One Health 19 (2024) 100913

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

Circulation of tick-borne pathogens in wildlife of the Republic of Korea

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ARTICLE INFO

Keywords:
Wild animal
Tick-borne pathogens
Zoonotic disease
One health
Natural environment

ABSTRACT

Habitat loss of wildlife and increased human activities in their habitat provide more opportunities for human-wild animal contact. These artificial environments influence humans by facilitating the transmission of tick-borne pathogens. Therefore, we aimed to detect and understand circulating tick-borne pathogens in the natural environment by analyzing blood and spleen samples of wild animals admitted to wildlife rescue centers in the Republic of Korea. In total, 376 samples were collected from 355 rescued wild animals immediately after their arrival or death. After DNA deoxyribonucleic acid and RNA extractions, reverse transcription polymerase chain reaction (RT-PCR) and nested PCR were conducted to detect target tick-borne pathogens. This study detected six positive samples of severe fever with thrombocytopenia syndrome virus (SFTSV), 146 *Anaplasma phagocytophilum*, 55 *Anaplasma bovis*, 19 *Rickettsia* spp., 45 *Borrelia theileri*, and 4 *Bartonella schoenbuchensis*. Among the positive samples, SFTSV was detected in one spleen sample from a Korean water deer, from which SFTSV was successfully isolated. After full genome sequencing, the L, M, and S segments all belonged to genotype B-3 and indicated 99.84 % ~ 99.94 % similarity with SFTSV isolated from human serum. In conclusion, wild animals are potential reservoirs of tick-borne pathogens. Therefore, surveillance systems to prevent transmission among ticks, animals, and humans must be developed using the One Health concept.

1. Introduction

Modern environmental changes due to human influences, such as urbanization, deforestation, and technological development, have led to the introduction of tick species (*Haemphysalis* spp., and *Amblyomma* spp.) and tick-associated pathogens [1]. These anthropogenic factors directly or indirectly affect wild predators and can have cascading effects on wild prey species that play essential roles in enzootic cycles [2]. Ticks are hematophagous obligate parasites that interact with their vertebrate hosts and play central roles in tick-borne pathogen dynamics [3]. Ticks may transmit pathogens (i.e., viruses, bacteria, protozoa, and helminths) to vertebrate wild animals [4]. The abundance of ticks and tick-borne pathogens is determined by humidity, temperature, and the presence of hosts. The increasing presence of animal reservoirs and tick vectors have resulted in the global emergence of novel infections [5]. Wild animals play various roles in maintaining and spreading pathogens and zoonotic diseases. A previous study demonstrated that anthropogenic influences could disrupt enzootic cycles and cause epizootic outbreaks of tick-borne diseases [3].

According to a recent study, Black-faced Bunting (*Emberiza spodocephala*) and Olive-backed Pipit (*Anthus hodgsoni*) birds in the Republic of Korea (ROK), that migrate through East Asian flyways, were infected with severe fever with thrombocytopenia syndrome virus (SFTSV), also known as *Bandavirus dabieense* [6]. These birds migrate from China to the ROK; consequently, inflow tick-borne pathogens such as SFTSV have a zoonotic risk if they steadily circulate in the natural environment,

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https://doi.org/10.1016/j.onehlt.2024.100913
Received 12 August 2024; Received in revised form 29 September 2024; Accepted 7 October 2024
Available online 10 October 2024

Table 1
Nucleotide sequences and conditions of PCR primers for identification of target genes of tick-borne pathogens.

Target genes	Primer names and conditions	Primer sequences (5′ → 3′)				PCR product size (bp)	References
		Denaturation (°C/min)	Annealing (°C/min)	Extension (°C/min)	Cycles		
SFTSV S segment	NP-2F	CATCATTGTCTTTGCCCTGA					
	NP-2R	AGAAGACAGAGTTCACAGCA				461	[39]
	Condition	94/0.33	52/0.67	72/0.5	40		
	N2-F	AAYAAGATCGTCAAGGCATCA					
	N2-R	TAGTCTTGGTGAAGGCATCTT				346	[40]
	Condition	94/0.33	55/0.67	72/0.5	25		
TBEV E gene	TBE913F	TGCACACAYYTGGAAAACAGGGA					
	TBE1738R	TGGCCACTTTTCAGGTGGTACTTGGTTCC				854	[41]
	Condition	94/0.5	59/0.5	72/1	30		
	TBE1192F	CAGAGTGATCGAGGCTGGGGYAA					
	TBE1669R	AACACTCCAGTCTGGTCTCCRAGGTTGTA				506	[42]
	Condition	94/0.33	62/0.17	68/0.33	30		
CCHFV S segment	F1	TGGACACYTTCACAAACTC					
	R1	GRYRAAYTCCCTRCACCA				536	[43]
	Condition	94/0.5	55/1	72/0.17	35		
	F2	GAATGTGCWTGGGTYAGCTC					
	R2	GACATCACAATTTCACCAGG				260	
	Condition	94/0.5	55/1	72/0.17	35		
YZ S segment	F1	TGCTCCAATCCCAGAATGTGCCTGG					
	R1	CCTGTGCCTTCTCTTGCTCCTCATGTC				322	[44]
	Condition	94/0.5	60/0.5	72/0.5	35		
	F2	TCTTCACGGAGGGTATGGCA					
	R2	CTTCGGGGCAATGTAGACCT				221	In this study
	Condition	95/0.33	54/0.5	72/1	30		
LV L segment	MJ_F1	TGAGCATGAACTGCTAAAGAGC					
	MJ_R1	ATTCCTTCGTCTGGCACATC				577	[45]
	Condition	95/0.33	62/0.66	72/1	35		
	MJ_F2	CGGCCTCATCAGTTCCAAAG					
	MJ_R2	AAAGGCGGTGGACAGTAAGGA				510	
	Condition	95/0.33	62/0.5	72/0.5	30		
A. phagocytophilum 16S rRNA	AE1-F	AAGCTTAACACATGCAAGTCGAA					
	AE1-R	AGTCACTGACCCAACCTTAAATG				1406	[40]
	Condition	94/1	56/1	72/1.5	35		
	EE3	GTCGAACGGATTATTCTTTATAGCTTGC					
	EE4	CCCTTCCGTTAAGAAGGATCTAATCTCC				926	[46]
	Condition	94/0.83	56/0.83	72/1.17	25		
A. bovis 16S rRNA	AE1-F	AAGCTTAACACATGCAAGTCGAA					
	AE1-R	AGTCACTGACCCAACCTTAAATG				1406	[40]
	Condition	94/1	56/1	72/1.5	35		
	ABKf	TAGCTTGCTATGGGGACAA					
	AB1r	TCTCCCGGACTCCAGTCTG				547	[47]
	Condition	94/0.83	59/0.83	72/0.83	25		
E. chaffeensis 16S rRNA	AE1-F	AAGCTTAACACATGCAAGTCGAA					
	AE1-R	AGTCACTGACCCAACCTTAAATG				1406	[40]
	Condition	94/1	56/1	72/1.5	35		
	HE3	TATAGGTACCGTCATTATCTTCCCTAT					
	HE1	CAATTGCTTATAACCTTTTGGTTATAAAT				390	[48]
	Condition	94/0.5	56/0.5	72/0.5	25		
E. canis 16S rRNA	AE1-F	AAGCTTAACACATGCAAGTCGAA					
	AE1-R	AGTCACTGACCCAACCTTAAATG				1406	[40]
	Condition	94/1	56/1	72/1.5	35		
	HE3	TATAGGTACCGTCATTATCTTCCCTAT					
	ECAN5	CAATTATTTATAGCCTCTGGCTATAGGA				350	[49]
	Condition	94/0.5	56/0.5	72/0.5	25		
Borrelia spp. 16S rRNA	B1	CAGTGCGTCTTAAGCATGC					
	B8	CCTTAAATACCTTCCTCCC				1427	[50]
	Condition	94/1	58/1	72/1.5	30		
	B3	GCAGCTAAGAATCTTCCGCAATGG					
	B6	CAACCATGCAGCACCTGTATAT				714	
	Condition	94/0.5	59/0.75	72/0.75	25		
Rickettsia spp. 16S rRNA	gltA	GGGGACCTGCTCACGGCGG					
	gltA	ATTGCAAAAAGTACAGTGAACA				381	[51]
	Condition	95/0.75	54/0.75	72/0.75	35		
	gltA	CTAATGAAGCAGTGATAA					
	gltA	GCGACGGTATACCCATAGC				337	
	Condition	95/0.5	58/0.5	72/0.5	25		
Bartonella grahamii Internal transcribed spacer	QHVE1	TTCAGATGATGATCCCAAGC					
	QHVE4	AACATGTCTGAATATATC				735	[52]
	Condition	94/0.75	55/0.75	72/0.75	30		
	QHVE12	CCGGAGGGCTTGTAGCTCA					
	QHVE14	CACAATTTCAATAGAAC				484	[53]
	Condition	94/0.5	55/0.75	72/0.75	30		

(continued on next page)

Table 1 (*continued*)

Target genes	Primer names and conditions	Primer sequences (5′ → 3′)				PCR product size (bp)	References
		Denaturation (°C/min)	Annealing (°C/min)	Extension (°C/min)	Cycles		
Bartonella schoenbuchensis Internal transcribed spacer	JEN1F	CTCTTTCTTCAGATGATGATCC					
	B1623R	AACCAACTGAGCTACAAGCC				317	[54]
	Condition	95/1	60/1	72/0.5	20		
	2F	GCTTGCCGCCTTCATTTCTC					
	2R	ACCAACTGAGCTACAAGCCC				206	In this study
	Condition	95/1	60/1	72/0.5	20		
Theileria spp. MPSP	MPSP-F	CACGCTATGTTGTCCAAGAG					
	MPSP-R	TGTGAGACTCAATGCGCCTA				875	[55]
	Condition	93/0.66	62/0.5	72/1.5	35		

ultimately infecting humans through wild animals.

In the ROK, various tick-borne diseases and pathogens associated with viral, bacterial, or protozoal agents have been identified, including SFTSV, tick-borne encephalitis virus (TBEV), anaplasmosis, rickettsioses and bartonellosis have been identified [7]. In particular, SFTSV causes clinical symptoms in humans, including fever, thrombocytopenia, and multiple organ failure, with a mortality rate of 12–30 % [8]. There have been no human cases of TBEV in the ROK; however, the protein E gene of the western subtype of TBEV has been detected by reverse transcription (RT)-nested polymerase chain reaction (PCR) and isolated from the lung and spleen tissues of striped field mice captured in the ROK, as well as being isolated from *Haemaphysalis longicornis* and *Ixodes nipponensis* [9]. Such findings are especially significant considering that alterations in wild animal populations may result in the spread of tick-borne infections, and environmental changes can stimulate the migration of vertebrate tick hosts, leading to the distribution of pathogens into new territories [10].

This study aimed to detect tick-borne pathogens in the blood and spleens of rescued wild animals in the ROK. The findings of this study can contribute to the understanding of tick-borne pathogens circulating in the natural environment, thus enhancing the management of tick-borne diseases, protecting against secondary transmission to humans.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU) (IACUC nos. SNU-220708-4 and SNU-220708-4-1) and Jeonbuk National University Institutional Biosafety Committee (IBC) (IBC no. JBNU2022–03-002), conducted in strict accordance with the recommendations of the national guidelines.

2.2. Sample collection

Samples were collected from six wild animal rescue centers in the ROK, including the Gangwon Wildlife Rescue Center, Northern Gyeonggi Wildlife Rescue Management Center, Gyeonggi-do Wildlife Rescue Center, Wildlife Center of Chungbuk, Ulsan Wildlife Rescue Center, and Wildlife Medical Center in the Nakdong Estuary Eco Center in Busan from July 2022 to October 2023.

Fresh blood samples were taken immediately after rescue, or in case of death, a blood sample was taken at death and where possible a spleen sample was collected at autopsy. In total, 280 blood and 96 spleen samples were collected from rescued wild animals ($n = 376$). Among the collected samples, 21 blood and spleen samples were collected from the same animal. The animals included 160 birds; 131 Korean water deer (*Hydropotes inermis*), 49 raccoon dogs (*Nyctereutes procyonoides*), 11 Siberian roe deer (*Capreolus pygargus*), 2 leopard cats (*Prionailurus bengalensis*), and 2 Asian badgers (*Meles leucurus*). Individual blood and spleen samples were placed in sterile 1.5-mL micro-centrifuge tubes and stored at −20 °C before DNA/RNA extraction.

2.3. DNA and RNA extraction

Regarding blood samples, 200 μL of each specimen underwent 10-min RT incubation with 300 μL of lysis buffer and 20 μL of proteinase K. As for spleen samples, 20 mg of the spleen was incubated in a water bath at 56 °C with 300 μL of lysis buffer and 20 μL of proteinase K for 30 min. After incubation, DNA and RNA were extracted using the Patho Gene-spin DNA/RNA Extraction Kit (iNtRON Biotechnology, Seongnam, ROK), according to the manufacturer's instructions. The final elution volume of the blood and spleen was 60 μL, with 40 and 20 μL of it respectively being used for detecting RNA viruses and detecting bacteria and protozoa.

2.4. Detection of pathogens

In this study, 13 tick-borne pathogens were tested, including five RNA viruses (SFTSV, TBEV, Crimean-Congo hemorrhagic fever virus, Yezo virus, and Langya henipavirus), seven bacteria (*Anaplasma bovis*, *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Ehrlichia chaffeensis, Rickettsia* spp., *Borrelia* spp., and *Bartonella* spp.), and one protozoan (*Theileria* spp.).

In this study, only *Theileria* spp. were detected by RT-PCR, and the remaining 12 pathogens were detected by nested PCR. To detect the amplified genome, RT-PCR or nested PCR were performed using 10 pmol of specific primers for all 13 pathogens (Table 1). To avoid contamination, only the negative control was used as TE buffer without a positive control. After DNA and RNA extraction, sample concentration was evaluated through agarose gel electrophoresis and spectrophotometry (NanoPhotometer; Implen, Munich, Germany) to determine the template amount. For the first round of PCR, 4 μL or 2 μL of the template was used without dilution, respectively for blood and spleen samples. The analyses were carried out using DiaStar™ 2× One step RT-PCR Pre-Mix (Solgent, Daejeon, ROK) for virus detection, and BioFACTTM 2× Taq PCR Pre-Mix (BioFACT, Daejeon, Korea) and EZPCRTM XO 5× Pre-Mix (Elpis Biotech, Daejeon, ROK) for bacteria and protozoa detection.

For the second of round PCR, 1 μL of the template from first round was used for all samples along with BioFACTTM 2× Taq PCR Pre-Mix (BioFACT, Daejeon, ROK) for virus and bacteria detection and EZPCRTM XO 5× Pre-Mix (Elpis Biotech, Daejeon, ROK) for bacteria and protozoa detection. PCR amplicons were identified on a 1.2 % agarose gel using a 100-bp ladder molecular weight DNA size marker (GeNet Bio, Nonsan, ROK). All PCR experiments were performed using filter tips and sterile tubes.

2.5. Sequencing and phylogenetic analysis

All positive PCR amplicons were sequenced (Bionics, Seoul, ROK). The obtained sequences were analyzed using the Chromas software (version 2.66) and aligned with BioEdit (version 7.2). After alignment, phylogenetic trees were constructed using the Molecular Evolutionary

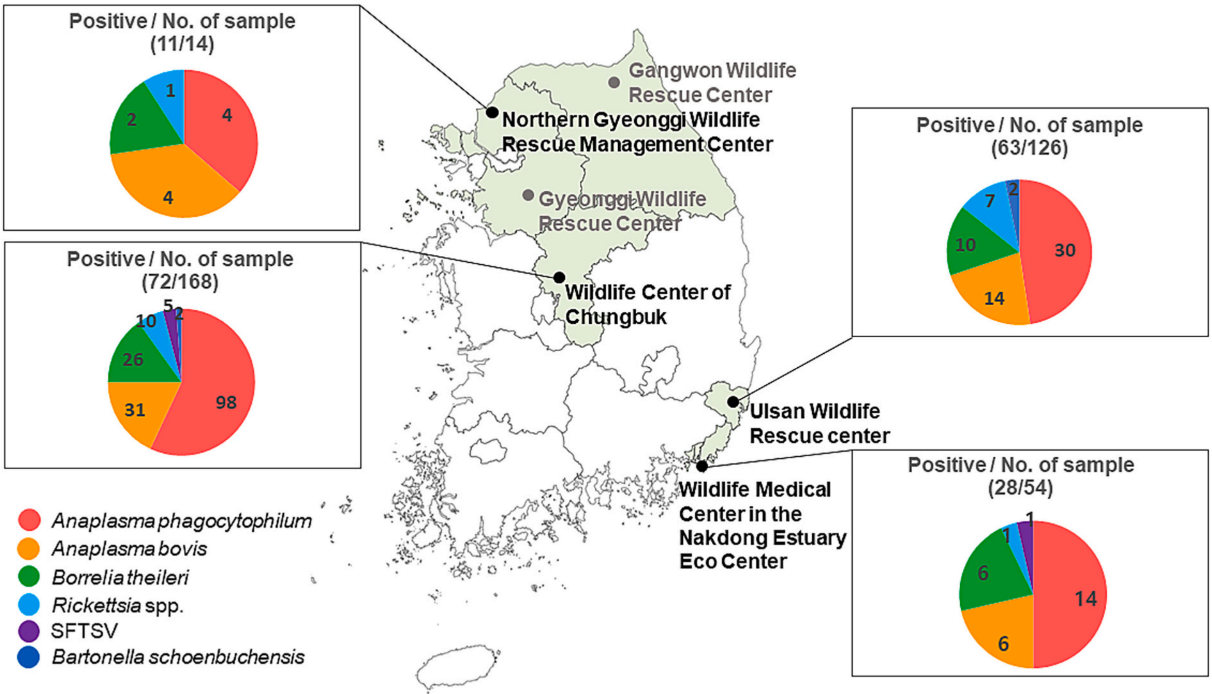

Fig. 1. Overall results of detected positive pathogens with nested polymerase chain reaction (PCR) in the Republic of Korea.

Genetics Analysis version 7.0 software based on maximum likelihood 1000 bootstraps.

2.6. SFTSV isolation

After the detection of SFTSV in the spleen of Korean water deer, Vero E6 cells were seeded in a T-75 flask at a concentration of 3×10^4 cells per 14 mL of Dulbecco's modified Eagle's medium (DMEM; HyClone™, USA), supplemented with 2 % fetal bovine serum (FBS; HyClone™, USA). Using blading scissors, 10 mg of the spleen of Korean water deer was prepared in a 1.5-mL tube, followed by two times phosphate-buffered saline (PBS) washing. Moreover, 300 μL of medium prepared previously was added to the SFTSV-positive sample. The spleen was ground well using an autoclaved homogenizer, and 300 μL of the supernatant was separated. The sample was prepared by filtering through a 5-mL syringe and a 0.45 mm filter. After confirming the formation of Vero cell monolayers, 300 μL of positive serum was added to the T-75 flask. The flask was incubated at 37 °C with 5 % CO_2 for 5–10 days. The presence of the virus was confirmed by RT-nested PCR and immunofluorescence assay (IFA) using the supernatants of infected cells [11]. In the ROK, SFTSV is classified as a biosafety level (BL)-3 pathogen; therefore, experiments using SFTSV were conducted in BL-3 laboratories.

2.7. Indirect immunofluorescence assay (IFA)

IFA slides were prepared using SFTSV-infected Vero E6 cells. The Vero E6 cells were seeded in T-75 flasks at a concentration of 3×10^4 cells per 14 mL of DMEM supplemented with 2 % FBS. Each well was added to a 24-well slide and incubated in 5 % CO_2 for 16 h. These slides were fixed with 100 % acetone for 10 min at −20 °C. Next, 5 % rabbit serum was added with PBS for 90 min. After washing with PBS, fluorescent-labelled antibody against deer immunoglobulin G (IgG) (H + L) (Sera Care, Milford, MA, USA) was added and incubated at 5 % CO_2 for one hour. The IFA slides were visualized using the EVOS M7000 Imaging System (Invitrogen, Frederick, MD, USA).

2.8. Virus titration

Vero cells seeded onto a 96-well microplate were inoculated with virus samples (serially diluted 10-fold) and incubated at 37 °C/5 % CO_2 for 4 days. The cells were fixed in 80 % acetone. To detect cells containing replicating SFTSV, viral antigens were stained with a mouse anti-SFTSV nucleocapsid (N) antibody, followed by a goat anti-mouse IgG (H + L) secondary antibody, and fluorescein isothiocyanate (ThermoFisher Scientific, MA,USA). The viral titer was determined as the 50 % tissue culture infection dose ($TCID_{50}$) [12]. To calculate the 50 % endpoint using serial dilutions, we used the following moderate statistical/mathematical formula [13]: $\log_{10}50$ % endpoint dilution = ([total number of dead animals/number of animals inoculated per dilution] +0.5) × log dilution factor.

2.9. Confirmation of isolated SFTSV by real-time PCR

To quantify the isolated SFTSV titers, 10-fold serial dilutions of SFTSV were titrated using real-time PCR. A One-Step RT qPCR Kit (Enzynomics, Daejeon, ROK) was used, according to the manufacturer's instructions, using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA).

2.10. Full genome sequencing

For the complete genetic sequencing of SFTSV from the spleen of Korean water deer, isolated SFTSV PCR amplicons were sequenced (Macrogen, Seoul, ROK). Nucleotide analysis of the full S, M, and L segments was performed using SPAdes 3.15.5. Nucleotide sequences were obtained from GenBank (National Center for Biotechnology Information, USA).

3. Results

3.1. Overall PCR results

In the 376 wildlife blood and spleen samples, the positivity rates

Table 2
Detection results of tick-borne pathogens with PCR in blood and spleen collected from wildlife rescue centers in the Republic of Korea between 2022 and 2023.

Samples	Pathogen	Gangwon			Northern Gyeonggi			Gyeonggi			Chungbuk			Ulsan			Busan			Total		
		N	P	PR	N	P	PR	N	P	PR	N	P	PR	N	P	PR	N	P	PR	N	P	PR
	SFTSV	1	0	0	12	0	0	1	0	0	119	4	3.4	93	0	0	54	1	1.9	280	5	1.8
	CCHFV	1	0	0	12	0	0	1	0	0	119	0	0	93	0	0	54	0	0	280	0	0
	TBEV	1	0	0	12	0	0	1	0	0	119	0	0	93	0	0	54	0	0	280	0	0
	YezoV	1	0	0	12	0	0	1	0	0	119	0	0	93	0	0	54	0	0	280	0	0
	LangyaV	1	0	0	12	0	0	1	0	0	119	0	0	93	0	0	54	0	0	280	0	0
	A. phagocytophilum	1	0	0	12	4	33.3	1	0	0	119	70	58.8	93	30	32.3	54	14	26.0	280	118	42.1
Blood	*A. bovis*	1	0	0	12	4	33.3	1	0	0	119	29	24.4	93	13	14.0	54	6	11.1	280	52	18.6
	E. chaffeensis	1	0	0	12	0	0	1	0	0	119	0	0	93	0	0	54	0	0	280	0	0
	E. canis	1	0	0	12	0	0	1	0	0	119	0	0	93	0	0	54	0	0	280	0	0
	Rickettsia spp.	1	0	0	12	1	8.3	1	0	0	119	10	8.4	93	7	7.5	54	1	1.9	280	19	6.8
	Borrelia theileri	1	0	0	12	2	16.7	1	0	0	119	26	21.9	93	10	10.8	54	6	11.1	280	44	15.7
	Bartonella schoenbuchensis	1	0	0	12	0	0	1	0	0	119	2	1.7	93	2	2.2	54	0	0	280	4	1.4
	Theilaria spp.	1	0	0	2	0	0	1	0	0	119	0	0	93	0	0	54	0	0	280	0	0
	SFTSV	12	0	0	2	0	0				49	1	2.0	33	0	0	–			96	1	1.0
	CCHFV	12	0	0	2	0	0				49	0	0	33	0	0				96	0	0
	TBEV	12	0	0	2	0	0	–			49	0	0	33	0	0				96	0	0
	YezoV	12	0	0	2	0	0				49	0	0	33	0	0				96	0	0
	LangyaV	12	0	0	2	0	0				49	0	0	33	0	0				96	0	0
	A. phagocytophilum	12	0	0	2	0	0				49	28	57.1	33	0	0				96	28	29.2
Spleen	*A. bovis*	12	0	0	2	0	0				49	2	4.1	33	1	3.0				96	3	3.1
	E. chaffeensis	12	0	0	2	0	0				49	0	0	33	0	0				96	0	0
	E. canis	12	0	0	2	0	0	–			49	0	0	33	0	0	–			96	0	0
	Rickettsia spp.	12	0	0	2	0	0				49	0	0	33	0	0				96	0	0
	Borrelia theileri	12	0	0	2	0	0				49	1	2.1	33	0	0				96	1	1.0
	Bartonella schoenbuchensis	12	0	0	2	0	0				49	0	0	33	0	0				96	0	0
	Theilaria spp.	12	0	0	2	0	0				49	0	0	33	0	0				96	0	0
Total No. of positive pathogens		0			4			0			6			5			5			6		

Note: -, no sample collected; N, number of tested samples; P, positive; PR, positive rate.

Table 3
Detection results of positive animal species in blood and spleen samples collected from wildlife rescue centers in the Republic of Korea.

Animal rescue center	General name (scientific name) of animals	No. of animals	Type of samples	Pathogens (No. of detected pathogen)
Northern Gyeonggi	Korean water deer (*Hydropotes inermis*)	3	Blood	*A. phagocytophilum* (3) *A. bovis* (3) *Rickettsia* spp. (1) *Borrelia theileri* (1)
	Siberian roe deer (*Capreolus pygargus*)	1	Blood	*A. phagocytophilum* (1) *A. bovis* (1) *Borrelia theileri* (1)
	Eurasian eagle-owl (*Bubo bubo*)	2	Blood	*A. phagocytophilum* (6)
	Spot-billed duck (*Anas poecilorhyncha*)	1	Blood	
	Rock dove (*Columba livia domestica*)	1	Blood	
	Grey heron (*Ardea cinerea*)	2	Blood	
Chungbuk	Korean water deer (*Hydropotes inermis*)	58	Blood	SFTSV (4) *A. phagocytophilum* (58) *A. bovis* (27) *Rickettsia* spp. (8) *Borrelia theileri* (24) *Bartonella schoenbuchensis* (1)
		9	Spleen	SFTSV (1) *A. phagocytophilum* (28) *A. bovis* (2) *Borrelia theileri* (1)
	Siberian roe deer (*Capreolus pygargus*)	4	Blood	*A. phagocytophilum* (4) *A. bovis* (2) *Borrelia theileri* (1) *Bartonella schoenbuchensis* (1)
	Raccoon dog (*Nyctereutes procyonoides*)	2	Blood	*A. phagocytophilum* (2) *Borrelia theileri* (1)
	Rock dove (*Columba livia domestica*)	1	Blood	*A. phagocytophilum* (4)
	Oriental turtle dove (*Streptopelia orientalis*)	1	Blood	
	Common kestrel (*Falco tinnunculus*)	1	Blood	
	Spot-billed duck (*Anas poecilorhyncha*)	1	Blood	
	Common gull (*Larus canus*)	1	Blood	*Rickettsia* spp. (1)
Ulsan	Korean water deer (*Hydropotes inermis*)	2	Blood	*A. phagocytophilum* (19) *A. bovis* (12) *Rickettsia* spp. (6) *Borrelia theileri* (7)
	Raccoon dog (*Nyctereutes procyonoides*)	6	Blood	*A. phagocytophilum* (3) *Borrelia theileri* (3)
	Siberian roe deer (*Capreolus pygargus*)	4	Blood	*A. phagocytophilum* (4) *A. bovis* (1) *Bartonella schoenbuchensis* (2)
	Spot-billed duck (*Anas poecilorhyncha*)	1	Spleen	*A. bovis* (1)
	Jungle crow (*Corvus macrorhynchos*)	1	Blood	*A. phagocytophilum* (1)
Busan	Korean water deer (*Hydropotes inermis*)	13	Blood	SFTSV (1) *A. phagocytophilum* (13) *A. bovis* (6) *Rickettsia* spp. (1) *Borrelia theileri* (4)
Chungbuk	Korean water deer (*Hydropotes inermis*)	9	Spleen	SFTSV (1) *A. phagocytophilum* (28) *A. bovis* (2) *Borrelia theileri* (1)

were 3.0 % (11/376) in Northern Gyeonggi, 46.0 % (173/376) in Chungbuk, 16.8 % (63/376) in Ulsan, and 7.4 % (28/376) in Busan. No positive samples were detected in Gangwon and Gyeonggi (Fig. 1, Table 2).

3.2. Detection of tick-borne viruses

The positive rates of viruses were 1.6 % for SFTSV (6/376) from the blood and spleen of Korean water deer in Chungbuk ($n = 5$) and Busan ($n = 1$) (Table 2). All obtained sequences were deposited in GenBank under the accession numbers PP974330–PP974335. Samples from Chungbuk (PP974330–PP974334) belonged to genotype b-3 (99.71 % identity with human or horse serum), and samples from Busan (PP974335) belonged to genotype b-1 (99.42 % identity with human serum).

3.3. Detection of tick-borne bacteria

The positivity rates were 38.9 % for *A. phagocytophilum* (146/376), 14.6 % for *A. bovis* (55/376), 0.1 % for *Rickettsia* spp. (19/376), 12.0 % for *Borrelia theileri* (45/376), and 1.1 % for *Bartonella schoenbuchensis* (4/376) (Table 2). However, *Rickettsia* spp. were only observed on the 1.2 % agarose gels.

The animals that tested positive were Korean water deer, raccoon dog, Siberian roe deer, rock dove (*Columba livia domestica*), oriental turtle dove (*Streptopelia orientalis*), Spot-billed duck (*Anas poecilorhyncha*), common kestrel (*Falco tinnunculus*), northern goshawk (*Accipiter gentilis*), common pheasant (*Phasianus colchicus*), common gull (*Larus canus*), Eurasian eagle-owl (*Bubo bubo*), grey heron (*Ardea cinerea*), and jungle crow (*Corvus macrorhynchos*) (Table 3).

The obtained sequences were deposited in GenBank under the following accession numbers: PP236887–PP263895 (*A. phagocytophilum*), PP236897–PP236903 (*A.bovis*), PP236943–PP236947 (*Borrelia theileri*), and PP968763–PP968766

Table 4

Mixed infections of tick-borne pathogens in blood and spleen samples of rescued wild animals in the Republic of Korea in 2022 and 2023.

Classification	Infected tick-borne pathogens	No. of samples		Regions	Animals
		Bloods	Spleens		
Double infection	SFTSV + *A. phagocytophilum*	1	1	CB	KWD
	A. phagocytophilum + *A. bovis*	16	1	NG, CB Ulsan, Busan	KWD, SRD
	A. phagocytophilum + *Bartonella schoenbuchensis*	2	0	Ulsan	SRD
	A. phagocytophilum + *Borrelia theileri*	15	0	CBD	KWD, SRD
	A. bovis + *Borrelia theileri*	2	0	CBD, Ulsan	KWD
Sub total		38			
Triple infection	SFTSV+ *A. phagocytophilum* + *A. bovis*	2	0	CB	KWD
	SFTSV+ *A. phagocytophilum* + *Borrelia theileri*	1	0	CB	KWD
	A. phagocytophilum + *A. bovis* + *Borrelia theileri*	17	0	NG, CB, Ulsan, Busan	KWD, SRD
	A. phagocytophilum + *A. bovis* + *Rickettsia* spp.	4	0	CB, Ulsan	KWD
Sub total		24			
Quadruple infection	SFTSV+*A. phagocytophilum* + *A. bovis* + *Borrelia theileri*	1	0	Busan	KWD
	A. phagocytophilum + *A. bovis* + *Borrelia theileri* + *Rickettsia* spp.	6	0	NG, CB, Ulsan	KWD
	A. phagocytophilum + *A. bovis* + *Rickettsia* spp. + *Bartonella schoenbuchensis*	2	0	CB	KWD, SRD
Sub total		9			
Grand total		71			

Note: NG, Northern Gyeonggi; CB, Chungbuk; KWD, Korean water deer; SRD, Siberian roe deer.

(*Bartonella schoenbuchensis*).

3.4. Mixed infections

A total of 71 mixed infections were identified, including 38 with double infections, 24 with triple infections, and nine with quadruple infections (Table 4). These infections were observed in Korean water deer and Siberian roe deer from Northern Gyeonggi, Chungbuk, Ulsan, and Busan.

3.5. Isolation of SFTSV

SFTSV was isolated from a Korean water deer spleen sample collected after death in a car accident at the Chungbuk Wildlife Rescue Center. To confirm the isolated SFTSV, RT-nested PCR was used to observe segments of SFTSV target bands in the first and second rounds of PCR, 461 bp and 346 bp, respectively (Fig. 2A, B). To detect anti-SFTSV antibodies by IFA, SFTSV antibodies were observed at a 1:50 dilution (Fig. 2C).

3.6. Evaluation of the 50 % tissue culture infection dose ($TCID_{50}$)

With SFTSV dilution started from Log-1 to Log-5 in the 96 well plate, the total number of infected wells was 30.5 (inoculated SFTSV into eight plates per dilution), and the death score of Vero E6 cell was 3.8125.

Using the formula $\log_{10}$ 50 % endpoint dilution $= -(30.5/8 + 0.5) \times 1 = -3.8125$, the 50 % endpoint dilution was $10^{-3.8}$ and the titer of the virus was $10^{3.8}$ $TCID_{50}$/mL. In this study, the virus inoculation was 50 ul /well; thus, the titer of the virus was $10^{3.8} \times 20 = 2 \times 10^{4.8}$.

The viral titer was calculated using the following formula: $\log(2 \times 10^{4.8}) = 10^{5.1}$ $TCID_{50}$/mL. The viral titer was quantified by a 10-fold dilution of $10^{5.1}$ $TCID_{50}$/mL using real-time PCR. Moreover, the result showed detection until $10^{0.1}$ $TCID_{50}$/mL (Fig. 2D).

3.7. Phylogenetic analysis

The nucleotide sequences obtained in this study had the following accession numbers: PP790964 (L segment, 6368 bp) (Fig. 2E), PP790965 (M segment, 3378 bp) (Fig. 2F), and PP790966 (S segment, 1674 bp) (Fig. 2G). These sequences were isolated from the spleen of Korean water deer. All of these full sequences belonged to sub-genotype B-3 and had 99.84 % ∼ 99.94 % similarity to human serum in the ROK (reference no. MK301480). The full sequences were of the same genotype as the partial sequences from the spleen samples (PP974334).

4. Discussion

Although wild animals are associated with ticks and pathogens, their roles are still not understood due to the difficulty of obtaining samples [14]. Wild animals act as reservoirs or amplification hosts for various human pathogens. In addition, they can migrate naturally, leading to the spread of ticks and tick-borne pathogens to new areas [15]. In some cases, a virus is transmitted from wildlife to humans via domestic animals, as in the case of Nipah virus. Bats are the primary reservoir for the Nipah virus, but in the epidemic in Malaysia and Singapore, humans were thought to be infected through close contact with pigs or their feces [16].

Several tick-borne pathogens have been detected in wild animals in the ROK, for instance, *A. bovis* infection in Korean raccoon dogs; *Theileria cervi* infection in Siberian roe deer; and *A. phagocytophilum*, *A. capra*, *Bartonella capreoli*, and *Coxiella burnetii* infections in Korean water deer [17,18]. In this study, SFTSV, *A. phagocytophilum*, *A. bovis*, *Borrelia theileri*, *Rickettsia* spp., and *Bartonella schoenbuchensis* were detected from 168 animals of 11 species (3 mammals and 8 birds). Among them, *Borrelia theileri* is an infectious agent of bovine borreliosis that is transmitted by hard ticks, such as *Rhipicephalus* species, with a mild disease, fever, anorexia, and hemoglobinuria [19,20]. *Borrelia theileri* is found in Africa, Australia, Europe, and South America [21]. Meanwhile, *Borrelia theileri* DNA in the ROK was recently detected in Korean cattle (*Bos taurus coreanae*), raccoon dogs, and ticks collected from domestic goats (*Capra hircus*) [22–24]. In this study, *Borrelia theileri* was detected in the blood of Korean water deer, Siberian roe deer, and raccoon dogs, suggesting that other wild animals may harbor *Borrelia theileri* in their natural habitats.

Bartonella schoenbuchensis causes deer ked dermatitis in humans and was identified in dissected developing larvae of wingless *Lipoptena cervi* from red deer (*Cervus elaphus*) [25]. In this study, *Bartonella schoenbuchensis* was detected in the blood of Korean water deer and Siberian roe deer, which were highly similar to *Lipoptena fortisetosa*'s gut (reference no. CP154603).

Together, this study proposes that tick-borne pathogens may circulate in wild animal environments and serve as potential vectors for transmission to humans. Coinfection indicates diagnostic problems, and pathogens may react indirectly or antagonistically within their respective hosts and modulate disease severity [26]. Animal model studies of tick-borne coinfections have reported that simultaneous infection with

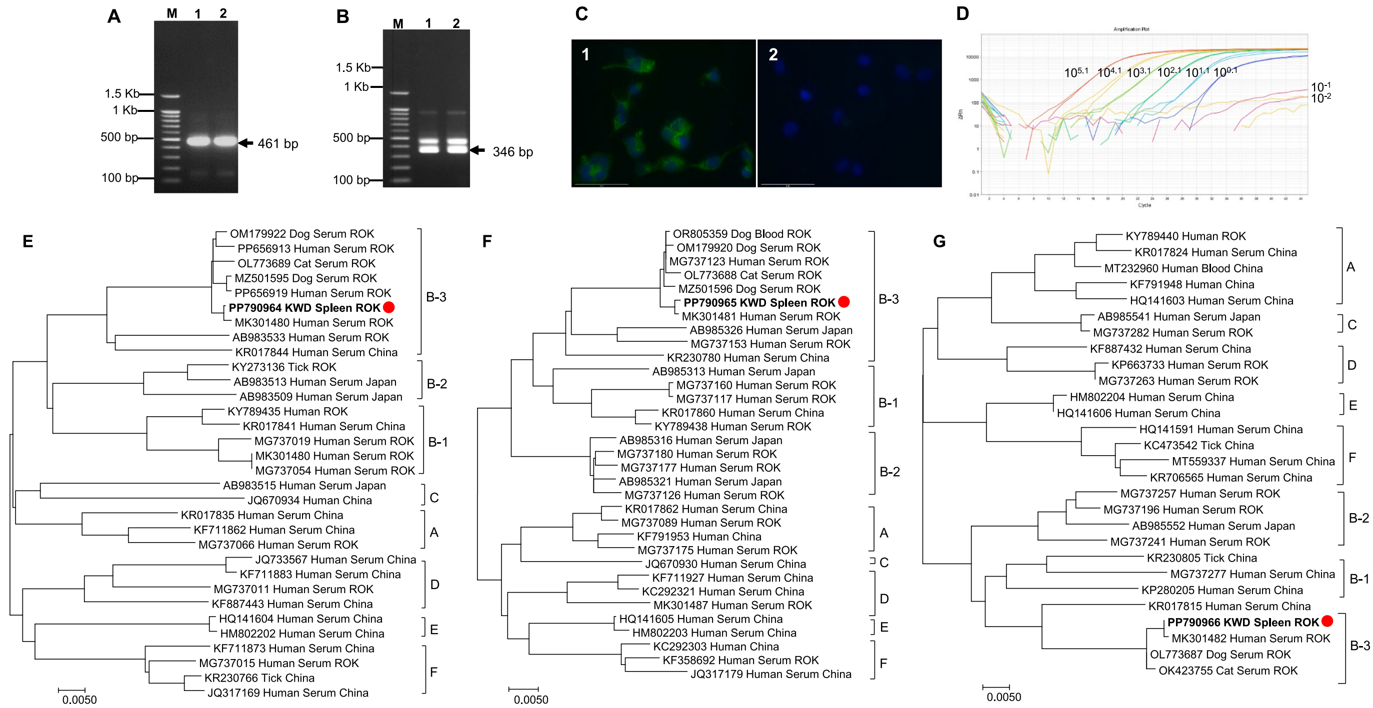

Fig. 2. Overall results of severe fever with thrombocytopenia syndrome virus (SFTSV) isolation confirmed by reverse transcription (RT)-nested polymerase chain reaction (PCR) and immunofluorescence assay (IFA) and quantified at $10^{5.1}$ 50% tissue culture infection dose ($TCID_{50}$)/mL using real-time PCR. A, Gel electrophoresis of first-round PCR results for detecting small segments (461 bp) of SFTSV; M, 100 bp deoxyribonucleic acid (DNA) ladder marker; lane 1, 2; result of first-round PCR. B, Gel electrophoresis of second-round PCR results for detecting small segments (346 bp) of SFTSV; M, 100 bp DNA ladder marker; lane 1, 2; result of second-round PCR. C, Results of IFA for SFTSV antibody detection in the isolated virus; 1, 1:50 dilution ratio in SFTSV; 2, negative control, the blue color represents 4′, 6-diamidino-2-phenylindole, and the green color represents green fluorescent protein. Scale bar = 75 μm. D; Real-time PCR results for quantification, starting with $10^{5.1}$ $TCID_{50}$/mL, and 10-fold dilution was conducted until $10^{4.1}$, $10^{3.1}$, $10^{2.1}$, $10^{1.1}$, and $10^{0.1}$ $TCID_{50}$/mL. A negative result showed 10^{-1} and 10^{-2} $TCID_{50}$/mL. Phylogenetic relationships for SFTSV detected from Korean water deer's spleen based on full nucleotide sequences; The tree indicates the comparison between the SFTSV sequences of the present study and reference sequences. The sequences identified from SFTSV-positive Korean water deer's spleen are shown in boldface and red dot. Maximum likelihood analysis was used to construct the phylogenetic tree, based on the Kimura two-parameter model (1000 bootstrap replicates). KWD, Korean water deer; ROK, Republic of Korea. E, SFTSV full nucleotides sequences of the L segment (6368 bp); F, SFTSV full nucleotide sequences of the M segment (3378 bp); G, SFTSV full nucleotide sequences of the S segment (1647 bp). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Borrelia burgdorferi and *A. phagocytophilum* promotes the pathogenesis of Lyme disease in laboratory mice, as *A. phagocytophilum* functionally damages neutrophils during the early defense against infection by *Borrelia burgdorferi* [27]. In this study, a quadruple infection of SFTSV with *A. phagocytophilum, A. bovis*, and *Borrelia theileri* was detected in the blood of Korean water deer in Busan. There remains a lack of coinfection reports with tick-borne pathogens in wild animals; thus, exploration of this field is required to prevent severe infections in humans.

The number of Korean water deer is steadily increasing owing to the absence of higher predators in the ROK, and they can act as a major reservoir of several tick-borne pathogens that infect humans and domesticated animals [18]. In a recent study, the presence of SFTSV was evaluated in Korean water deer tissues, including the spleen, heart, kidney, lung, liver, and intestine, and it was detected only in the spleen by RT-PCR [28]. Previous studies have indicated that the main organ of SFTSV infection is the spleen, as colocalization of SFTSV with platelets was observed in the cytoplasm of macrophages, which adhered to platelets and stimulated platelet clearance with phagocytosis of host splenic macrophages [29].

The SFTSV comprises three negative-strand segments; the large (L) segment encodes RNA-dependent RNA polymerase (RdRP), medium (M) encodes two glycoproteins, Gn and GC, and the small (S) segment encodes two proteins, Np (nucleoprotein) and Ns (non-structural protein) [30]. In this study, we successfully isolated SFTSV from the spleen of Korean water deer and conducted full genome sequencing of the L, 6368 bp; M, 3378 bp and S segments; 1674 bp. The SFTSV isolated from the spleen sample was collected from a Korean water deer in Chungbuk, which died after a car accident. All three segments, L, M and S, of SFTSV belonged to sub-genotype B-3 and were highly similar to SFTSV isolated from a human serum in the ROK. Furthermore, the partial and full genome sequences were of the same genotype and matched those of human isolates.

In the ROK, six genotypes (A, B, C, D, E, and F) and three sub-genotypes (B-1, B-2, and B-3) of SFTSV have been identified, with A, B-1, and B-2 being the predominant genotypes [31–34]. A recent study showed that genotype B (B-1, B-2, and B-3) caused 100 % mortality in aged ferrets, which exhibited clinical signs similar to those of humans infected with SFTSV [33].

In this study, we isolated SFTSV from the spleen of Korean water deer using Vero E6 cells, and the viral titer was $10^{5.1}$ $TCID_{50}$/mL. Another study demonstrated that 10^5 $TCID_{50}$ SFTSV in C57/BL6 mice was a suitable animal model for investigating the pathogenesis of SFTSV infection by testing various rodent strains and infection routes and revealed that splenic macrophages were target cells for SFTSV infection [29]. This study has some limitations. First, *Rickettsia* spp. are not confirmed species; thus, there is not complete understanding of all tick-borne pathogens in the samples. Second, all rescued wild animals were rescued by car accidents, falling, being caught in a net, and injuries, such as dislocation, fracture, and paraplegia. Therefore, it is unknown whether they have clinical symptoms, even if tick-borne pathogens are detected. Serological tests for the presence of pathogens are also informative for seroprevalence assessment and diagnostic investigation, although this study focused on molecular methods. Recent studies have reported serological detection of tick-borne viruses, such as SFTSV and CCHFV, which have allowed early detection of disease burden and diagnosis [35,36].

Several tick-borne pathogens were detected in the present study; in particular, SFTSV has been reported in cases of person-to-person transmission by direct contact with the index patient's blood, oral cavity, or nasal cavity [37,38]. Among the infected individuals, some only came into contact with the index patient after death, thus, the SFTSV-infected blood of the dead patient, having extreme levels of viral copies, may have remained infectious [37].

To protect against secondary infections, veterinarians and animal care workers must wear personal protective equipment, including gloves, goggles, and face shields, under the risk factors assessment. Before the laboratory diagnosis of the disease, it is possible for rescued wild animals to have unknown pathogens; thus, it is important to maintain hygiene when in contact with animals.

Meanwhile, in the wildlife rescue centers of the ROK, the majority of rescued cases involve infections, anthropogenic accidents, or injuries. Consequently, a system is in place to facilitate the release of animals back into their natural habitats following comprehensive treatment. The diversity of rescued animal species and the distribution of wild animal species vary according to regional characteristics. This study demonstrates that the number and species of animals rescued differ across various centers.

To sum up, we detected several tick-borne pathogens, including SFTSV, *A. phagocytophilum, A. bovis, Rickettsia* spp., *Borrelia theileri,* and *Bartonella schoenbuchensis,* in rescued wild animals in the ROK, and for the first time, isolated SFTSV from the spleen of Korean water deer. Notably, the SFTSV isolated from the spleen of Korean water deer and its full genome (L, M and S segments) were all in sub-genotype B-3, which is highly similar to the SFTSV isolated from a human serum in the ROK.

5. Conclusions

This study showed that various tick-borne pathogens circulate and infect wild animals in the ROK. The role of wild animals in nature and the cases of transmission to humans remain unknown. In addition, investigation into of wild animal clinical symptoms is required in the future. Based on the one health approach, establishing national surveillance systems and steady analysis of the tick-borne pathogens from wild animals are required to prevent zoonotic spillover.

Funding

This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, and Forestry through the Animal Disease Management Technology Development Program funded by the Ministry of Agriculture, Food, and Rural Affairs under Grant (122062-2).

CRediT authorship contribution statement

Hye-ryung Byun: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. **Seong-Ryeong Ji:** Data curation, Formal analysis, Methodology, Resources, Visualization, Validation, Writing – review & editing. **Jun-Gu Kang:** Resources, Validation. **Chang-Yong Choi:** Funding acquisition, Writing – review & editing, Validation. **Ki-Jeong Na:** Resources, Validation. **Jong-Taek Kim:** Resources, Validation. **Joon-Seok Chae:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The nucleotide sequences obtained in this study were submitted to the GenBank database under Accession No. PP236887–PP236895, PP236943–PP236947, PP236897–PP236903, PP790964-PP790966, and PP968763–PP968766.

Acknowledgements

We are thankful to the Gangwon Wildlife Rescue Center, Northern Gyeonggi Wildlife Rescue Management Center, Gyeonggi Wildlife Rescue Center, Wildlife Center of Chungbuk, Ulsan Wildlife Rescue Center, and Wildlife Medical Center in the Nakdong Estuary Eco Center for sending samples.

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