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Original Research Article

DETECTION OF *Lawsonia intracellularis* DNA IN ILEAL TISSUES OF DEAD WILD BIRDS IN THE REPUBLIC OF KOREA

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Abstract: *Lawsonia intracellularis* is an etiological agent that causes proliferative enteropathy in various species. Little is known about the mechanisms of transmission of *L. intracellularis*, especially in wild bird species. The presence of *L. intracellularis* in dead wild birds in the Republic of Korea was investigated using the polymerase chain reaction method. *L. intracellularis* DNA was identified in the mucous membrane of the ileum in one Eurasian eagle-owl (*Bubo bubo*, Strigidae), two black-billed magpies (*Pica pica sericea*, Corvidae), and one jungle crow (*Corvus macrorhynchos*, Corvidae) among 745 dead wild birds examined. Although few wild birds in this study were exposed to *L. intracellularis*, the exposure was likely to be epidemiologically relevant. Regarding the ecological behavior of the bird species found to be exposed to *L. intracellularis* in the current study (Eurasian eagle-owl, black-billed magpie, and jungle crow), swine or horse farm facilities, which are reservoirs for *L. intracellularis*, might be easily accessed by such wild birds. Thus, these and similar species could have increased chances of exposure to *L. intracellularis* and could serve as biological vectors of proliferative enteropathy. Wild bird feeding patterns and previous reports of wild and feral animals exposed to *L. intracellularis* could be an alternative explanation for the association between *L. intracellularis* and wild birds.

Key words: *Lawsonia intracellularis*; gene; diagnosis; surveillance; infectious disease; PCR

Introduction

Proliferative enteropathy is an intestinal disease that is characterized by thickening of the distal small and proximal large intestinal mucosa due to enterocyte proliferation associated with the presence of an intracellular bacterium (1). One such bacterium is the highly fastidious, obligate intracellular Gram-negative bacterium *Lawsonia intracellularis* (2). A salient feature of the biology of *L. intracellularis* is its ability to produce a chronic infection that persists in the host, thus making control of proliferative enteropathy difficult in infected animals.

We previously reported an overall 4-year average true prevalence of *L. intracellularis* infection of 40.0% (CI: 39.4 - 40.6%) at the individual animal level and 71.9% (CI: 70.3-73.4%) at the herd level in 8,008 swine serum samples obtained from 1,001 herds (3). Although proliferative enteropathy is currently present in all swine-producing areas worldwide, including the Republic of Korea (ROK) (4), the epidemiology of proliferative enteropathy is still poorly understood. Although *L. intracellularis* has been most frequently recognized and studied by serology and molecular methods in swine and horses (5-8), diseases that closely resemble porcine

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proliferative enteropathy and are also caused by *L. intracellularis* have been described in a range of host species as single case reports, including rodents, deer, emus, wolves, foxes, non-human primates, and rhesus macaques (9-16). Much of the available information regarding *L. intracellularis* is still rudimentary, despite its worldwide spread, high prevalence, economic impacts on the swine industry, and newly identified susceptible animal hosts. The fastidious conditions required for the isolation and in vitro cultivation of this pathogen also impede the progress of discovery and make *L. intracellularis* difficult to study. Therefore, previous surveillance for proliferative enteropathy caused by *L. intracellularis* has focused on the swine and equine industries, while reports in other species are in the format of case studies (10, 15-17).

L. intracellularis has been reported in animals living in the wild, most frequently in wild pigs in the Czech Republic (15, 18) and in the feces of wolves (*Canis lupus*), red foxes (*Vulpes vulpes*), and red deer (*Cervus elaphus*) in the Slovak Republic (16). Recent studies of *L. intracellularis* in wild and feral animals caught on pig farms suggested a potential environmental spill-over from swine to wildlife (19, 20). However, little is known about the mechanisms of transmission of *L. intracellularis,* especially in wild bird species, which could be important vectors for this bacterium. Proliferative enteropathy has not been reported in chickens or other avian species, with the exception of ratite birds (11, 17). Although *L. intracellularis* DNA was recently detected in turkey flocks (21), the disease has been confirmed only in emus (11) and ostriches (17) and has been reported as being absent in chickens and wild birds (22-24). The aim of the present study was to screen for the presence of *L. intracellularis* in the ileal tissues of dead wild birds in the ROK.

Materials and methods

Samples

The study was carried out from 2010 to 2013. Carcasses of wild birds submitted to the Conservation Genome Resource Bank for Korean Wildlife (CGRB, Seoul National University, Seoul, ROK) and the Animal Disease Diagnostic Center of the Animal, Plant, and Fisheries Quarantine and Inspection Agency of the Ministry of Food, Agriculture, Forestry and Fisheries of the ROK, were used in the study. A total of 745 dead wild birds (belonging to 70 species) from all geographical regions of the ROK were submitted for examination, comprising 51 samples from 2010, 167 samples from 2011, 239 samples from 2012, and 288 samples from 2013. Table 1 shows the taxonomic families of the birds collected. All of the birds were received as carcasses, which were then stored at -20 ℃, until required. At necropsy, the gastrointestinal tracts were examined, with special attention paid to gross pathological findings, such as swelling, congestion, and hemorrhage of the ileum and cecum. The mucous membrane of the ileum was sampled by scraping the intestinal walls of each carcass; in addition, a 1-cm sample was taken from the middle of each ileum.

Polymerase Chain Reaction (PCR)

Ileal tissues were processed for nucleic acid purification within 48 hours of the carcasses having thawed. Phosphate-buffered saline (2 mL) was added to 1 g of ileal tissue in a conical tube. Each sample was vortexed for 10 sec. Nucleic acid purification from 180 µL of the supernatant was performed using an automated nucleic acid extraction system (BioRobot M48 Workstation, Qiagen, GmBH, Hilden, Germany) according to the manufacturer's recommendations. The purified DNA was then amplified by PCR using a previously described assay targeting an *L. intracellularis* gene, GenBank ID L08049 (25), and using a Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). The following primer sequences were used: LIA (5′-TATGGCTGTCAAACACTCCG-3) and LIB (5′-TGAAGGTATTGGTATTCTCC-3′). Positive (DNA from a pure culture of *L. intracellularis*) and negative (*L. intracellularis*-free DNA from ileal tissue samples) DNA controls were used in each run. After the PCR reaction, amplification products (5 µL) were analyzed by electrophoresis on a 3% agarose gel containing 0.5 µg/mL ethidium bromide. A 319-bp product indicated that *L. intracellularis* DNA was in the original sample. To prevent cross-contamination, the lab areas used for sample preparation, DNA extraction using automated nucleic isolation/processing, and amplification/post-PCR analysis

were physically separated. To prevent false positives, individual reagents and PCR-related consumables were screened before use to test for unknowns, especially oligonucleotides. Moreover, negative controls were run for every step using fresh reagents and disposables.

Cloning, Sequencing, and Analysis of Nucleotide Sequences

PCR and sequencing analysis of the 16S rRNA gene were performed. The amplified PCR products of DNA extracted from the ileal tissues were used for sequence analysis. Briefly, the PCR products were purified using a PCRquick- spin™ PCR Product Purification Kit (Intron Biotechnology, Seongnam-si, ROK) and cloned into the pGEM-T cloning system (Promega Corp., Madison, WI, USA). The plasmid clones were purified with a DNA- spin™ Plasmid DNA Extraction Kit (Intron Biotechnology), and the sequence analysis was conducted by Macrogen (Seoul, ROK). Nucleotide sequence homology searches of the cloned products of *L. intracellularis* in ileal tissues from the dead birds were analyzed by the National Center for Biotechnology Information (NCBI) BLAST network service.

Results and discussion

Of the 745 birds examined, *L. intracellularis* DNA was present in the mucous membrane of the ileum from one Eurasian eagle-owl (*Bubo bubo*; 5.0% among 20 samples), two black-billed magpies (*Pica pica sericea*; 2.0% among 96 samples), and one jungle crow (*Corvus macrorhynchos*; 3.0% among 33 samples) (Table 2). The amplified 16S rRNA gene sequences from all four infected wild birds were found to be 100% identical to a strain designated *L. intracellularis* PHE/MN1-00 (GenBank accession no. AM180252.1) by sequence analysis. In some wild birds, gross pathology, such as swelling, congestion, or intestinal hemorrhage, was found, but was not correlated with the molecular detection results. No pathological findings were observed in the intestines of wild birds that tested positive for *L. intracellularis*. The lack of molecular pathogen detection in those wild birds with pathological findings may be related to a potential intermittent mode of pathogen shedding or recovery from *L. intracellularis* infection.

All of the wild birds that were found to be positive for *L. intracellularis* in this study were collected in provinces where the prevalence of farm animals was relatively high. For example, the *L. intracellularis* DNA-positive Eurasian eagle-owl was collected in Gyeonggi province, where a 34.8% pig prevalence and 77.3% herd prevalence of *L. intracellularis* were previously reported (3). A black-billed magpie was found in Gangwon province (46.5% pig and 63.6% herd prevalence) and in Chungnam province (44.4% pig and 89.1% herd prevalence). In addition, the jungle crow was found in Jeju province (40.6% pig and 64.7% herd prevalence). All of the wild birds collected in areas with a lower prevalence of pigs and herds were negative in this study, e.g., Chungbuk (26.4% pig and 52.9% herd prevalence), Jeonbuk (30.8% pig and 38.2% herd prevalence), and Gyeongnam (20.3% pig and 47.9% herd prevalence).

The demonstration of *L. intracellularis* as a causative agent is difficult (4, 26), because its in vitro cultivation is complicated and not widely available. For these reasons, methods of molecular biology are widely used to detect this pathogen (25, 27, 38). Our surveillance method for the molecular detection of DNA demonstrated evidence of *L. intracellularis* in tissue samples of the small intestine in dead wild birds (Eurasian eagle-owl, black-billed magpie, and jungle crow) during the surveillance period. However, there are a few reports that detail a lack of evidence for the presence of *L. intracellularis* in other avian species, such as sparrows (*Passer domesticus*) and domestic poultry (*Gallus gallus*) (29, 24). McOrist *et al.* could not find evidence of *L. intracellularis* DNA in chickens with enteric disease and considered that the bacterium appears to be associated with malabsorption syndromes in these birds (23).

We previously reported that 40.0% (CI: 39.4-40.6%) of pigs and 71.9% (CI: 70.3-73.4%) of swine herds (3) were serologically positive for *L. intracellularis*. Lim et al. published that a total of 13/137 healthy rabbit feces were positive for *L. intracellularis* in the ROK (30). In addition, Hossain et al. reported that a total of 35 (25.74%) out of 136 sera and 36 (33.03%) out of 109 feces were positive for *L. intracellularis* in wild animals, such as the Korean water deer (*Hydropotes inermis*), Siberian roe deer (*Capreolus pygargus*), and raccoon dogs (*Nyctereutes procyonoides*), in

Table 1: Seven hundred forty-five dead wild birds from 70 species were tested for the presence of *Lawsonia intracellularis* infection

Family	Species	Common name	Samples
Accipitridae	*Aegypius monachus*	Cinereous vulture	1
	Buteo buteo	Common buzzard	8
	Accipiter nisus	Eurasian sparrowhawk	1
Alcedinidae	*Alcedo atthis*	Common kingfisher	3
Anatidae	*Anas formosa*	Baikal teal	6
	Anser fabalis	Bean goose	2
	Anas platyrhynchos	Mallard	60
	Aix galericulata	Mandarin duck	2
	Psittacidae	Parrot	1
	Anas acuta	Pintail	2
	Anas poecilorhyncha	Spot-billed duck	16
	Anser albifrons	White-fronted goose	6
Anatinae	*Anas crecca*	Common teal	7
Ardeidae	*Nycticorax nycticorax*	Black-crowned night heron	5
	Bubulcus ibis	Cattle egret	10
	Ardea alba	Great egret	6
	Ardea cinerea	Gray heron	9
	Mesophoyx intermedia	Intermediate egret	1
	Egretta garzetta	Little egret	8
	Butorides striatus	Striated heron	3
Caprimulgidae	*Caprimulgus jotaka*	Gray nightjar	5
Ciconiidae	*Ciconia boyciana*	Oriental white stork	1
Columbidae	*Columba livia*	Feral pigeon	3
	Columba rupestris	Hill pigeon	39
	Streptopelia orientalis	Rufous turtle dove	19
Coraciidae	*Eurystomus glaucurus*	Broad-billed roller	3
Corvidae	*Cyanopica cyanus*	Azure-winged magpie	1
	Pica pica sericea	Black-billed magpie	96
	Garrulus glandarius	Jay	6
	Corvus macrorhynchos	Jungle crow	33
Cuculidae	*Cuculus canorus*	Common cuckoo	1
	Cuculus optatus	Oriental cuckoo	1
Emberizidae	*Emberiza rustica*	Rustic bunting	2
Falconidae	*Falco tinnunculus*	Common kestrel	11
	Falco subbuteo	Eurasian hobby	10
Fringillidae	*Carduelis spinus*	Eurasian siskin	1
	Carduelis sinica	Gray-capped greenfinch	1

Family	Species	Common name	Samples
Gaviidae	*Gavia stellata*	Red-throated diver	1
Halcyonidae	*Halcyon pileata*	Black-capped kingfisher	2
Hirundinidae	*Hirundo rustica*	Barn swallow	1
Laridae	*Larus crassirostris*	Black-tailed gull	2
	Larus argentatus	Herring gull	1
Muscicapidae	*Cyanoptila cyanomelana*	Blue-and-white flycatcher	1
Oriolidae	*Oriolus chinensis*	Black-naped oriole	2
Paridae	*Parus major*	Great tit	2
Passeridae	*Passer montanus*	Tree sparrow	18
Phasianidae	*Gallus gallus domesticus*	Chick	1
	Chrysolophus pictus	Golden pheasant	1
	Gallus gallus var. domesticus	Korean black chicken	1
	Phasianus colchicus	Ring-necked pheasant	39
Picidae	*Dendrocopos major*	Great spotted woodpecker	1
	Picus viridus	Green woodpecker	1
	Dendrocopos kizuki	Japanese pygmy woodpecker	1
Procellariidae	*Calonectris leucomelas*	Streaked shearwater	1
Pycnonotidae	*Microscelis amaurotis*	Brown-eared bulbul	11
Rallidae	*Fulica atra*	Coot	1
	Gallinula chloropus	Moorhen	1
Scolopacidae	*Numenius phaeopus*	Whimbrel	1
	Scolopax rusticola	Woodcock	6
Strigidae	*Ninox scutulata*	Brown hawk owl	37
	Otus lettia	Collared scops owl	7
	Bubo bubo	Eurasian eagle-owl	20
	Otus scops	Eurasian scops owl	33
	Asio otus	Long-eared owl	2
	Strix aluco	Tawny owl	1
Sturnidae	*Sturnus cineraceus*	Gray starling	1
Sylviidae	*Paradoxornis webbiana*	Vinous-throated parrotbill	1
Turdidae	*Turdus hortulorum*	Gray-backed thrush	1
	Zoothera dauma	White`s thrush	13
Zosteropidae	*Zosterops japonicus*	Japanese white-eye	1
Unidentified			142
Total			745

the ROK (31). However, a molecular survey of *L. intracellularis* in wild birds was lacking.

This is the first report of the detection of *L. intracellularis* in wild birds in the ROK. In this study, very few wild birds were infected with *L. intracellularis*, but the infections are likely to have epidemiological relevance. One Strigidae (Eurasian eagle-owl) and two Corvidae (black-billed magpie and jungle crow) were exposed to *L. intracellularis*. The Eurasian eagle-owl is known to feed mainly on small mammals, such as voles, rats, mice, and hares. However, prey the size of foxes, marmots, and young deer (up to 17 kg) can also be killed, if taken by surprise (32). Another significant group of prey is other birds, and almost any type of bird is potential prey. Common avian prey includes corvids, grouse, woodpeckers, and other raptors. These feeding behaviors could be a reason why the Eurasian Eagle-owl had been exposed to *L. intracellularis*, given that recent studies have shown exposure to *L. intracellularis* among wild and feral animals, e.g., cats, rabbits, foxes, and wild rodents, that were caught on pig farms (16, 19, 20). The black-billed magpie is an opportunistic omnivore, known for eating many types of insects, carrion, seeds, rodents, berries, nuts, eggs, and garbage and food from pets that are fed outside (33). Its chicks are fed animal matter almost exclusively. Crows are also omnivorous (34), and will eat a variety of both plant and animal foods, whether alive or dead, including fruits, nuts, mollusks, earthworms, seeds, frogs, eggs, nestlings, mice and carrion (35, 36). In rural areas of the ROK, these two Corvidae species, the black-billed magpie and jungle crow, scavenge livestock feeding areas in large numbers, and obtain much of their food from grains spilled or wasted by livestock feeders or from undigested grain in horse manure (37). These foraging habits may be responsible for the positive PCR results for *L. intracellularis*, because horses are one of the most important susceptible animal species in the epidemiology of proliferative enteropathy. The feeding patterns of the Eurasian eagle-owl, black-billed magpie, and jungle crow and previous reports of wild and feral animals exposed to *L. intracellularis* could be possible alternative explanations for the association between *L. intracellularis* and wild birds. Further study will be necessary to determine the relationship between susceptible animal species and avian species, given that increasing numbers of new susceptible animal hosts being identified.

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DOLOČANJE DNK BAKTERIJE *Lawsonie intracellularis* V TKIVU VITEGA ČREVESA MRTVIH PTIC V REPUBLIKI KOREJI

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Povzetek: Vrsta bakterije *Lawsonia intracellularis* je vzrok proliferativne enteropatije pri različnih vrstah živali. O mehanizmih prenosa *L. intracellularis*, še zlasti pri divjih ptičjih vrstah, je na voljo malo podatkov. Prisotnost *L. intracellularis* pri mrtvih prosto živečih pticah v Republiki Koreji je bila raziskana z metodo verižne reakcije s polimerazo (PCR). DNK *L. intracellularis* smo dokazali v sluznici vitega črevesja pri veliki uharici (*Bubo bubo*, Strigidae), dveh korejskih srakah (*Pica pica sericea,* Corvidae) in eni velekljuni vrani (*Corvus macrorhynchos*, Corvidae) izmed 745 preiskanih mrtvih divjih ptic. Čeprav je bilo v tej študiji le nekaj prosto živečih ptic izpostavljenih *L. Intracellularis,* kaže, da je izpostavljenost epidemiološko pomembna. V povezavi z običajnim obnašanjem različnih vrst ptic je verjetno možno domnevati, da so vrste izpostavljene *L. intracellularis* (velika uharica, korejska sraka in velekljuna vrana) na različnih farmah zlahka dostopale do prašičev ali konj, ki pa so znani rezervoarji za *L. Intracellularis*. Tako je verjetno, da imajo te in podobne vrste visoko možnost izpostavitvi *L. intracellularis* in bi lahko služile kot biološki vektorji za proliferativno enteropatijo. Drugo možnost izpostavitve teh ptic *L. intracellularis* pa so lahko načini prehranjevanja divjih ptic in njihovi stiki z divjimi živalmi, okuženimi z *L. Intracellularis.*

Ključne besede: *Lawsonia intracellularis*; geni; diagnoza; nadzor; nalezljiva bolezen; PCR