Zoonotic pathogens linked with hedgehog diphtheric disease

ZOONOSES IN HEDGEHOGS

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Summary

Hedgehog diphtheric disease (HDD), an ulcerative skin disease with a high fatality rate is an emerging threat to European hedgehogs (*Erinaceus europaeus*). We explored the potential role of a panel of zoonotic pathogens in the presumed multifactorial nature of HDD in 188 hedgehogs from three wildlife rescue centers in Belgium. As expected, and with a prevalence of 67% in 57 hedgehogs with skin lesions, characteristic of HDD, the occurrence of *Corynebacterium ulcerans* was strongly associated with the disease. Remarkably, with a prevalence of 42% in affected animals, infections with *Borrelia burgdorferi sensu latu* were 3.92 times more likely to be detected in HDD (95% confidence interval: 1.650 – 9.880; p = 0.0024). Overall, 40 hedgehogs tested positive for the *Borrelia burgdorferi sensu latu* complex, including *B. afzelii* (n = 30)*, B. bavariensis* (n = 7) and *B. spielmanii* (n = 7). Other widely occurring pathogens included *Salmonella* (prevalence of 19%, with three PFGE profiles) and *Leptospira* sp. (prevalence of 11%, including *L. interrogans* and *L borgpetersenii*) but these were not associated with occurrence of HDD. These findings show that hedgehogs in Belgium represent a significant reservoir of multiple zoonotic bacteria, of which toxigenic *C. ulcerans* and *Borrelia* burgdorferi *sensu latu* are associated with widespread hedgehog skin pathology and mortality.

KEYWORDS

Borrelia burgdorferi; Corynebacterium ulcerans; diphtheria; *Erinaceus europaeus;* hedgehog; *Salmonella*

1 | INTRODUCTION

Corynebacterium ulcerans is an emerging pathogen in European hedgehogs (*Erinaceus europaeus*) (Berger et al., 2019; Martel et al., 2020). In the spring of 2020, around 6500 hedgehogs were admitted to different wildlife rescue centers in Flanders (Belgium). The majority of animals were presented with ulcerative skin lesions on their head and/or legs (further called hedgehog diphtheric disease, HDD). Although toxin producing *C. ulcerans* were isolated from a high proportion of HDD cases (Martel et al., 2020) their role as primary or secondary pathogens is unclear. The surprisingly high diversity of *C. ulcerans* isolates in affected hedgehogs suggests the current epidemic to have arisen from an endemic state (Martel et al., 2020), as opportunistic pathogens in a multifactorial disease. Other factors can be highly diverse, but may include co-infections.

We here focus on the occurrence of the major zoonotic pathogens (*C. ulcerans*, *Salmonella* sp., *Leptospira* sp. and *Borrelia* sp.) that are known to occur in hedgehogs as potential co-factors in the development of HDD. While these are pathogenic for humans, their relevance for hedgehog health is unknown.

In humans, *C. ulcerans* is a known cause of respiratory and cutaneous diphtheria and in recent years, despite available immunization, an increase in the *C. ulcerans* cases has been noted in Europe (Wagner et al., 2010; Both et al., 2015; Wyndham-Thomas et al., 2018; Martini et al., 2019; Gower et al., 2020).

With an estimated 93.8 million infections and 155000 deaths in humans yearly across the world (Majowicz et al., 2010), *Salmonella* is an important, global cause of (mainly) gastro-enteritis. The two clinically most important serovars in Europe (Enteritidis and Typhimurium) have been reported to occur in free living hedgehogs in Europe (Handeland et al., 2002; Keymer, Gibson, & Reynolds, 1991; Nauerby, Pedersen, Dietz, & Madsen, 2000).

A wildlife screening study in France (2012-2015) discovered a high prevalence (37.5%) of pathogenic *Leptospira sp. (L. interrogans* and *L. borgpetersenii*) in hedgehogs (Ayral et al., 2016). In humans,

infection with pathogenic *Leptospira* sp. can result in severe disease and even death (Adler & de la Peña Moctezuma, 2010; Bharti et al., 2003).

Lyme borreliosis, caused by the *Borrelia burgdorferi sensu lato* complex, is the most frequent tick-borne disease in Europe (Fingerle et al., 2008). Infection with any of the zoonotic genospecies can lead to skin lesions (erythema migrans) and multi-organ infections, including the heart, musculoskeletal - and nervous system (Stanek & Strle, 2008). Studies have shown the role of the European hedgehog and its tick species (*Ixodes ricinus* and *Ixodes hexagonus*) as a reservoir for multiple *Borrelia burgdorferi sensu latu* genospecies, including *B. afzelii, bavariensis*, *garinii* and *spielmanii* (Skuballa et al., 2007, 2012).

Exposure to any of the pathogens above may lead to transmission of infection to humans via direct or indirect (environmental contamination) contact with hedgehogs. Hedgehogs frequent (sub)urban areas, where contact with humans occurs through supplementary feeding and presentation of weak or injured individuals in wildlife rescue centers (Lawson et al., 2018). Especially since 2020, rescue centers have been reporting a significant increase in admitted hedgehogs, which coincides with the emergence of diphtheric disease. Daily care of these animals, including topical treatment of skin lesions, puts staff and volunteers at risk of zoonotic pathogen exposure.

In this study, we assessed to which extent the presence of *C. ulcerans, Salmonella sp., Leptospira sp.*, *Borrelia burgdorferi sensu latu* and *Borrelia miyamotoi* in hedgehogs is associated with the occurrence of HDD.

2 | MATERIALS AND METHODS

2.1 Animals and sample collection

A total of 188 carcasses of European hedgehogs (*Erinaceus europaeus*) were collected from three wildlife rescue centers in Flanders (Belgium): Oostende (n = 74), Geraardsbergen (n = 105) and Merelbeke (n = 9). The animals were stored at -20 degrees Celsius after natural death for a maximum

of six months before microbiological analysis. Animals were divided into two groups based on age (juvenile (less than 1 year of age) and adult). Organ (lung, liver, kidney, heart, urinary bladder), tissue (ear, skin lesions) and intestinal content samples were collected for pathogen detection (Table 1). Skin lesions were scored based on location, including head, legs and thorax/abdomen.

2.2 DNA extraction

DNA was extracted from the liver, kidney, heart, urinary bladder and ear with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). These DNA extracts were used for the molecular detection of *Leptospira* sp. and *Borrelia* sp..

2.3 Pathogen detection and isolation

C. ulcerans and *Salmonella* sp. were isolated following previously described culture protocols. *C. ulcerans* isolation was attempted from sterile cotton swabs (155C, COPAN, Italy) from the skin lesions, mouth and lungs as described in Martel et al., 2020. *C. ulcerans* was identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and sequencing of the *rpoB* gene (Martel et al., 2020). The presence of the diphtheria tox gene (toxE) in *C. ulcerans* isolates was determined via PCR (Martini et al., 2019).

Salmonella sp. isolation was attempted from the intestinal content using ISO 6579-1:2017/AMD 1:2020 (ISO, 2020). Confirmation of *Salmonella* at level was done using MALDI-TOF MS (Lay Jr, 2001). Serotyping of the isolates was executed by Check&Trace Salmonella DNA microarray hybridisation (Check-Points, Wageningen, the Netherlands)(Chui, Ferrato, Li, & Christianson, 2021). Distinction between the different isolates was done by Pulsed-Field Gel Electrophoresis (PFGE) using restriction enzymes XbaI and BlnI (PulseNet, 2017).

Molecular analyses were performed to detect *Leptospira* sp. and *Borrelia* sp.. The *lipl32* PCR was used to detect *Leptospira* DNA in the kidney-derived DNA preparations. PCR-positive samples were further analyzed by sequencing via the *Sec*Y PCR (Mayer-scholl et al., 2011). DNA of the heart, liver, urinary

bladder and ear was screened for the presence of *Borrelia burgdorferi sensu latu* complex, using the pan-*Borrelia* qPCR amplifying a 148-bp fragment of the *Borrelia* 16S rRNA gene and *Borrelia miyamotoi*–specific qPCR (only ear samples) assay specific to a region of the *flaB* gene (Cull et al., 2021). *Borrelia* positive samples were sequenced and identified to genospecies level via typing of the 5S-23S rRNA intergenic spacer region (Cull et al., 2021).

2.4 Statistical analysis

Generalized Linear Mixed Models (GLMM) were used to test for an association between the occurrence of abscesses and pathogens (*Salmonella, Leptospira* and *Borrelia*), using a binomial error distribution and specifying the wildlife rescue centers where hedgehogs were found as random effect. Statistical analysis was performed in R 4.0.3. using the 'lme4' package (Bates, Mächler, Bolker, & Walker, 2014). The extent to which *C. ulcerans, Salmonella, Leptospira and Borrelia* occur concomitantly was quantified using Mainali et al.'s (2022) affinity metric of co-occurrence, an odds ratio for co-occurrence that is independent of pathogen prevalence.

3 | RESULTS

3.1 Pathogen prevalence

Pathogen prevalence is summarized in Table 1 and 2. Fifty nine animals were positive for *C. ulcerans* in at least one sample of either skin, mouth or lung. In 38 of 57 hedgehogs with skin pathology, *C. ulcerans* was isolated from the lesions (66.67% positive detection rate). Head swabs were most often positive (73.68%) (28/38), followed by leg (44.74%) (17/38) and thorax/abdomen (7.89%) (3/38). The presence of *toxE* gene was demonstrated in 91.7% (44/48) of the isolates.

C. ulcerans was isolated from mouth swabs in 13.74% (18/131) of hedgehogs without skin pathology. Of these isolates, 72.2% (13/18) was positive for *toxE*.

Thirty five animals tested positive for *Salmonella* Enteritidis (18.62%) (35/188). Three very similar pulsotypes could be identified, with only small differences in the Blnl profiles.

Two pathogenic *Leptospira sp.* genospecies were identified in 21 hedgehogs (11.17%) (21/188), *L. interrogans* (95.24%) (20/21) and *L. borgpetersenii* (4.76%) (1/21).

The occurrence of one or several of three genospecies of the *Borrelia burdorferi sensu latu* complex (n = 44) was identified in 40 hedgehogs, *B. afzelii* (68.18%) (30/44), *bavariensis* (15.91%) (7/44) and *spielmanii* (15.91%) (7/44). They were most often detected in the ear (82.50%) (33/40), followed by the urinary bladder (n = 16) (40.00%) (16/40), heart (15.00%) (6/40) and liver (5.00%) (2/40). Multiple organs were positive in nine hedgehogs. Co-infections with multiple genospecies were demonstrated in four hedgehogs. All positive hedgehogs could be identified with a combination of ear and urinary bladder samples. No hedgehogs tested positive for *Borrelia miyamotoi*.

3.2 Pathogen co-occurrence

Both *Borrelia* and *C. ulcerans* occurrence were negatively related to *Salmonella* occurrence (odds-ratio's 0.19 and 0.10, and p-values of 0.018 and 0.004 respectively). *C. ulcerans* and *Borrelia* tended to occur together (odds ratio of 2.37, p-value of 0.063); all other comparisons had p-values > 0.14.

These associations likely arise because *C. ulcerans* and *Borrelia* are predominately found in adult hedgehogs (respectively 89 and 85% of positive cases were adults) whereas *Salmonella* is typically found in juvenile animals (only 20% were of *Salmonella* positive hedgehogs were adults)

3.3 Pathogens associated with HDD

Of the 188 hedgehogs sampled in this study, 57 were presented with skin pathology. All the affected hedgehogs originated from the rescue centers in Geraardsbergen or Merelbeke. Skin lesions were most often found on the head (n = 43), followed by legs (n = 32) and thorax/abdomen (n = 6). In 20 hedgehogs, lesions were present on both head and legs. Pathogen prevalence in hedgehog with and without HDD is presented in Table 3. Two hedgehogs with skin pathology (3.50%) (2/57) tested positive

for *Salmonella sp.* in the intestinal content, with a weak significant odds ratio of 0.12 (95% confidence interval: 0.0134 – 0.543; p = 0.017). Ten hedgehogs with skin lesions (17.54%) (10/57) were positive for *Leptospira sp.* in the kidney tissue (odds ratio of 1.75; 95% confidence interval: 0.586 – 5.200; p = 0.32). Twenty four animals were positive for skin lesions and the *Borrelia burgdorferi sensu latu* complex, with a significant odds ratio of 3.92 (95% confidence interval: 1.650 – 9.880; p = 0.0024).

4 | DISCUSSION

We found high prevalences for all zoonotic agents studied, with a significant association of the occurrence of *Borrelia sp.* with hedgehog diphtheric disease (HDD). *Corynebacterium ulcerans* was detected in a high proportion (66.7%) of affected hedgehogs, corroborating its widespread involvement in the disease. The high prevalence of *Borrelia sp*. in affected hedgehogs, with the skin as a clear predilection site, demonstrates the multifactorial nature of the disease. Whether any of these infections is the actual cause of the disease, or rather secondary to a currently unknown underpinning cause(s) remains to be determined. Factors like other pathogens, diet, pesticides or other toxicants and stress are worthwhile to explore in follow-up studies.

A hypothesis for the observed association of borreliosis with HDD is that hedgehogs are often infested with ticks, *Ixodes ricinus* and *I. hexagonus*, which are both competent vectors for *Borrelia burgdorferi sensu latu* (Skuballa et al., 2012). For a blood meal, ticks penetrate the skin barrier by inserting their hypostome and two chelicerae into the host's skin. Afterwards, they secrete saliva which contains immunosuppressive compounds (lytic activity against fibroblasts and anti-inflammatory molecules against a range of immune cells) facilitating the feeding of the ticks and pathogen transmission, including *Borrelia burgdorferi sensu latu* (Bernard, Grillon, Lenormand, Ehret-Sabatier, & Boulanger, 2020). Such breaches in the skin barrier can be a portal of entry for *C. ulcerans*. We here demonstrate that *C. ulcerans* can be present in the mouth of asymptomatic hedgehogs and might be a constituent

of the commensal oral flora. Licking, grooming, self-anointing or fighting behavior of hedgehogs could inoculate *C. ulcerans* in skin lesions of the paws and head of the animals.

The high prevalence of *Salmonella* Enteritidis, belonging to a limited and very similar pulsotypes, suggests endemism of potentially hedgehog associated strains. These findings are in line with previous reports of *Salmonella sp.* in hedgehogs across Europe (Ayral et al., 2016; Badudieri & Ghysels, 1966; Lawson et al., 2018). We found a weak negative association between *Salmonella sp.* and HDD, but this was deemed irrelevant due to age bias (*Salmonella* was more present in juvenile animals; HDD was more present in adult hedgehogs). *Leptospira sp.*, consisting of two pathogenic genospecies (*interrogans* and *borgpetersenii*), occurred in considerable numbers as well, confirming previous studies in Europe (Ayral et al., 2016; Badudieri & Ghysels, 1966). However, no significant association with HDD could be found. From a public health perspective, it is unclear to which extent infections with *Salmonella* and *Leptospira* in hedgehogs contribute to disease burden in humans. Given the high prevalence of these pathogens, combined with potential exposure of humans (e.g. via infected pets), these findings warrant further studies.

In conclusion, hedgehogs are reservoirs of multiple zoonotic pathogens, at least some of which are involved in hedgehog diphtheric disease. People that come into contact with diseased hedgehogs, should be aware of the potential risks and take preventative measures accordingly. The emerging, largely unexplained threat of HDD in hedgehogs, especially, will require the attention of researchers, stakeholders and the public alike. This disease may have a major impact on hedgehog populations.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal , as noted on the journal's author guidelines page have been adhered to. The authors confirm that, following the EU legislation, no ethical approval was required as this work was carried out on naturally died animals.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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AUTHOR CONTRIBUTION STATEMENT

A.M. and F.P. designed the research. A.M., E.G., G.R., S.B. and S.W. carried out the research. A.M., D.S., E.G., F.B., F.P. and G.R. interpreted the data. A.M., D.S., F.P., K.V., L.B. and L.L. conceived the framework, attracted funding and provided resources. A.M., D.S., E.G., F.P. and N.T. wrote the paper with input from all other authors.

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