciprofloxacin	5	15	S	S	S
Sulphonamides					
Sulfamethoxazole+trimethoprim	23.75+1.25	10	S	S	R
Tetracyclines					
Tetracycline	30	14	R	R	R
Oxytetracycline	30	14	R	R	R
Doxycycline	30	12	R	R	R
Macrolids					
Erythromycin	15	13	R	R	R
Lincomycin	15	14	R	R	R
Clindamycin	2	14	R	R	R

well as in the potential spillover of this pathogen to other wildlife species, domestic animals, and human populations (Andrés et al. 2012). The acquisition of this and other potentially pathogenic bacteria and the opportunities for cross-infection from wild birds should be higher at carcass and refuse dumps where many individuals congregate, compared to locations where birds hunt or scavenge alone or in smaller groups (Hiraldo et al. 1991, Blanco 2014a). In particular, pathogen acquisition may be enhanced during the winter, when naive individuals may be more susceptible to disease due to lowered immune function resulting from migration from north and central Europe, harsh weather conditions and high density promoting disease transmission in communal roosts and foraging areas

(Hubálek 1994, 2004, Benskin et al. 2009). Given the large-scale and seasonal movements of Red Kites and other avian scavengers, their potential role as vectors in the dissemination of pathogens, as well as natural reservoir hosts, may be significant and may influence disease epidemiology.

Whether the population decline of breeding and wintering Red Kites was partially caused by negative effects on mortality rates and survivor health by infections with pathogens acquired from carrion remains unknown. The presence of the emergent monophasic variant of the Typhimurium serovar in a proportion of wintering kites, along with the potential ingestion of veterinary drugs (Margarida et al. 2014) and bacterial resistance to these

drugs, should be cause for concern and further research. Importantly, more research is required to determine whether infection with this *Salmonella* serotype and other pathogens potentially acquired in carcass dumps can affect the health of wintering Red Kites and other avian scavengers.

SEROTIPO TYPHIMURIUM MONOFÁSICO DE *SALMONELLA* MULTIRRESISTENTE EN INDIVIDUOS INVERNANTES *MILVUS MILVUS* EN SEGOVIA, ESPAÑA CENTRAL

RESUMEN.—Las aves silvestres que se alimentan en vertederos y muladares pueden adquirir microorganismos asociados a la carne en descomposición y otros residuos, así como agentes patógenos de ganado, y dispersarlos a larga distancia durante la migración, con implicaciones para la salud pública y la conservación de la fauna salvaje. Se evaluó la presencia de *Salmonella* y su resistencia frente a antimicrobianos en muestras fecales de individuos de *Milvus milvus* que se alimentan de una mezcla de presas silvestres y carroña de ganado durante el período de invernada en el centro de España. El único serotipo aislado fue *Salmonella enterica* subespecies *enterica*, serotipo Typhimurium monofásica 4,5,12:i:-. Estos aislados presentaron resistencia a aproximadamente la mitad de los antimicrobianos probados, incluyendo aquellos que son típicos de este serotipo en ganado porcino y sus derivados. La carroña de cerdo y de otro ganado estabulado podría ser la fuente de la infección con este serotipo emergente de potencial transmisión zoonótica y resistente a múltiples antibióticos, aunque otras fuentes de infección como agua contaminada o presas salvajes son también posibles. Se requiere más investigación para determinar si los muladares y vertederos pueden influir en la difusión de éste y otros serotipos en el ganado y los seres humanos, y su impacto en la salud de individuos invernantes de *M. milvus* y de otros animales salvajes.

[Traducción del equipo editorial]

ACKNOWLEDGMENTS

I thank J.L. Gonzalez, Ó. Frías, and P. Alcázar for their help with fieldwork, and J.A. Diaz de Tuesta for help in the interpretation of laboratory analysis. Funds were provided by the projects CGL2009-12753-C02-01/BOS and CGL2010-15726 of Spanish Ministerio of Economía y Competitividad. I acknowledge the efforts of five anonymous reviewers and P. López-López in improving earlier versions of this report.

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Received 9 June 2014; accepted 30 January 2015
Associate Editor: Pascual López-López