LISTERIA MONOCYTOGENES INFECTION OF FREE-LIVING WESTERN EUROPEAN HEDGEHOGS (*ERINACEUS EUROPAEUS*)

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Abstract: *Listeria monocytogenes* is a ubiquitous environmental bacterium that causes disease in a wide range of species. Infection with this pathogen is most frequently diagnosed in ruminant livestock, but is also known to infect people and occasionally wildlife. Post-mortem examinations of Western European hedgehogs (*Erinaceus europaeus*) in Great Britain (2011-2017) identified five (5/266, 2%, 95% confidence interval: 0.8-4.3%) animals with *L. monocytogenes* infection. The *L. monocytogenes* isolates comprised three serogroup 1/2a and two serogroup 4 from three Multilocus Sequence Types (2, 37 and 121), all of which were different by single nucleotide polymorphism analysis indicating they were distinct and epidemiologically-unrelated. These findings are consistent with hedgehogs contracting sporadic infection from the environment, perhaps through eating soil-dwelling invertebrates. Examination of data from scanning surveillance programs focused on other British wildlife species indicates that the hedgehog is one of the wildlife species from which *L. monocytogenes* has been most frequently identified to date in Great Britain. However, further studies of multiple taxa with comparable sampling efforts are required to assess the relative frequency of *L. monocytogenes* infection in different wildlife species. The bacterium was isolated from extra-intestinal sites in multiple hedgehogs which may indicate septicemia. However, histological examination was limited and could not discriminate subclinical infection from disease (i.e. listeriosis). Whilst *L. monocytogenes* is a source of zoonotic infection, disease in people is typically contracted from the ingestion of contaminated foods. The risk to immunocompetent people of contracting listeriosis from hedgehogs is considered very low to negligible.

Key words: Erinaceidae, Erinaceus europaeus, Listeria monocytogenes, Western European hedgehog, whole genome sequencing, zoonosis.

INTRODUCTION

Listeria monocytogenes, the causative agent of listeriosis, is a Gram-positive rod to coccobacillus. This bacterium is widely distributed in the environment, particularly in soil and decomposing plant material.[5] Listeriosis is primarily reported in domestic ruminants, but is also known to affect people and occasionally wildlife including species present in Great Britain.[7,11,19,20] Disease presentation can be similar across mammalian species and includes septicemia, encephalitis, and abortion.[5,7,19] *Listeria monocytogenes* can also be carried in the gastrointestinal tract of individuals without apparent disease.[5,7]

In people, listeriosis typically affects pregnant women, neonates, and other immunocompromised individuals.[13] It is usually contracted through ingestion of contaminated food and rarely via contact with infected animals or their feces.[5,17] In England and Wales, the number of human cases per annum is relatively low (143 cases in 2017) in comparison to other food-borne zoonoses (Public Health England (PHE), unpubl. data). Although a rare disease, the case fatality rate in people is 20-30%.[13]

The Western European hedgehog (*Erinaceus europaeus*), hereafter hedgehog, is a nocturnal insectivorous mammal found throughout Western and Northern Europe.[1] *Listeria monocytogenes* infection has previously only been reported from a single hedgehog in France as part of a targeted survey for this bacterium.[2] No information was provided on the animal's history or whether evidence of listeriosis was detected.

A scanning surveillance program for disease in free-living hedgehogs in Great Britain has been conducted since 2011. To date, this program has detected infection of hedgehogs with multiple zoonotic pathogens, e.g. *Cryptosporidium parvum* and *Salmonella* Enteritidis.[9,18]

The aims of this study were three-fold: first, to investigate the findings of hedgehogs examined *post mortem* from which *L. monocytogenes* was isolated, and to further appraise the clinical significance of the bacterial infection, through microscopic examination where possible. Second, to characterize the *L. monocytogenes* isolates from hedgehogs using molecular methods and compare them with those which most frequently affect people and livestock in Great Britain. Third, to compare our findings with data on *L. monocytogenes* infection from other British wildlife species to appraise the occurrence of this pathogen across different taxa.

MATERIALS AND METHODS

Post-mortem examination

Systematic post-mortem examinations were conducted on 266 hedgehogs found dead in the wild or in a wildlife rehabilitation center in Great Britain, 2011-2017, as described by Franklinos et al. (2015).[8] Carcasses were examined fresh where possible, or stored at -20°C until examination. For each animal, information was recorded on the location and habitat type where found, sex, age class (based on dentition), body weight, subjective assessment of body condition (based on fat deposits) and the amount of time spent in a wildlife rehabilitation center (if applicable).

Parasitology and microbiology

Microscopic examination of 0.85% saline wet mount preparations (E & O Laboratories Ltd., Bonnybridge, FK4 2HH, United Kingdom) of bronchial and small intestinal contents was routinely conducted. Microbiological testing on a skin swab, a throat swab (from June 2015 onwards only), liver tissue, small intestinal tract contents, and on any macroscopic lesions detected was also routinely conducted from each hedgehog. Each sample was cultured onto Columbia 5% horse blood agar plates and incubated aerobically and anaerobically. Samples of small intestinal tract contents were cultured directly onto *Campylobacter* selective blood free agar plates and incubated micro-aerophilically, cultured directly onto xylose-lysine-desoxycholate agar plates and incubated aerobically, and enriched in selenite broth for 48 hours at 37 °C prior to being sub-cultured onto xylose-lysine-desoxycholate agar plates and incubated aerobically. Lung tissue when sampled was cultured onto a chocolate horse blood agar plate and incubated CO_2 (all from Oxoid Ltd., Basingstoke, RG24 8PW, United Kingdom). All plates were incubated at 37 °C and observed after 1, 2 and 5 days. The identification of *L. monocytogenes* was based on Gram's stain (Pro-Lab Diagnostics Inc., Wirral, CH62 3QL, United Kingdom), colonial morphology and biochemical reaction profile using API CORYNE identifications strips (bioMérieux UK Ltd., Basingstoke, RG22 6HY, United Kingdom).

Whole genome sequencing

DNA was extracted from purified colonies of *L. monocytogenes* using the QIAsymphony® automated extraction platform following a pre-lysis step with lysozyme and Proteinase K (all Qiagen Ltd., Manchester, M15 6SH, United Kingdom). Whole genome sequencing (WGS) was

performed as described by Dallman et al. (2015).[4] The serogroups, inferred from the sequence, were assigned according to the Doumith et al. (2004) classification.[6] The Multilocus Sequence Type (MLST), as defined by the Pasteur Scheme,[15] was extracted from WGS data (Institut Pasteur MLST and MLST whole genome databases, http://bigsdb.pasteur.fr/listeria/). Single nucleotide polymorphism (SNP) analyses were performed using SnapperDB, a set of tools to store bacterial variant data and facilitate reproducible and scalable analysis of bacterial populations.[3]

Histopathology

When tissues fixed in neutral buffered 10% formalin (Genta Medical, York, YO26 7QF, United Kingdom) were available from hedgehogs with *L. monocytogenes* infection, they were processed for histological examination and stained with hematoxylin and eosin using routine methods (IDEXX Laboratories Ltd., Wetherby, LS22 7DN, United Kingdom). Gram, Ziehl-Neelsen and periodic acid-Schiff staining (all IDEXX Laboratories Ltd.) was conducted on a subset of tissue sections where indicated by the presence of lesions. Tissues from one case were not saved (case 1).

RESULTS

Listeria monocytogenes was isolated from the liver and a range of other tissues from five (2%, 95% confidence interval: 0.8-4.3%) of 226 hedgehogs examined (Tables 1-4). The majority of the *Listeria*-positive samples yielded pure/confluent/mixed isolates of the bacterium (Table 2). Infected animals comprised a mix of age class (two juvenile, three adult), sex (two female, three

male) and body condition (three thin, two normal) (Table 1). The five *Listeria*-positive hedgehogs died March to November, 2013-2016; three had died in wildlife rehabilitation centers. Two of the hedgehogs were observed by members of the public showing signs of ill health, before being found dead in the field. Three of the hedgehogs were found by members of the public, and presented for treatment at wildlife rehabilitation centers. The clinical signs observed in all of the *Listeria*-positive hedgehogs included lethargy and/or acting abnormally (e.g. active during the day). Four were from different sites in rural habitats and one from a suburban area. In each case, a single hedgehog was affected per site. Parasitology and microbiology results are presented in Table 2. Results from the macroscopic and microscopic examinations are presented in Table 3. No bacteria consistent with *L. monocytogenes* were observed on histopathological examination of tissues with microscopic lesions (cases 4 and 5). Case 4 had a single focus of necrotizing hepatitis; no infectious organisms were noted on Gram, Ziehl-Neelsen, or periodic acid-Schiff staining. Case 5 had pleuritis with Gram-negative intralesional bacteria. Concurrent disease considered sufficient to contribute to the cause of death, was diagnosed in four of the five hedgehogs (Table 3). The cause of death was undetermined for the fifth hedgehog.

WGS identified *L. monocytogenes* serogroup 1/2a, MLST 37 from two hedgehogs, *L. monocytogenes* serogroup 1/2a, MLST 121 from one, and *L. monocytogenes* serogroup 4, MLST 2 from the remaining two animals (Table 4). The two MLST 37 isolates had between 10 and 25 SNPs and the two MLST 2 isolates had between 50 and 100 SNPs.

DISCUSSION

In this study, *L. monocytogenes* infection from multiple hedgehogs in Great Britain was identified and the isolates were characterized. Infected hedgehogs had a wide geographical

distribution and both age classes and sexes were affected. In each case, the *L. monocytogenes* isolate was genetically different by MLST typing and/or by SNP analysis indicating that the cases were epidemiologically unrelated.

Meaningful histopathological interpretation was precluded for most of the infected animals due to autolysis and/or freeze-thaw artifact, therefore it was not possible to assess whether *L. monocytogenes* was causing listeriosis. That the bacterium was isolated from extra-intestinal sites in all cases could indicate dissemination of the bacterium and septicemic listeriosis, which is the most common disease presentation in small mammals.[11] Case 4 had *L. monocytogenes* in the small intestine. Since no bacteria were found associated with the liver lesions in this animal, it is possible that it was a subclinical carrier of the bacterium, with liver invasion occurring post-mortem. The etiology of the hepatic lesions in this animal remains undetermined. Optimal tissue preservation and immunohistochemistry would increase the likelihood of detecting listeriosis, if present.

Infections with pathogens other than *L. monocytogenes* were considered sufficient to have contributed to the cause of death in four of the *Listeria*-positive hedgehogs. *Listeria monocytogenes* tends to affect immunocompromised individuals,[5] thus concurrent disease might have predisposed the hedgehogs to disseminated *L. monocytogenes* infection. Three of the hedgehogs had been admitted to wildlife rehabilitation centers prior to death. The two cases with available data on length of time in rehabilitation (cases 2 and 3) died within 48 hours of admission, therefore the findings are likely to represent the wild status rather than be a consequence of stress in captivity or nosocomial infection.

In addition to the hedgehog surveillance program, post-mortem and routine microbiological examinations of other free-living wild animals in Great Britain are conducted at the Institute of

Zoology. To date, *L. monocytogenes* has not been isolated from circa 4,000 wild birds of circa 60 species (1992-2017) or from 728 cetaceans of 16 species (found stranded around the coast of England and Wales, 2004-2016) examined (unpubl. data). This pathogen was, however, isolated from two (7%) of 29 Eurasian red squirrels (*Sciurus vulgaris*), where macroscopic lesions led to microbiological testing, 2004-2013. These two were emaciated adults from separate incidents. *Listeria monocytogenes* was recovered from consolidated lung tissue in one squirrel, and from an enlarged spleen and intussuscepted large intestine in the other (unpubl. data). Histological examination was not possible in either case, therefore, the significance of this bacterial infection through assessment of the presence of microscopic lesions could not be further investigated.

The Animal Plant and Health Agency also conducts scanning surveillance of British wildlife. Of circa 1,000 wild mammal post-mortem examinations of 23 species, 2008-2017, *L. monocytogenes* was identified in five free-living Eurasian red squirrels, two captive European water voles (*Arvicola amphibious*) and one captive fallow deer (*Dama dama*) but from none of circa 90 hedgehogs (unpubl. data).

Based on our study and available data from the different British wildlife disease surveillance programs, the hedgehog is one of the species from which *L. monocytogenes* infection has been most frequently detected to date. Given that the study utilizes a convenience sample (i.e. non randomized sampling) and comparison with data from other taxa and surveillance schemes is limited by heterogeneity in microbiological examination and sampling effort, further research is required to investigate the relative frequency of *L. monocytogenes* infection in the hedgehog as compared with other British wildlife species to determine whether differential species susceptibility or exposure occurs. Hedgehogs typically eat soil-dwelling invertebrates,[16] such as earthworms, thus providing a mechanism for exposure to *L. monocytogenes*.

The hedgehog *L. monocytogenes* isolates were of the same serogroups and MLSTs detected in human and livestock listeriosis cases.[5,12] PHE considers isolates of *L. monocytogenes* linked within the same 0 to 5 SNP clusters to likely be from the same point source.[12] The hedgehog isolates within each MLST had ≥ 10 SNPs difference indicating that a common source was unlikely. No strains similar (i.e. within a 5 SNP cluster) to those isolated from the hedgehogs were identified in PHE surveillance database of human *Listeria* isolates.

Hedgehogs are the mammal species most frequently admitted to wildlife rehabilitation centers in Great Britain.[10] Hygiene precautions are recommended as a routine (e.g. wearing disposable, protective gloves during handling and washing hands afterwards), particularly for hedgehog carers who frequently handle hedgehogs. Immunocompromised people and pregnant women should take particular care when in contact with hedgehogs. Supplementary feeding of free-living hedgehogs is an increasingly common practice;[14] routine hand washing after feeding hedgehogs is similarly recommended when this takes place. The risk to immunocompetent people of contracting *L. monocytogenes* infection from infected hedgehogs is considered very low to negligible.

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Table 1. History and description of Western European hedgehogs (*Erinaceus europaeus*) isolated with *Listeria monocytogenes* in Great Britain 2013-2016.

Case	Month and year found dead, carcass storage and carcass condition status	Age class and sex	Body condition class and bodyweight	Habitat type and location	History of time in rehabilitation center?
1	09/2013 Frozen Mild autolysis	Juvenile Female	Thin 245 g	Rural, Wiltshire, England	No
2	08/2015 Frozen Moderate autolysis	Adult Male	Thin 441 g	Suburban, Merseyside, England	Yes, < 48 hours
3	11/2015 Frozen Moderate autolysis	Adult Female	Normal 744 g	Rural, Bedfordshire, England	Yes, < 48 hours
4	03/2016 Frozen Moderate autolysis	Juvenile Male	Normal 452 g	Rural, Denbighshire, Wales	No
5	06/2016 Frozen Moderate autolysis	Adult Male	Thin 574 g	Rural, East Sussex, England	Yes, length of time unknown

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Table 2. Parasitology and microbiology results from Western European hedgehogs (*Erinaceus europaeus*) isolated with *Listeria monocytogenes* in Great Britain 2013-2016.

Case	Parasitology	Organs tested on routine microbiology (* denotes organs culture-positive for *L. monocytogenes*) Other significant pathogens isolated
1	Ixodid ticks (skin)	Liver*[b], lung*[b], urine, brain, small intestine, large intestine.
	Crenosoma striatum adults and larvae (lung)	
	Capillaria sp. ova (lung and intestinal contents)	
	Acanthocephalans (mesenterium and intestinal serosa)	
2	*Capillaria* sp. ova (intestinal contents)	Skin*[a], liver*[b], mandibular abscess*[b], small intestine.
		Pasteurella multocida (isolated from skin, liver, mandibular abscess)
3	Ixodid ticks (skin)	Liver*[c], throat*[c], skin, small intestine.
4	*Crenosoma* sp. adults and larvae (lung)	Liver lesion*[a], small intestine*[c], liver, throat, skin, mesenteric lymph node
	Capillaria sp. adults and ova (small intestine contents)	*Staphylococcus aureus* (isolated from skin)
5	Helminth larvae (lung and small intestinal contents)	Liver*[d], heart*[a], lung, small intestine, throat, skin.
	Capillaria sp. ova (lung)	*Candida albicans* (isolated from throat)

330 [a] Few *Listeria monocytogenes* colonies.

[b] Moderate mixed isolate with *Listeria monocytogenes* colonies.

[c] Confluent mixed isolate with *Listeria monocytogenes* predominating.

[d] Pure *Listeria monocytogenes* isolate.

335 **Table 4.** *Listeria monocytogenes* isolate serogroup, Multilocus Sequence Type (MLST) and Single-Nucleotide Polymorphism (SNP) address from Western European hedgehogs (*Erinaceus europaeus*) isolated with *Listeria monocytogenes* in Great Britain 2013-2016.

Case	**Serogroup**	Multilocus Sequence Type (MLST)	Single-Nucleotide Polymorphism (SNP) address
1	4	2	1.1.4.305.323.339.376
2	4	2	1.1.283.297.313.329.365
3	1/2a	37	1.2.2.2.123.126.130
4	1/2a	121	1.1.17.42.473.489.605
5	1/2a	37	1.2.2.2.16.16.16

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