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Prevalence of *Anaplasma* sp. in Thrushes (Family Turdidae) in Jeju Island, Republic of Korea

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Abstract : Anaplasmosis is a rickettsial zoonosis mediated by blood-sucking arthropods, such as ticks, flies, and mosquitos. Migratory birds are common hosts of ticks that are mediators of anaplasmosis, in particular, the tick infection rate in thrushes (family Turdidae) has been known to be high. The main purpose of this study is to survey the occurrence and prevalence of *Anaplasma* spp. from the migratory thrushes in Jeju island. We collected blood samples from 6 thrushes rescued at the Jeju Wildlife Rescue Center and from 34 wild-caught thrushes on Mara island which is a satellite island of Jeju. As a result, the nested PCR confirmed that seven out of 40 individuals (17.5%) were infected by *Anaplasma* spp. and all of them were identified as *A. phagocytophilum* based on sequences obtained from partial 16S rRNA. All the infected birds were on their northward migration in spring, our results suggest that the Turdidae family, which is a common and abundant migrant group passing through Jeju island, may act a role as active reservoir and disperser of *A. phagocytophilum* causing potential influx of the zoonotic pathogens from its wintering grounds in lower latitude to the mainland Korea as well as Jeju.

Key words **:** Turdidae, *Anaplasma phagocytophilum*, migratory birds, Jeju island, Mara island.

Introduction

Anaplasmosis is a rickettsial zoonosis mediated by blood-sucking arthropods, such as ticks, flies, and mosquitos, and it is caused by obligate intracellular tick-borne bacterium which belongs to the family Anaplasmataceae. Anaplasmataceae includes the genus *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Wolbachia*, and six species have been known in the genus *Anaplasma*: *A. phagocytophilum*, *A. bovis*, *A. centrale*, *A. margimale*, *A. ovis*, and *A. platys* (5).

In general, migratory birds may enable ticks to disperse over a long distance as common hosts (3). Thrushes (family Turdidae), common and abundance migrants in the Republic of Korea, are medium-sized songbirds, and they are often highly infested by ticks due to their ground-feeding habits (1,3) In Europe, thrushes, especially the Blackbirds (*Turdus merula*), seem to be the most important host harboring ticks (8). Pathogens are often associated with ticks; previous studies in Korea reported that *Anaplasma* and *Ehrlichia* spp. were identified from *Haematophysalis flava* ticks collected from migratory birds and *A. phagocytophilum, A. bovis, A. centrale, A. platys, E. chaffeensis, Borrelia* spp. and *Batronella* spp. were detected in *Ixodes* spp. and *H. longicornis* ticks from migratory birds and vegations (12,19). Outside Korea, various tick-borne pathogens such as *A. phagocytophilum* (1,6,9,10,18), *Borrelia garinii* (20), *B. valaisiana* (20) and *B. burgdorferi* (1,6,11,18) were reported from ticks on migratory or sedentary birds. Along with the studies on the pathogen prevalence in collected ticks, direct detections of *A. phagocytophilum* (4,15,22) and *E. chaffeensis* (15) from avian hosts like Blackbirds and Chaffinches (*Fringilla coelobs*) have been reported. All of these results suggest that wild birds are hosts of ticks and reservoirs of associated pathogens, and migratory birds in particular may be dispersers carrying tick-borne pathogens over a long distance.

There have been several studies on the connection between birds and tick-borne pathogens in Korea, but most of them have focused on the tick infestation (3) and the pathogen detection in bird ticks, not from avian hosts themselves (12). Therefore, the information on the prevalence of tick-borne pathogens in wild birds is rarely available. This study aims to detect and identify *Anaplasma* spp. in migratory thrushes that represent the highest tick infestation rate in order to understand the possible influx of tick-borne pathogens to Jeju island through bird migration.

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Materials and Methods

Study area and sample collection

Study area is in the jurisdiction of Jeju Special Self-governing Province, including main island of Jeju and its satellite island. From 2012 to 2013, a total 34 migratory thrushes were trapped using mist nets (2.5 × 12 m) on Mara island in Seogwipo-si, Jeju. We also examined six rescued thrushes admitted to the Jeju Wildlife Rescue Center, Jeju National University. We collected blood samples (< 10 μl) from brachial veins of each thrush caught or rescued, comprising nine species: Brown-headed Thrush (*Turdus chrysolaus*, 3 birds), Dusky Thrush (*Turdus eunomus*, 1), Eye-browed Thrush (*Turdus obscurus*, 3), Grey-backed Thrush (*Turdus hortulorum*, 3), Japanese Thrush (*Turdus cardis*, 5), Naumann's Thrush (*Turdus naumanni*, 2), Pale Thrush (*Turdus pallidus*, 13), Siberian Thrush (*Zoothera sibirica*, 1), and White's Thrush (*Zoothera aurea*, 9) (Table 1).

DNA Extraction and 16S rRNA gene amplification

Total DNA was extracted from 5 μl of each heparin-treated blood sample using the DNeasy Blood & Tissue kit (Qiagen, Santaclarita, CA, USA). The DNA concentration was measured by spectrophotometer (NanoVue, GE Healthcare, USA) and adjusted about 100 ng/ul.

PCR was carried out in two stages. Primary PCR amplification was performed with the primer set AE1-F (5'-AAG CTT AAC ACA TGC AAG TCG AA-3') and AE1-R (5'-AGT CAC TGA CCC AAC CTT AAA TG-3') that was specific to the 16S rRNA gene of both *Anaplasma* and *Ehrilchia* species. After then, a nested PCR was performed with EE3F (5'-GTC GAA CGG ATT ATT CTT TAT AGC-3') and EE4R (5'-CCC TTC CGT TAA GAA GGA TCT AAT CTC C-3') that was specific to the 16S rRNA gene of *A. phagocytophilum.*

The primary PCR product was diluted to 1/20 and then the nested PCR was conducted. The PCR reaction consisted of 100 ng of genomic DNA, 10 μM of each primer, PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs and 1.5 U *Taq* polymerase (2X TOP simple TM DyeMIX aliquot-n*Taq* Kit, Enzynomics, Korea), making up to a final volume of 20 μl. The PCR cycling conditions were as shown in Table 2 and the PCR products were visualized using a 1.5% ethidium bromide-stained agarose gel.

Sequencing and phylogenetic analysis

The PCR products were used for the following sequence analysis (Solgent, Korea), and the sequences obtained (partial 16S rRNA gene) were compared with the GenBank database by nucleotide sequence homology searches made at the network server of the National Center for Biotechnology Information (NCBI) using BLAST. Multiple sequence alignments were performed using the program Clustal W and Phylogenetic trees were constructed by neighbor-joining method with distance matrix calculation by Kimura's two parameters, operated by MEGA (Molecular Evolutionary Genetics Analysis) software version 5.1 (17).

Table 1. Thrush species and information tested for *Anaplasma phagocytophilum* in this study

Date of colletion	Thrush (Common name)	Thrush (Scientific name)	ID no.
2012.04.01	Dusky Trush	Turdus eunomus	040-12144
2012.04.01	Pale Thrush	Turdus pallidus	040-12152
2012.04.01	Pale Thrush	Turdus pallidus	040-12164
2012.04.01	Pale Thrush	Turdus pallidus	040-12173
2012.04.01	White's Thrush	Zoothera aurea	050-07121
2012.04.07	White's Thrush	Zoothera aurea	050-07103
2012.04.07	White's Thrush	Zoothera aurea	050-12132
2012.04.07	White's Thrush	Zoothera aurea	050-12145
2012.04.07	Japanese Thrush	Turdus cardis	050-12878
2012.04.08	Pale Thrush	Turdus pallidus	040-12176
2012.04.08	Pale Thrush	Turdus pallidus	040-12822
2012.04.08	Pale Thrush	Turdus pallidus	040-12826
2012.04.08	Pale Thrush	Turdus pallidus	040-12837
2012.04.08	Pale Thrush	Turdus pallidus	040-12894
2012.04.08	Pale Thrush	Turdus pallidus	040-12899
2012.04.14	Pale Thrush	Turdus pallidus	020-12879
2012.04.14	Japanese Thrush	Turdus cardis	040-12836
2012.04.14	White's Thrush	Zoothera aurea	050-12166
2012.04.15	Pale Thrush	Turdus pallidus	040-12287
2012.04.15	Japanese Thrush	Turdus cardis	040-12857
2012.04.22	Grey-backed Thrush	Turdus hortulorum	040-12834
2012.05.30	Grey-backed Thrush	Turdus hortulorum	040-12845
2013.03.15	Naumann's Thrush	Turdus naumanni	040-12806
2013.03.31	Naumann's Thrush	Turdus naumanni	040-15403
2013.04.23	Japanese Thrush	Turdus cardis	2013-149
2013.05.04	Brown-headed Thrush	Turdus chrysolaus	040-15456
2013.05.11	Eye-browed Thrush	Turdus obscurus	040-15417
2013.05.25	Siberian Thrush	Zoothera sibirica	040-12816
2012.09.22	White's Thrush	Zoothera aurea	050-07135
2012.09.22	White's Thrush	Zoothera aurea	050-07152
2012.10.06	Grey-backed Thrush	Turdus hortulorum	040-12198
2012.10.09	White's Thrush	Zoothera aurea	2012-448
2012.10.13	Eye-browed Thrush	Turdus obscurus	040-12829
2012.10.19	Japanese Thrush	Turdus cardis	040-12842
2012.10.20	Eye-browed Thrush	Turdus obscurus	040-12195
2012.11.16	Brown-headed Thrush	Turdus chrysolaus	040-12831
2013.10.25	Brown-headed Thrush	Turdus chrysolaus	2013-516
2012.12.09	Pale Thrush	Turdus pallidus	2012-562
2012.12.31	White's Thrush	Zoothera aurea	2012-586
2013.01.17	Pale Thrush	Turdus pallidus	2013-033

Table 2. PCR condition for the detection of *Anaplasma phagocytophilum* in blood samples of migratory thrushes in Jeju Island, Korea

Primer	Species and target gene	PCR condition				Product size (bp)
		Denaturation	Annealing	Extension	cycles	
AE1-F AE1-R	*Anaplasma/Ehrilchia* spp. 16S rRNA	94°C/30s	59°C/30s	72°C/30s	35	1406
EE3F EE4R	*A. phagocytophilum* 16S rRNA	94°C/30s	56°C/30s	72°C/45s	35	926

Results

The nested PCR using the EE-16S primer set amplifies an ~1000 bp band in case of the infection of *A. phagocytophilum,* otherwise no band appears. As a result, seven blood samples had clear bands at 1000 bp site (Fig 1), and thus we detected *A. phagocytophilum* in seven out of 40 blood samples collected. Bird species with seven positive results were White's (*Zoothera aurea*; one bird), Japanese (*Turdus cardis*; two birds), and Pale Thrushes (*Turdus pallidus*; four birds) (Table 1). Among the seven infected thrushes, six were wild caught thrushes on Mara island while the rest was a rescued Japanese Thrush in main island of Jeju. All infected thrushes were caught or rescued during their northward migration in spring, not in autumn nor winter.

Sequencing and Phylogenetic analysis

By nucleotide sequence analysis of seven positive samples, we obtained the 926 bp sequence of each (Table 3). Though several mutations were identified in the nucleotide sequence of each individual, but all sequences corresponded to the sequence of *A. phagocytophilum* (> 99% identical), indicating that all the pathogens detected are *A. phagocytophilum.*

The sequences were compared with other gene sequence of *Anaplasma* and *Ehrlichia* species obtained from the GenBank database. sequence comparisons were as follow : *A. phagocytophilum* (GU046565) from Korea, *A. phagocytophilum* (GU064896) from Korea, *A. phagocytophilum* (GU556621) from Korea, *A. phagocytophilum* (AB196721) from Japan, *A. phagocytophilum* (AY055469) from USA, *A. phagocytophilum* (AY527213) from Sweden, *A. phagocytophilum* (AY969012) from Japan, *A. bovis* (GU556626) from Korea, *A. bovis* (EU181143) from Korea, *A. marginale* (DQ341370) from China, *A. platys* (AF536828) from Japan, *A. platys* (AF156784) from China, *A. centrale* (AF318944) from Netherlands, *A. marginale* (FJ226454) from Japan, *A. ovis* (EF587237) from China, *Ehrlichia sp.* (AJ242784) from Sweden, *E. canis* (M73226) from USA, *E. chaffeensis* (AF-416764) from USA, *E. chaffeensis* (M73222) from USA, *E. canis* (AF373613) from Venazuela (Fig 2) (19).

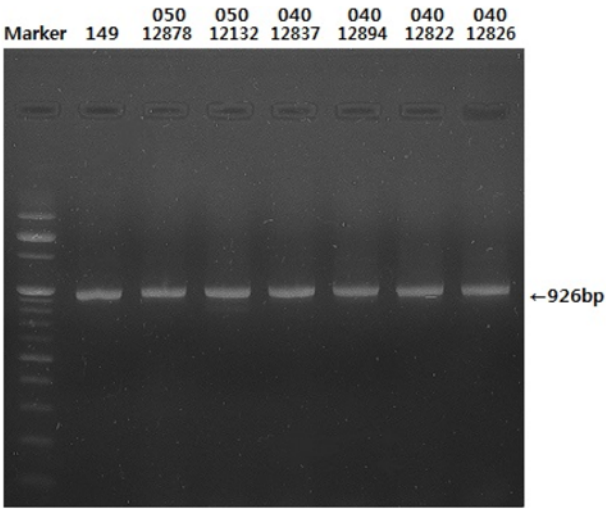

Fig 1. Agarose (1.5%) gel electrophoresis of the nested PCR products representing positive results about *A. phagocytophilum.*

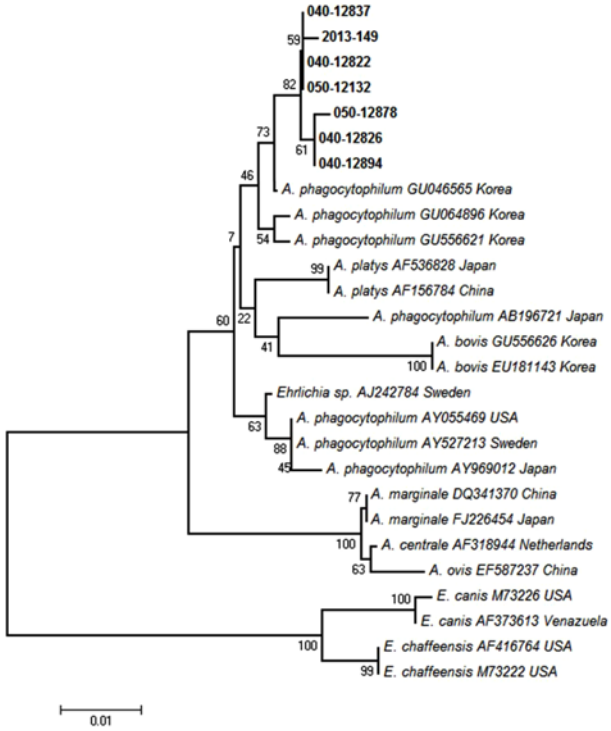

Fig 2. Phylogenetic tree of partial 16s rRNA gene sequences of various *Anaplasma* and *Ehrlichia* species and PCR-amplified 16s rRNA products the Family Turdidae collected in this study. Scale bar indicates the number of mutations per sequence position. The number at the each node represents the percentage of 1,000 bootstrap resamplings.

Table 3. Sequence alignments of the 16s rRNA genes in the positive samples. Dots indicate identical to those of the reference sample (050-12878).

	sequence																																					
												1	1	1	2	2	2	3	3	3	4	4	4	5	5	5	6	6	7	7	7	8	8	8	8	9	9	9
Sample				1	1	2	2	3	4	6	9	2	6	8	1	5	8	2	5	8	0	5	9	3	5	7	2	7	0	4	8	2	4	8	9	2	2	2
	1	2	3	0	5	0	5	0	4	7	5	0	8	5	3	7	5	7	6	4	1	4	3	3	2	8	2	3	3	8	5	6	8	7	2	4	5	6
050-12878	G	T	C	A	T	A	T	T	T	A	C	A	A	G	A	C	T	C	T	A	A	T	T	C	A	A	C	G	T	T	G	G	C	T	T	G	G	G
050-12132	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
040-12837	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
040-12894	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
040-12822	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
040-12826	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
2013-149	.	.	.	G	C	G	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.

Discussion

In this study, *Anaplasma phagocytophilum* causing the zoonotic anaplasmosis was detected in bloods of Turdidae birds. Up to now, *A. phagocytophilum* has been detected to diverse animals including racoon (7,13), red fox (7), mouse (16), dog (21), deer (22), etc, and it is known as a possible cause of human granulocytic anaplasmosis (HGA)(2). There was also a case report that *A. phagocytophilum* was identified from Blackbirds in the family Turdidae (4,22). Other studies about the *A. phagocytophilum* infection rate were 6.3% and 4.2% in ticks collected from Redwings (*Turdus iliacus*) and Blackbirds (9). Compared with such results, this study shows the relatively high infection rate of *A. phagocytophilum* (17.5%) in the Family Turdidae. It is unclear that such higher prevalence in thrushes was partially caused by different detection methods (blood vs bird tick tests), but it must be clearly related with the high tick infestation rate due to their ground-dwelling habits of Turdidae birds (3). Therefore, we may suggest that Turdidae plays a role as carriers of a tick-borne disease caused by *A. phagocytophilum.*

In this study, all of infected White's, Japanese, and Pale Thrushes were on their northward migration from their wintering grounds to Jeju, while no bird on southward migration was infected. This clear contrast between two seasons indicates that the tick-borne disease is more prevalent in the wintering grounds of thrushes in lower latitude than in breeding grounds in Jeju and the mainland Korea. As typical migratory birds in Mara island as well as Jeju, most of Turdidae birds pass through Mara island, Jeju island, and move to the northern breeding grounds like Korea, China, and Russia in spring (14), suggesting the influx of *A. phagocytophilum* into the mainland Korea as well as Jeju Island by migratory Turdidae.

The northward expansion of tick-borne diseases is recently one of special interests in terms of the effect of global climate change on public and animal health concerns (3). This study demonstrates a confirmed role of Turdidae, incoming from tropics and subtropics (such as Southeast Asia and Southern China), in the influx or transmission of tick-borne pathogens (*A. phagocytophilum*), though the detailed process and robust effect of the role are still unknown. Although we only investigated Turdidae in this study, we are able to suggest that other migratory birds may be reservoirs and carriers of any type of tick-borne diseases. In the future, combined and comprehensive surveillances on pathogens, vectors, and migratory hosts are recommended rather than separate studies confined to a specific group of interest.

Conclusion

We identified that seven out of 40 thrushes were infected by *A. phagocytophilum* in Jeju island in the Republic of Korea. This result indicates that the family Turdidae, common and abundant migratory birds passing through Jeju, may cause the influx of *A. phagocytophilum* into the mainland Korea as well as Jeju island during their northward migration.

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제주도와 마라도내 지빠귀과 조류에서 *Anaplasma* spp. 감염 조사

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요 약 : Anaplasmosis 은 흡혈성 절지동물인 진드기, 이파리, 모기 등에 의해 매개되는 리케차성 인수공통 전염병이다. 철새는 anaplasmosis의 매개체인 진드기의 숙주이다. 제주도의 다양한 철새 분류군 중에서 지빠귀과 조류의 진드기 감염률이 높다. 특히 마라도는 봄철 남방구에서 북방구로 이동하는 철새의 중간기착점으로 지리적으로 중요한 위치에 있다. 따라서 본 연구에서는 제주도의 대표적 이동철새인 지빠귀과 새들의 *Anaplasma* spp. 감염여부를 조사하였다. 우리는 마라도에서 34마리의 혈액과 제주야생동물구조센터에서 구조 채취된 6개의 혈액 시료를 대상으로 하였다. 그 결과, 40개체의 지빠귀 중 7개체가 감염이 확인되었으며, 감염률은 17.5%로 나타났다. 7개체 모두 *Anaplasma phagocytophilum*으로 동정되었다. 이러한 결과는 제주도를 통과하는 대표적인 철새인 지빠귀과 새들이 *A. phagocytophilum*을 육지로 전파할 수 있음을 시사하며, 다른 이동성 철새들간의 질병전파의 보균자로 작용할 수 있기에 철새, 텃새 및 가축으로의 전파여부를 지속적으로 모니터링할 필요가 있다.

주요어 : 지빠귀과, *Anaplasma phagocytophilum*, 철새, 제주도, 마라도