The Journal of Veterinary Medical Science

NOTE

Wildlife Science

DNA-based detection of *Leptospira wolffii*, *Giardia intestinalis* and *Toxoplasma gondii* in environmental feces of wild animals in Korea

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J. Vet. Med. Sci.
83(5): 850–854, 2021
doi: 10.1292/jvms.20-0596

Received: 8 October 2020
Accepted: 18 March 2021
Advanced Epub:
26 March 2021

ABSTRACT. *Leptospira*, *Giardia intestinalis* and *Toxoplasma gondii* infections are reported in humans and animals worldwide, but molecular surveillance of these pathogens in Korean wildlife is still limited. Here, we examined the prevalence of these pathogens in environmental feces of Eurasian otters, leopard cats and raccoon dogs using nested PCR followed by DNA sequencing. *G. intestinalis* was detected in all of three animals, while *T. gondii* was detected only in leopard cats. *Leptospira wolffii* was detected in raccoon dog and Eurasian otter. Our results suggest that these animals can act as a reservoir of these zoonotic pathogens. Consistent monitoring of these pathogens in wildlife is needed to prevent from their infections in humans and livestock in Korea.

KEY WORDS: *Lutra lutra*, *Nyctereutes procyonoides*, One Health, *Prionailurus bengalensis*, zoonosis

Leptospirosis, giardiasis and toxoplasmosis are reemerging zoonotic diseases caused by *Leptospira* spp., *Giardia intestinalis* and *Toxoplasma gondii*, respectively [10, 12, 27]. These pathogens are distributed worldwide and can exist in non-infectious form in environment or can cause asymptomatic to severe infections in both humans and animals. Leptospirosis is the prevalent bacterial zoonosis, as it affects most of the mammalian species [4]; hence, the burden and risk of leptospirosis to human health is high [31]. Giardiasis is another common parasitic gastrointestinal disease in humans [13], and about 280 million global people have reported to have symptomatic giardiasis annually [9]. Toxoplasmosis caused by the intracellular protozoan parasite is prevalent in both human and livestock populations [7]. The estimated global seroprevalence of toxoplasmosis is 30–50% [23], though, the infections vary widely by region [12].

Wild animals are considered to be an important reservoir of zoonotic pathogens [18]. The Korean peninsula is recognized as one of the "hotspots" for emergence of zoonotic diseases [15], as rapid urbanization in this region intensifying the interactions between wildlife, livestock and humans, which increases the risk of transmission of zoonotic pathogens to domestic animals and humans. Multiple outbreaks of various zoonotic diseases have been reported in Korea, including leptospirosis [17], giardiasis [5] and toxoplasmosis [6]. The presence of zoonotic pathogens in feces of wildlife might be an important epidemiological indicator of the risk of zoonoses to human and livestock populations, hence fecal samples are often used to detect various zoonotic pathogens in animals, such as stray domestic cats [16, 32] and stray domestic dogs [28]. However, surveillance of zoonotic pathogens in feces of wildlife is still lacking in Korea.

Here, we investigated the prevalence of *Leptospira*, *G. intestinalis* and *T. gondii* in fecal samples of three medium-sized carnivore or omnivore animals inhabiting Korea, namely, Eurasian otters (*Lutra lutra*), leopard cats (*Prionailurus bengalensis*) and raccoon dogs (*Nyctereutes procyonoides*), using nested PCR followed by DNA sequencing. The results of this study would be useful for evaluating the risk of exposure of these zoonotic pathogens from wildlife to livestock and to humans in Korea.

The fecal samples of Eurasian otters (n=7), leopard cats (n=22) and raccoon dogs (n=15) were collected from different sites in Korea. The otters' spraints were previously collected in an estuary area in Ansan-si in Gyeonggi-do, with a centroid position at

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

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37°16'22.6"N and 126°50'24.7"E, for their diet analyses on June 2017 [19]. The scat samples of leopard cats were collected in the Gongju-si and Yeongi-gun area in Chungcheongnam-do (n=14) at a centroid position at 36°32'2.86"N and 127°10'13.25"E, the Sangju-si area in Gyeongsangbuk-do (n=1) at 36°17'55.8"N and 128°7'27.12"E, and the Dalseong-gun and Goryeong-gun area in Daegu-si or Gyeongsangbuk-do (n=7) at a centroid position at 35°44'0.94"N and 128°24'10.24"E during February 2019. The raccoon dogs' feces were collected in the Seosan-si area in Chungcheongnam-do at a centroid position of 36°39'52.05"N and 126°28'41.02"E on May 2017. We used a sterile wooden spatula to collect about 10 g of each feces into a 50 m*l* conical tube. The samples were stored in laboratory at −80°C until DNA extraction.

Total genomic DNA extraction of all fecal samples was performed using a PowerMax1 Soil DNA Isolation Kit (Mobio Laboratory, Inc., Carlsbad, CA, USA) by following a modified protocol reported previously [1, 19]. First, each Mobio tube was filled with approximately 250 mg of each homogenized fecal sample together with additional 0.1 mm (300 mg) and 0.5 mm (100 mg) diameter glass beads. Next, sample homogenization was carried out on a bead beater (BioSpec Products, Inc., Bartlesville, OK, USA) for 3 min. Finally, DNA was extracted and eluted into 50 µl of Tris-ethylenediaminetetraacetic acid buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0) according to the protocol of manufacturer. For each fecal sample, DNA extractions were performed in triplicate and recombined at the elution step to obtain sufficient DNA quantity.

Each fecal sample was verified for its identity by Eurasian otter-, leopard cat- or raccoon dog-specific PCR assay with a primer pair LutcytF/LutcytR targeting the cytochrome b gene [26], PrioF/PrioR targeting the 12S rRNA gene [11] or spRdg/prH targeting the D-loop region of mitochondrial DNA [25], respectively. All fecal samples of Eurasian otters and leopard cats were positive in PCR amplification, whereas, 11 out of 15 samples of raccoon dog feces showed PCR amplification. The four negative samples were excluded from analyses.

The positive control DNA templates were obtained after *in silco* PCR in SerialCloner v2.6.1. These DNA templates containing priming sites of *Leptospira*, *G. intestinalis* and *T. gondii* were synthesized from 18th to 541th nucleotide positions of the 16S rRNA gene (AB758738.1), 1st to 292th nucleotide positions of the 18S rRNA gene (MK573336.1), and 1st to 230th nucleotide positions of the B1 gene (KC607827.1), respectively, and cloned into the pTOP Blunt V2 vector (Enzynomics Co., Ltd., Seoul, Korea) at Macrogen Incorporation, Seoul, Korea.

Each of the nested PCR assays was carried out in triplicate using each primer set described in Supplementary Table 1. The first round of PCR was performed in reaction mixture (30 µl) containing approximately 50 ng of fecal DNA template and 15 µl of Premix Taq™ (Takara Bio Inc., Kusatsu, Japan). The following nested PCR was carried out using 1 µl of the first PCR product as template. The primers concentrations and thermal conditions were as described previously [14, 22, 24]. After the reaction, the amplified products were purified and analyzed by gel electrophoresis on 3% agarose (OmniPur Agarose, Merck & Co., Inc., Kenilworth, NJ, USA) prepared in 1 × Tris base, acetic acid and ethylenediaminetetraacetic acid buffer followed by staining with SYBR Green I Nucleic Acid Gel Stain (Invitrogen, Waltham, MA, USA). Each sample was considered positive for the specific pathogen if two of three nested PCR replicates showed similar amplification length compared to the respective positive control.

For positive samples by nested PCR assays, the targeted bands obtained after second nested PCR were purified from the agarose gels using MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced using the respective forward primers on an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at Macrogen Incorporation, Seoul, Korea. The detection of *Leptospira* was not conclusive due to ambiguity in obtained DNA sequence reads, therefore, the PCR products were cloned into pCR2.1 TOPO TA vector (Invitrogen, Waltham, MA, USA), and from each clone library five representative clones were sequenced using the M13 F primers (Bionics Co., Seoul, South Korea). The taxonomic assignments of resulting sequences were performed by BLASTN search against the National Center for Biotechnology Information (NCBI)'s nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The results of nested PCR assays are shown in Supplementary Figs. 1–3 and Supplementary Tables 2–4. For the positive samples by nested PCR assays, DNA sequencing of the PCR products was performed (Supplementary Data 1). DNA sequencing validated that all of the detection of *G. intestinalis* and *T. gondii* by nested PCR assays was due to DNA of *G. intestinalis* and *T. gondii* (Table 1). *G. intestinalis* was detected in all of three animals, while *T. gondii* was detected only in leopard cats. *G. intestinalis* is one of the most common enteric parasites with a broad host range [10]. The first outbreak of human giardiasis in Korea was reported due to water contamination in 2010 [5]. The prevalence of *G. intestinalis* was similar to the prevalence in wildlife by a previous study [3], but lower than the prevalence in domesticated animals reported by previous studies [20, 28].
T. gondii was only detected in fecal samples of leopard cats, and the presence of *T. gondii* has been recorded in fecal samples of stray domestic cats in Korea [16]. *T. gondii* oocysts are known to persist and remain viable for a long duration in soil [8, 21], hence the risk of oocyst-borne infections in humans and animals may be high, especially following the intense rainfall events that mobilize the oocysts from soils to water bodies.

However, the detection of *Leptospira* by DNA sequencing of PCR products was not conclusive due to ambiguity in obtained DNA sequence reads (Table 1). *Leptospira wolffii* was identified in one samples of Eurasian otter (O01_Ls) and one sample of raccoon dog (R13_Ls). However, the sequence identities were low, i.e., 84% and 74%, respectively, which is due to mixed sequence reads in a PCR product by the *Leptospira*-specific nested PCR assay. Indeed, this nested PCR assay is not designed for specific *Leptospira* species. Therefore, it is possible that the sequences of multiple species within the genus *Leptospira* existed in a PCR product, which might have led to ambiguity in obtained DNA sequence reads. It is also probable that *Leptospira* DNA was cross-amplified with other bacterial taxa as we identified non-*Leptospira* bacterium in one sample of leopard cat (L15_Ls) (Table 1).

DNA sequencing of fragments subcloned from the PCR products was performed for samples O1 and R13 (Supplementary Data 2). We confirmed that the PCR products contained DNA fragments of *Leptospira wolffii* with sequence identities of more than 99.5%

Table 1. Closest BLASTN match sequences in GeneBank for PCR products of each pathogen-specific nested PCR assay [a)]

Pathogen	Sample ID [b)]	Read quality	Query length (bp)	Query coverage (%)	Identity (%)	BLASTN match in GeneBank	Accession number
Leptospira spp.	O01_Ls	Low signal	276	72	83.8	*Leptospira wolffii*	KX245334.1
	O02_Ls	Mixed	285	88	73.4	Uncultured bacterium	KF247583.1
	O03_Ls	Mixed	241	43	82.1	Uncultured bacterium	KM200387.1
	O07_Ls	Mixed	236	39	80.4	Uncultured delta proteobacterium	EF075352.1
	L01_Ls	Mixed	280	44	81.3	Uncultured bacterium	KT769478.1
	L02_Ls	Mixed	279	45	78.3	Uncultured bacterium	KT769478.1
	L03_Ls	Mixed	280	93	97.7	Uncultured bacterium	KF247583.1
	L04_Ls	Mixed	233	24	88.1	Uncultured delta proteobacterium	EF075352.1
	L05_Ls	Mixed	280	94	98.5	Uncultured bacterium	JQ370007.2
	L07_Ls	Mixed	301	69	88.2	Uncultured bacterium	KF909666.1
	L08_Ls	Mixed	231	38	76.7	Uncultured bacterium	HM700763.1
	L10_Ls	Mixed	235	39	80.2	Uncultured delta proteobacterium	EF075352.1
	L12_Ls	Mixed	275	24	84.5	Uncultured bacterium	JF229060.1
	L14_Ls	Mixed	306	75	97.4	Uncultured bacterium	HM270596.1
	L15_Ls	Mixed	269	95	83.5	*Hymenobacter* sp.	MN833060.1
	L19_Ls	Mixed	229	34	77.2	Uncultured bacterium	KU931195.1
	L20_Ls	Mixed	294	94	98.6	Uncultured bacterium	KF909683.1
	R04_Ls	Mixed	267	96	97.7	Uncultured bacterium	KU929667.1
	R05_Ls	Mixed	225	39	77.5	Uncultured bacterium	JQ171440.1
	R06_Ls	Mixed	266	98	97.0	Uncultured bacterium	KU929667.1
	R07_Ls	Mixed	276	89	81.8	Uncultured rumen bacterium	AB614901.1
	R09_Ls	Mixed	260	53	83.1	Uncultured rumen bacterium	GU304251.1
	R12_Ls	Mixed	296	95	96.5	Uncultured organism	HQ785908.1
	R13_Ls	Mixed	273	81	74.4	*Leptospira wolffii*	MT611937.1
Giardia intestinalis	O06_Gi	High	120	99	99.2	*Giardia intestinalis*	MF153911.1
	L03_Gi	High	120	97	98.3	*Giardia intestinalis*	MF153911.1
	L11_Gi	High	128	96	99.2	*Giardia intestinalis*	MN263894.1
	L14_Gi	High	176	69	98.4	*Giardia intestinalis*	MT129481.1
	L15_Gi	High	236	52	100.0	*Giardia intestinalis*	MN263894.1
	L17_Gi	High	220	55	100.0	*Giardia intestinalis*	MT129481.1
	L19_Gi	High	106	95	98.0	*Giardia intestinalis*	KT595655.1
	L20_Gi	High	135	93	96.1	*Giardia intestinalis*	MT129481.1
	R09_Gi	High	138	98	95.7	*Giardia intestinalis*	MN263894.1
Toxoplasma gondii	L03_Tg	High	75	69	96.2	*Toxoplasma gondii*	AB743594.1
	L04_Tg	High	76	69	98.1	*Toxoplasma gondii*	AB743594.1

a) Raw sequence reads are shown in Supplementary Data 1. b) The letters before underscores indicate 'O' for Eurasian otters, 'R' for raccoon dogs and 'L' for leopard cats. The numbers indicate the sample IDs for each animal. The letters after underscores indicate 'Ls' for *Leptospira*, 'Gi' for *Giardia intestinalis* and 'Tg' for *Toxoplasma gondii*.

(Table 2). *L. wolffii*, which was first isolated from an infected patient in Thailand [29], belongs to the group of intermediate species [2]. The detection of DNA of *L. wolffii* was also reported in domestic animals in Iran [33] and wildlife in Malaysia [30]. In Korea, wild rodents (*Apodemus agrarius*) are reported as a primary natural reservoir of *Leptospira* spp. [17], indicating that infection might be common among their predatory animals (e.g., raccoon dogs).

In conclusion, we found that *Giardia intestinalis* was prevalent across the three medium-sized carnivore and omnivore animals inhabiting Korea, while *Toxoplasma gondii* was detected only in leopard cats (Table 3). *Leptospira wolffii* was identified in feces of Eurasian otter and raccoon dog. These results may not be representative of the overall prevalence of *L. wolffii*, *G. intestinalis* and *T. gondii* in Korea because of the limited sample size. However, these results still provide important insights into the prevalence of zoonotic pathogens in fecal samples of wildlife, and highlight the need of close monitoring of zoonotic pathogens to prevent the possible outbreaks of zoonotic diseases in Korea.

Table 2. Closest BLASTN match sequences in GeneBank for fragments subcloned from PCR products of the *Leptospira*-specific nested PCR assay [a)]

Sample ID [b)]	Subclone ID	Query length (bp)	Query coverage (%)	Identity (%)	BLASTN match in GeneBank	Accession number
O01_Ls	clone01	289	99	100.0	*Leptospira wolffii*	MK567972.1
	clone02	289	99	99.7	*Leptospira wolffii*	MK567972.1
	clone03	289	99	100.0	*Leptospira wolffii*	MK567972.1
	clone04	289	99	100.0	*Leptospira wolffii*	MK567972.1
	clone05	289	99	99.7	*Leptospira wolffii*	MK567972.1
R13_Ls	clone01	289	99	100.0	*Leptospira wolffii*	MK567972.1
	clone02	289	99	100.0	*Leptospira wolffii*	MK567972.1
	clone03	289	99	100.0	*Leptospira wolffii*	MK567972.1
	clone04	289	99	100.0	*Leptospira wolffii*	MK567972.1
	clone05	289	99	100.0	*Leptospira wolffii*	MK567972.1

a) Raw sequence reads are shown in Supplementary Data 2. b) The letters before underscores indicate 'O' for Eurasian otters and 'R' for raccoon dogs. The numbers indicate the sample IDs for each animal. The letters after underscores indicate 'Ls' for *Leptospira*.

Table 3. Summary statistics of the number of detection of *Leptospira*, *Giardia intestinalis* and *Toxoplasma gondii* in fecal samples of Eurasian otters, leopard cats and raccoon dogs

Pathogen	Nested PCR			DNA sequencing [a)]		
	Eurasian otter (n=7)	Leopard cat (n=22)	Raccoon dog (n=11)	Eurasian otter (n=7)	Leopard cat (n=22)	Raccoon dog (n=11)
Leptospira spp.	4 (57%)	13 (59%)	7 (64%)	1 (14%) [b)]	0 (0%) [b)]	1 (9%) [b)]
G. intestinalis	1 (14%)	7 (32%)	1 (9%)	1 (14%) [c)]	7 (32%) [c)]	1 (9%) [c)]
T. gondii	0 (0%)	2 (9%)	0 (0%)	0 (0%)	2 (9%) [c)]	0 (0%)

a) The samples positive by nested PCR assays were further analyzed by DNA sequencing of the PCR products and/or subcloned fragments of the PCR products. b) The number of samples identified as *Leptospira* by DNA sequencing of fragments subcloned from PCR products of the *Leptospira*-specific nested PCR assay. Only the samples identified as *Leptospira* by DNA sequencing of the PCR products were included for further analysis by DNA sequencing of their subcloned fragments. c) The number of samples identified as target pathogens by DNA sequencing of PCR products of each pathogen-specific nested PCR assay.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENTS. This work was supported by Seoul National University Research Grant in 2018 (W-SL, JK and NY) and by the Midcareer Researcher Program of the National Research Foundation of Korea (2020R1A2C1004903) (NY).

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