Short Communication

Novel *Salmonella* variant associated with mortality in two great spotted woodpeckers (*Dendrocopos major*)

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Abstract:

Two adult great spotted woodpeckers (*Dendrocopos major*) from separate sites in Great Britain were examined *post mortem* in 2013 and 2016. A *Salmonella* sp. was isolated from multiple tissues in both birds. Histopathology and immunohistochemistry confirmed disseminated salmonellosis. Whole genome sequencing and biochemical analyses putatively identified both isolates as a novel variant of *Salmonella enterica* subsp. *enterica* serovar Hessarek (*S*. Hessarek). Salmonellosis has seldom been reported in Piciformes, and never before in association with *S.* Hessarek infection. These findings, therefore, add to current knowledge regarding the range of wild bird species susceptible to this *Salmonella* serovar, and our understanding of the pathogens affecting great spotted woodpeckers, in particular.

Key words: disease, *Salmonella*, surveillance, woodpecker

Salmonellosis, resulting from infection with avian host-adapted biotypes of *Salmonella enterica* subsp. *enterica* serovar Typhimurium, is a well-known cause of mortality in passeriform species in multiple continents (Hall and Saito, 2008; Lawson et al., 2010). Conversely, *Salmonella enterica* subsp. *enterica* serovar Hessarek (*S*. Hessarek) has been isolated from only a small number of wild Passeriformes. First identified in a common raven (*Corvus corax*; Corvidae) found dead in Hessarek, Iran (Neel et al., 1953), *S.* Hessarek has been isolated from infrequent disease outbreaks (range 10 - >1000 dead birds) in song thrushes (*Turdus philomelos*; Turdidae)

(Velarde et al. 2012) and blackbirds (*Turdus merula*; Turdidae) (MacDonald et al., 1968) in Europe, and starlings (*Sturnus vulgaris*; Sturnidae) in Europe (Magistrali et al., 2008) and the Middle East (Singer et al., 1977; Dakman et al., 2017).

Unlike Passeriformes, salmonellosis has rarely been reported in Piciformes: a study by Hall et al. (2018) described four cases in the USA (Hall and Saito et al., 2008), although details of the species affected were not provided. We present evidence of disseminated salmonellosis in two great spotted woodpeckers (*Dendrocopos major*; Picidae) (GSW) in Great Britain (GB), identifying the causative agent as a novel *S.* Hessarek variant.

Scanning disease surveillance of garden birds is conducted across GB by obtaining reports of morbidity and mortality from the public and performing post-mortem examinations (PMEs). Of 4,340 wild birds examined, 1992–2017, the majority were Passeriformes (86%: n=3,719), principally Fringillidae (49%: n=2,105), Passeridae (8%: n=348) and Paridae (7%: n=311). Piciformes constituted only 0.7% (n=33) of this convenience sample and comprised GSW (n=26) and green woodpecker (*Picus viridis*; Picidae) (GW) (n=7). Trauma was the commonest cause of death in Piciformes (63%), with infectious disease diagnosed in only two GSW and one GW (Table 1.).

Due to the paucity of knowledge regarding *Salmonella* sp. infection in Piciformes, we further investigated two cases of salmonellosis diagnosed in GSWs, characterizing the strain of the bacterium involved in each.

The GSWs were found dead in separate domestic gardens (approximately 15 kilometers apart) in Surrey, England, in December 2013 (GSW1) and June 2016 (GSW2). Systematic PMEs were conducted following a standardized protocol (Lawson et al., 2010). Bacteriological culture of the liver, small intestinal contents, and macroscopic lesions was performed: streaking samples onto *Salmonella*-selective enrichment media, incubating aerobically at 37 °C and observing after one, two and five days. Colonial and Gram's stain morphology, and slide agglutination using poly-O antisera (Pro-lab diagnostics, Neston, UK), were used to confirm the isolation of *Salmonella* spp.. Biochemical analysis of *Salmonella* sp. isolates was undertaken using the 20E Enterobacteriaceae biochemical test (API-BioMerieux, Marcy l'Etoile, France). Whole genome sequence (WGS) data was obtained from each *Salmonella* sp. isolate, from which the multi-locus sequence types (MLST) and single nucleotide polymorphism (SNP) profiles were determined using the methods of Ashton et al. (2016). The MLST, SNP and biochemical profiles were compared to a reference human isolate of *S.* Hessarek (Short read archive reference SRR7293657).

A suite of tissues from each GSW, comprising brain, heart, kidney, liver, lung, pectoral muscle, small intestine, spleen, and others with gross lesions (where

present), was fixed in 10% neutral buffered formalin and embedded in paraffin before being sectioned, stained with Haematoxylin and Eosin or Gram Twort, and subjected to routine histopathological examination. Immunohistochemistry, targeting the *Salmonella* common structural antigen (CSA-1), was performed using the methods of Lawson et al. (2018).

Both GSWs were adults in poor body condition, based on reduced pectoral muscle mass and absence of fat deposits. GSW1 was male, GSW2 was female. Macroscopic abnormalities comprised a full thickness skin wound with tendon exposure on the the right fourth digit (GSW1), circa 15 yellow-coloured skin nodules (1-3 mm diameter) on the ventrum and caudo-dorsum (GSW2), and pale discolouration of liver and lung tissue (GSW2). *Salmonella* sp. was isolated from the liver, lung and small intestinal contents of both birds. The most significant histopathological findings were acute to subacute bacterial dermatitis (GSW1 and GSW2) and necrotising myositis (GSW1). Gram-negative rod-shaped bacteria were observed, both within tissues (i.e. skin, muscle and lung (GSW1 and GSW2); brain and meninges (GSW1); liver, heart, spleen and kidney (GSW2)) and intravascularly in multiple organs (i.e. heart (GSW1 and GSW2); liver, lung, brain and kidney (GSW2)). Immunohistochemistry demonstrated bacteria with *Salmonella*-specific immunoreactivity in all tissues examined, co-localising *Salmonella* with inflammatory lesions in the skin and pectoral muscle (GSW1 and GSW2), and identifying the presence of *Salmonella* bacteria, without detectable inflammatory infiltrates, in the heart, gizzard and meninges (GSW2) (Figure 1.).

Biochemical analyses identified both GSW isolates as *Salmonella* sp. with the same biochemical profile, of which pronounced latent production of hydrogen disulfide was a notable feature. Serotyping showed they shared the same antigenic profile (4,5,12 : a : 1,5), which differed from the reference *S.* Hessarek isolate (4,12,[27] : a : 1,5) (SRR7293657), indicating that both GSW isolates are an 0:5 variant of *S.* Hessarek. The MLST was identical for both isolates and identified as ST 4896, a single locus variant of the MLST of *S*. Hessarek, ST 255 (SRR7293657). SNP analyses revealed that, across the whole genome, the isolates from GSW1 (SRR7293661) and GSW2 (SRR7293656) differed by only 2 SNPs, and from *S.* Hessarek (SRR7293657) by 2690 SNPs. These results show that both birds were infected with the same strain of *S*. Hessarek and represent the first identification of this novel *Salmonella* variant, and of *S.* Hessarek, in a member of the Piciformes.

Passeriform species have been proposed as a reservoir for *S.* Hessarek and bird-associated biotypes of *S.* Typhimurium, with occasional zoonotic spillover (Singer et al., 1977; Lawson et al., 2014). Whilst avian and human infection with *S.* Hessarek has been diagnosed in England and Wales, this serovar is uncommonly detected in GB. Only five human isolates of *S*. Hessarek have been confirmed by Public Health England (PHE), 2004-2017 inclusive, one of which was included in this study, and two of which were isolated from people with a history of international travel (MacDonald et al., 1968; PHE unpublished data). This indicates that any public health risk from *S.* Hessarek is likely to be very low.

In previous avian disease incidents with *S.* Hessarek involvement, the most consistent features were multiple mortalities (MacDonald et al., 1968; Singer et al., 1977; Velarde et al., 2012; Dakman et al., 2016), hepatomegaly and/or splenomegaly (MacDonald et al., 1968; Velarde et al., 2012; Dakman et al., 2016), and detection of intravascular and intralesional bacteria where histopathology was performed (Velarde et al., 2012). Migratory stress and high population densities have been proposed as predisposing factors for some of the events (Velarde et al., 2012; Dakman et al., 2016).

Whilst histopathological findings were similar between GSW1, GSW2 and those reported for passerines that died from *S*. Hessarek-associated disease, in each of the GSW incidents there was an absence of organ enlargement and only single birds were found dead. The latter may be the result of detection and/or reporting bias, or a function of species ecology since the GSW is predominantly non-migratory and solitary.

Several contrasting features of *S*. Hessarek and *S*. Typhimurium-associated mortality in wild birds are noteworthy. Passerine salmonellosis associated with *S*. Typhimurium infection in GB principally affects Fringillidae and Passeridae species; typically occurs in proximity to anthropogenic supplementary food sources; exhibits winter seasonality, and is characterised by granulomatous lesions in the oesophagus, liver, spleen and caecal tonsils (Lawson et al., 2018). Greenfinch (*Chloris chloris*; Fringillidae) and house sparrow (*Passer domesticus*; Passeridae)

are the proposed reservoirs of passerine-associated biotypes of *S*. Typhimurium in GB and the species most-often documented with salmonellosis, perhaps due to granivorous diet and flocking behaviour that result in frequent opportunities for faeco-oral pathogen transmission at garden feeding stations (Lawson et al., 2018).

In contrast, the reservoir and/or source of *S*. Hessarek infection in wild birds remain unknown. Mortalities have occurred in various habitats, across the year with no seasonality, and exhibited different macroscopic and histopathological lesions. Life histories of the wild bird species documented with *S*. Hessarek infection differ from those most frequently affected by *S*. Typhimurium, and anthropogenically provisioned food has not been a common factor in recorded disease incidents.

The high similarity of the GSW *S*. Hessarek isolates, alongside their close geographical-proximity are indicative of a single source. Faeco-oral transmission, typical of *Salmonella* sp. infection (Finlay 1994), is considered most likely but histological appraisal of the gastrointestinal tract was limited by autolysis in both birds. As GSW are known to utilise garden feeding stations, these cannot be ruled out as a site for pathogen transmission. However, the GSW's predominantly insectivorous diet (Robinson 2018) reduces the probability that anthropogenically provisioned food was involved in the incidents reported here. Although the skin lesions observed in both birds present another possible route of infection, this could not be determined by histopathological examination and is deemed unlikely given the pathogenesis of the majority of *Salmonella* sp. infections (Finlay 1994). Rather,

we suspect that dermatitis and myositis were the result of haematogenous bacterial spread following initial infection. Ultimately, enhanced surveillance of GSW may aid understanding, and analysis of any further *S*. Hessarek isolates from this species, e.g., using in vivo expression technology and WGS, would enable investigations into host-adaptation to the GSW (Uzzau et al., 2000).

Little is known about the disease conditions affecting Piciformes worldwide. Amongst those examined in GB, just one further case of infectious disease was diagnosed with yersiniosis as the cause of death (Table 1). Globally, few infectious causes of mortality have been reported in GSW; these include Usutu virus infection (Garigliany et al., 2014) in Belgium and toxoplasmosis (Jokelainen and Vikøren 2014) in Norway. Our findings add to current understanding of the pathogens affecting GSW.

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Table 1. Piciformes species examined *post-mortem* 1992-2017 inclusive as part of a wildlife health surveillance project, with a summary of the cause of death assigned, and infectious disease agent(s) isolated.

Species	**Cause of death category**	**Number of birds (number of sites)**	**Infectious agents identified* (number of birds)**
Great spotted woodpecker *Dendrocopos major*	Infectious disease	2 (2)	*Salmonella* Hessarek variant (2)
	Trauma	18 (16)	*Staphylococcus aureus* (1)
	Predation	2 (2)	*Pasteurella multocida* (1)

	Undetermined	3 (3)	Negative
	Euthanasia	1 (1)	Negative
Green woodpecker *Picus viridis*	Infectious disease	1 (1)	*Yersinia pseudotuberculosis* (1)
	Trauma	3 (3)	Negative
	Predation	3 (3)	Negative

***based on microbiological culture of the liver in all cases, and a combination of small intestinal contents, and/or lung, and/or spleen in the majority of cases.**

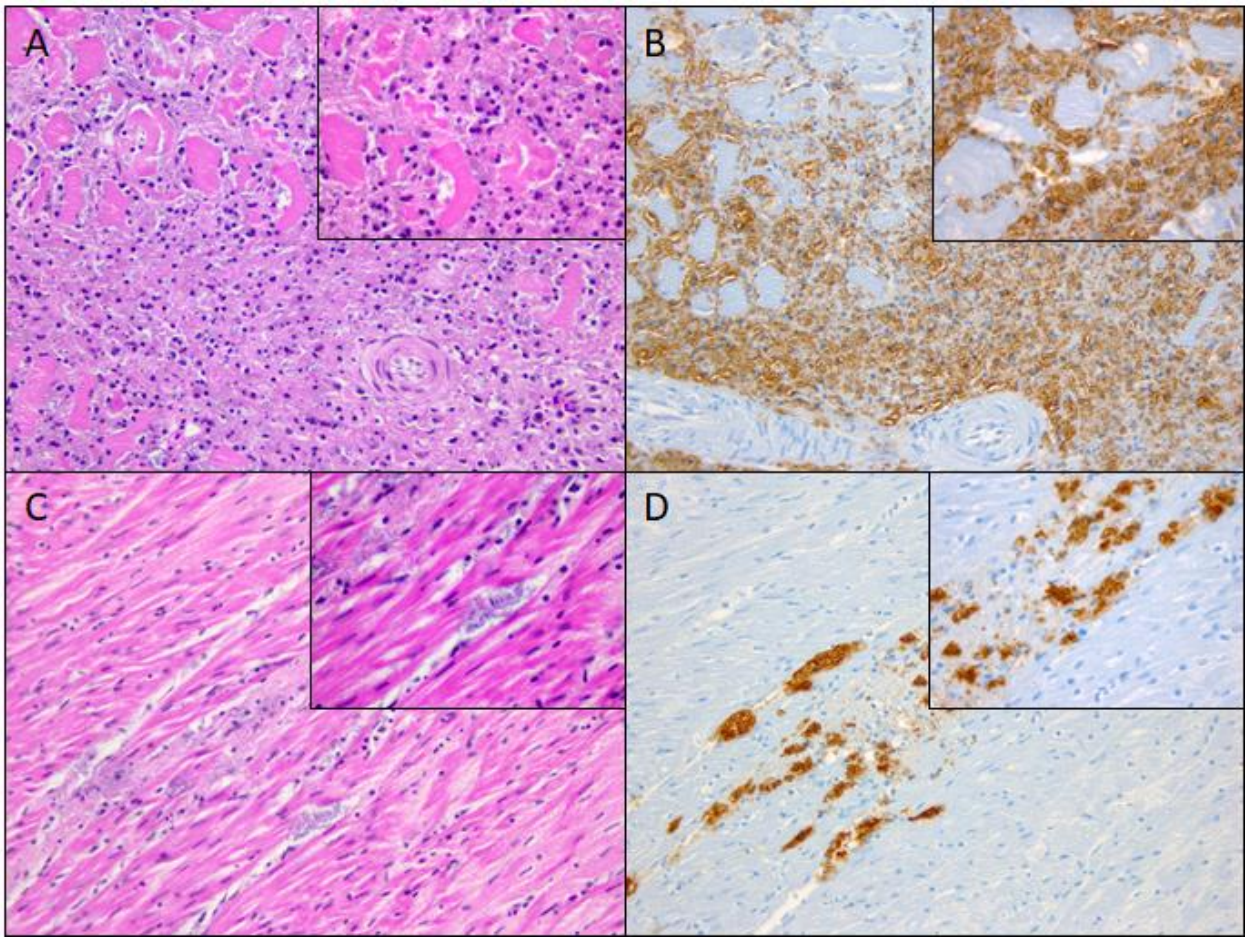

Figure 1. Histopathology and immunohistochemistry of superficial pectoral muscle from great spotted woodpecker (*Dendrocopos major*) 1 (A, B) and of heart from great spotted woodpecker 2 (C, D). (A) Necrotizing myositis with abundant intralesional bacteria. Haematoxylin and Eosin. 40x (Inset 100x) (B) The bacteria show immunoreactivity for *Salmonella* common structural antigen (CSA-1). IHC. 40x (Inset 100x) (C) Intravascular bacterial colonies. Haematoxylin and Eosin. 40x (Inset 100x) (D) The bacteria show immunoreactivity for *Salmonella* CSA-1. IHC. 40x (Inset 100x)