Identification of Amblyomma Javanense and Detection of Tick-Borne Ehrlichia Spp. In Confiscated Malayan Pangolins

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Research

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Abstract

Background

Due to habitat destruction and illegal hunting and trade, the number of pangolins has been sharply reduced. To prevent their extinction, relevant departments are combined and active action have been taken. A total of 21 confiscated Malayan pangolins were rescued in 2019, but died continuously for unknown reasons. This study aimed to investigate the reasons for the death of these pangolin and rescue them.

Methods

Recused Malayan pangolins were checked for clinical symptoms. Ticks on the surface of pangolins were collected and identified using morphological and molecular biological methods. Dead pangolins were autopsied for the gross lesion and tissues were collected for microscopic lesion using HE staining. Viral and protozoa pathogens' detection were carried on ticks firstly and further confirmed in dead pangolin pathological tissues using PCR.

Results

When rescued, pangolins were in bad situation. More than 90% (19/21) of the rescued pangolins had ticks on their body surfaces. A total of 303 ticks were removed and identified as *Amblyomma javanense (A. javanense)* according to their morphology and the sequences of PCR product of 16S rRNA and internal transcribed spacer 2 (ITS2). Autopsy of the dead pangolins showed multiorgan damage, especially congestion and hemorrhage in lung, heart and kidney. Histopathological analysis revealed marked presence of inflammation in tissues. Pathogens' nucleic acid detection showed ticks were only positive for *Ehrlichia spp*, with 56.7% positive rate of collected ticks (127/224), which was further confirmed in tissues from dead pangolins. Sick pangolins reduced clinical symptoms after medical treatment of intramuscular injection with doxycycline and kept alive before transfer.

Conclusions

Our findings confirm that ehrlichiosis caused by *Ehrlichia spp.* from *A. javanense* might be one of the reasons for the confiscated pangolins' death. More attention should be payed to tick-elimination work and the diagnoses and treatment of tick-borne diseases in the follow-up rescue operation.

Background

Ticks (Acari: Ixodidae) are parasites of obligate blood-sucking arthropod on the body surface of animals and are important vectors for many kinds of pathogens because of its extensive host range (1). When ticks bit into the nude skin, they can not only cause animal or human itch, anemia and emaciation, but also spread pathogens such as bacteria, viruses, nematodes and protozoa, which would seriously

threaten human health, animal husbandry production and wildlife survival. Tick-borne diseases mostly occur in temperate, tropical and subtropical regions (2-6).

The genus *Ehrlichia* belongs to the family *Anaplasmataceae* and consists of six recognized species, *Ehrlichia minasensis* (*E. minasensis*), *E. canis, E. muris, E. chaffeensis, E. ruminantium* and *E. ewingii*. All members of *Ehrlichia* are tick-borne pathogens for ruminants (*E. ruminantium*), dogs (*E. canis, E. chaffeensis and E. ewingii*), mice (*E. muris*) and humans (*E. canis, E. ruminantium, E. chaffeensis* and *E. ewingii*) worldwide (7, 8). *Ehrlichia* infection in mammal host usually induces a kind of zoonosis ehrlichiosis, which can parasite in the cell of spleen, lymph node, bone marrow and peripheral blood and causes multiorgan lesions and dysfunction. The clinical symptoms of ehrlichiosis were deemed to be caused by host inflammatory reaction instead of direct damage caused by bacteria (9). Pangolin ehrlichiosis has been reported in the past (6, 7), but didn't cause much serious consequences.

The pangolin is a toothless mammal that are famous for their full armor of scales. In the past days, pangolin armor was considered a valuable Chinese medicinal material and its meat was also regarded as a nourishing treasure. Coupled with the serious destruction of habitats, their survival is threatened, and the number of wild pangolins has continued to decline. In addition, the decrease of pangolin may also be related to diseases. They are extremely susceptible to diseases such as blood parasites, bacteria and viruses in the wild, which further exacerbates the decrease of pangolin population (10). There are limited reports about the diseases of pangolins caused by ticks, bacteria, virus and other pathogens (11, 12), such as *Anaplasmaspp.*, Sendai virus and coronavirus in Malayan pangolin (*Manis javanica*) (9, 13, 14). *Rickettsia africae* in African giant pangolin (15), *Trypanosoma brucei gambiense* in long tailed pangolins (*M. tetradactyla*) and tree pangolins (*M. tricuspis*) (16), fatal canine parvovirus-2 (CPV-2) and canine distemper virus (CDV) in Taiwanese pangolins (*M. pentadactyla pentadactyla)* (17). All kinds of pangolin are included in the Appendix I to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Chinese authorities are also actively taking action to protect and rescue these species to prevent population extinction (17).

Malayan pangolin mainly distributing in Asia and Africa is one of the eight existing pangolin species (18). According to the study of Heinrich in 2016, there were 1485 pangolin trade events between 1977 and 2014, of which approximately 809,723 pangolins were traded internationally (19), which increased the risk of pangolin infectious pathogens. During the transfer process, pangolins probably carrying parasite ticks and other infectious pathogens were normally crowed in hostile environments, therefore increasing the risk of tick-borne diseases transmission among individuals. Recently, pangolin has become a research hotspot, but little research has been done on the disease of pangolins and its etiology.

In this study, to investigate the reasons for the death of this batch of confiscated Malayan pangolin, ticks and tissues from the dead pangolins were collected and were detected for reported viral and protozoa pathogens. The results showed more than 90% pangolins were parasitized by ticks, which were further identified as *Amblyomma javanense (A. javanense)* by their morphology analysis and nucleotide sequences of 16S rRNA and internal transcribed spacer 2 (ITS2). In addition, Gross and microscopic

lesions of the dead pangolins were observed after autopsy and HE staining. In addition, *Ehrlichia spp*, were detected to be positive in tick and pangolin tissue. Pangolins were recovered after medical treatment of intramuscular injection with doxycycline and kept alive before transfer. Our findings confirmed that ehrlichiosis caused by *Ehrlichia spp.* from *A. javanense* might be the reasons for the rescued pangolins' death. Through the study of this batch of confiscated Malayan pangolins, more attention should be payed to tick-elimination work and the diagnoses and treatment of tick-borne diseases in the follow-up rescue operation. This research fills the gaps in pangolin rescue operation at home and abroad, and provides an important reference for the diagnosis and prevention of pangolin-related diseases in the future.

Methods

Customs Anti-smuggling Bureau ferreted out many live pangolins on 24th March 2019. Subsequently, Guangdong Wildlife Rescue Center received 21 live pangolins in the afternoon of March 25th, and carried out active rescue work cooperating with Guangzhou Zoo and Guangdong Institute of Applied Biological Resources.

Recused pangolins were observed for the clinical symptoms. The ticks collected were identified using morphological and molecular biological methods. Morphological identification referred to the comparative classification of ticks by morphological analysis. Sequence of 16S ribosomal RNA (rRNA) and internal transcribed spacer 2 (ITS2) of ticks were amplified using specific primers and sequenced, based on which phylogenetic trees were drawn to classify ticks on the body of Malayan pangolin. Post-mortem examinations of dead pangolins were performed to observe the pathological changes of organs, and pathological sections were taken from the diseased tissue and stained with hematoxylin-eosin dyes to observe the pathological changes.

Pangolin tissues and ticks also were tested for pathogens nucleic acids. Tissues and ticks were homogenized and extracted for nucleic acids using Axygen® AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit and OMEGA EZNA® Tissue DNA Kit respectively. According to the experiment purpose, nucleic acids were reverse-transcribed (RT) using Takara PrimeScript 1st strand cDNA Synthesis Kit or not, and were detected using PCR for viral pathogens, including encephalomyocarditis virus (ECMV), parainfluenza virus type 5 (PIV5), avain influenza virus (AIV), canine distemper virus (CDV), canine herpes virus (CHV) and canine parvovirus (CPV), and parasitic pathogens, including *Ehrlichia spp.,Babesia, Theileria* and *Hepatozoon*. Primers used in this study were listed in Table 1. Primers were synthesized and specific PCR products were sequenced by Ruibiotech company (China). Then sequences were analyzed using BLAST on NCBI website. Phylogenetic tree analyses were conducted using MEGA 6.0 with the Maximum Likelihood algorithm. Bootstrap values were calculated with 1,000 replicates.

Results

When confiscated, most Malayan pangolins were in poor health, part of which had severe trauma, and infested with dozens of adults and subadult ticks. Then rescue work measures were carried out. Firstly, ticks on the body surface were gently removed (Figure 1A) and the wounds were cleaned and applied with antibiotics. Afterwards, they were kept in clean rooms specially adapted for pangolins. Finally, pangolins were fed with the artificial diets that consisted of silky ants, mealworms, bee pupa and vitamin by intragastric gavage until they can eat on their own. After 5 ~ 7 days, some pangolins began to eat independently.

Clinical symptoms showed that rescued pangolins were in bad situation, including poor spirit, drowsiness, anorexia, edema of extremities, snotty and coughing. Some pangolins had inexplicable wounds and bleeding on their body surface for about 10 ~ 26 days. A small group of pangolins even excreted tarry stool and hematuria, and erythrocyte and leucocyte could be found in the urine in the later course. There were nervous symptoms such as convulsions before they died.

Then ticks collected from the surface of pangolins were identified using morphological method (Figure 1B) and molecular biotechnology (Figure 2). Based on the presence of eyes, inornate dark brown scutum and stubby round spur in the coxae II, III and IV, which were different from the other species of the *Amblyomma,* morphologic analysis implied that these ticks collected from the confiscated Malayan pangolins were *Amblyomma javanense* (*A. javanense*) (Figure 1B). Subsequently, specific DNA sequences were amplified from whole DNA genomes of 6 individual ticks and sequenced for 16S rRNA (4/6) and ITS2 (6/6) (Supplementary Figure 1A). BLAST results showed that 16S rRNA gene of ticks was close to *Amblyomma javanense* (*A. javanense*) with a similarity of more than 99%, and ITS2 region was close to *Amblyomma paulopunctatum* with a similarity of nearly 92%. Genetic evolution analysis showed that the selected sequences of 16S rRNA gene belong to the same branch as *A. javanense* (Figure 2A) and ITS2 region sequences formed an individual branch (Figure 2B). Based the results above, the ticks were finally defined as *A. javanense.* and ITS2 sequences identified in this study were uploaded as *A. javanense* (Accession number: MK928428.1 and MK928429.1).

In the autopsy of pangolins, ulcerations and blood on the skin surface, edematous body and pale mucous membranes were observed (Figure 3A). Congestion and oedema were shown in most internal organs (Figure 3B-D), including lung, pancreas, spleen, kidney and bladder mucosa. Bleeding points could be seen on the surface of lung, trachea, bronchus and kidney, and the cut surface of the lungs was infiltrated with foamy fluid. In the heart, there were signs of myocardial edema, pericardium effusion and ventricular congestion (Figure 3C). There were congestion and jaundice in the serosa of omasum of the gastrointestinal system, many *Angiostrongylus cishonensis* adsorbed in the duodenum (data not shown). Part of the interstina parva mucosa was congested, and the mesenteric lymph nodes were highly swollen, congested, edematous and hemorrhagic as well. Infiltrating hemorrhage was seen in the node section. In the kidneys, corticomedullary differentiation was not clear and blood-like fluid accumulated in the renal calyes (Figure 3).

Histopathological analysis revealed marked presence of inflammation in tissues (Figure 4). Microscopic lesions included myocardial failure, sinus hepaticus and lymphoid nodule dilatation with blood stasis, widened splenic cord, multiply lymphocytes, collapsed alveoli, glomerular capillary hyperemia and increased volume, epithelial cells of mucosa necrosis and submucosa congestion in the sialaden, and bladder mucosa folds inward (Figure 4).

The organs of dead pangolins were collected for the detection of viral pathogens using PCR or reverse transcription-PCR (RT-PCR). The results showed the specimens were negative for EMCV, PIV5, AIV, CDV, CHV and CPV (data not shown). Also, pathogens of ticks like *Babesia*, *Hepatozoon*, *Theileria* and *Ehrlichia spp*. were detected as described above and the results showed that only *Ehrlichia spp*. was positive in this study (Supplemental Figure 1B). Self-designed primers were used to detect *Ehrlichia spp.* of 224 individuals of ticks (Male: 140, Female: 84) using PCR and nested PCR in which 56.7% of the specimens were shown to be positive (Table 2), most of which were detected in lung, spleen and blood of dead pangolins. The autopsy and histopathological changes were consistent with the changes in the case of *Ehrlichia spp.* infection. *Ehrlichia spp.* infection might be one of the reasons for the death of rescued Malayan pangolins.

According to BLAST result, the 16S rRNA sequences of 3 randomly selected samples were identified as *Ehrlichia spp.* and were closest to *E. chaffeensis* and *E. ruminantium,* with a similarity of above 98%. This observation was consistent to a phylogenetic tree (Figure 5). However, the obtained sequences (about 550 bp) were not long enough to be identified to the species level. When *Ehrlichia spp.* had been detected, all live pangolins were injected with broad-spectrum tetracycline-class antibiotic doxycycline for medical treatment. Finally, three pangolins were alive before transfer.

Discussion

In the rescue operation of 21 Malayan pangolins confiscated by the -Customs, 14 of them were female and 7 were male. More than 90% of them (19/21) were carrying ticks on their surfaces, which was much higher than the parasitology survey of Formosan pangolins and previous Malayan pangolin. In a parasitological survey of 52 Taiwan pangolins (25 males, 27 females), 25% of them were found to be infested with ticks (1). A survey of 16 Malayan pangolins carried by Hassan et al in 2013 showed a rate of 68.8% of the tick infection (18). In this study by Hassan et al., a higher rate of tick was found in male pangolins rather than female pangolins, which might as a result of the difference behavior of male and female pangolin, especially during the spawning season (18). In this study, 19 of the 21 Malayan pangolins were found to bearing ticks, 13 of the 14 female pangolins and 6 of the 7 male pangolins. Due to the small number of pangolins rescued, the obtained data were not representative. As a result of the lack of suitable cages during the smuggling process, pangolins were squeezed into restricted space, and individuals got too close to each other, which may help ticks moving from one individual to another and causing cross-infection of ticks among pangolins.

Amblyomma javanense (*A. javanense*) is commonly associated with pangolins, but is also found in other reptiles and mammals (24) According to previous reports, a total of 12 species of mammals and 4 species of reptiles were infected with *A. javanense*, the 12 species of mammals are Sunda pangolin, Chinese pangolin, Indian pangolin, Palawan pangolin, wild boar, bat, hyena, 'bear', sambar deer, Indian crested porcupine, mouse deer and human; 4 species of reptiles are water monitor, 'python', long-tailed skink and hill turtle (18, 24, 25). According to the research by Mihalca and Hassan., *A. javanense* was the most common tick parasitic on pangolins and may also be a common endangered species on Asian pangolins (18, 26, 27). In 1981, Nandi reported that the ticks captured from Indian pangolins were *A. javanense* in 1981, and Kwak also reported the same tick caught on Sunda Pangolin from Singapore in 2018 (24, 27). *A. javanense* was found in Malayan pangolins and wild boars from Thailand in 2000 (25), and *A. javanense* was successfully identified on Chinese pangolin through species DNA barcode in 2019 (28). In this study, the ticks collected from Malayan pangolin were finally determined as *A. javanense* through morphological and molecular biology analysis.

In this study a total of 224 ticks of 303 ticks collected were firstly tested for viral and protozoa pathogens, 56.5% of which were found to be positive for *Ehrlichia spp.* only. *Ehrlichia spp.* was also found in the tissues of host pangolins of ticks. Sequencing and alignment analysis of partial sequence of 16S rRNA an ITS2 further confirmed that the bacteria detected in ticks and pangolin tissues in this study belong to *Ehrlichia spp.* Phylogenetic analysis using 16S rRNA of *Ehrlichia spp.* as the standard, these *Ehrlichia spp.* from ticks and pangolins in this study were most closely related to *E. ruminantium* (99.50%, 98.84% and 98.66%, respectively), and they formed a clade together with *E. chaffeensis*, *E. muris*, *E. ewingii*, *E. minasensis* and *E. canis* (Figure 5).

Common clinical symptoms of ehrlichiosis are anorexia, lymphadenopathy, cardiopulmonary dysfunction, leucopenia, neural and ocular lesions, and pathological lesions include pale mucous membranes, hepatomegaly, splenomegaly, edema, glomerulonephritis, interstitial mononuclear infiltration (29). The symptoms and lesions of the pangolins were highly consistent with the above description of ehrlichiosis. In addition, lung, spleen and blood of the dead Malayan pangolins had also been confirmed to be infected with *Ehrlichia spp.* It was further confirmed that the infection of *Ehrlichia spp.* may be one of the reasons for the death of this batch of rescued pangolins.

Ehrlichia spp. are obligate intracellular parasitic bacteria and tends to favor hematopoietic cells. In dogs, the infection lasts a life time even after injecting doxycycline (11). Although rickettsiosis is a fatal disease and the estimated death rate is 1% to 10% in human (30), prompt treatment for rickettsiosis can dramatically reduce mortality. However, the death rate of confiscated pangolins is staggering in the case. pangolins might be extremely susceptible to *Ehrlichia spp.* In addition to the bad situation before rescue, low immunity of themselves and the delay of treatment, On the whole, pangolins were dead because of ehrlichiosis caused by *Amblyomma javanense* when crowded in a small space in the process of smuggling and trafficking. Pangolins would further undergo stress and disturbance, aggravating pangolin disease development during the transfer. The low immunity of pangolins and physical trauma also

aggravated the disease. In addition, *Ehrlichia spp*. was resistant to most drugs, and the treatment effect was very poor, which might also be the reason for the high mortality after pangolin rescued.

Conclusion

Our findings confirm that ehrlichiosis caused by *Ehrlichia spp.* from *A. javanense* might be one of the reasons for the rescued pangolins' death. Through the study of this batch of confiscated Malayan pangolins, more attention should be payed to tick-elimination work and the diagnoses and treatment of tick-borne diseases in the follow-up rescue operation. This research fills the gaps in pangolin rescue work at home and abroad, and provides an important reference for the diagnosis and prevention of pangolin-related diseases in the future.

Abbreviations

PCR: polymerase chain reaction; 16S rRNA: 16 Svedberg ribosomal ribonucleic acid; BLAST, basic local alignment search tool.

Declarations

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Tables

Table 1: Primers used for detection of pathogens in this study

Primer name	Primer sequence	Product length	Reference*
Tick-16S-F	CTGCTCAATGATTTTTTAAATTGCTGTGG	450 bp	(20)
Tick-16S-R	CCGGTCTGAACTCAGATCAAGT		
ITS2-F	CGAGACTTGGTGTGAATTGCA	1231 bp	(21)
ITS2-R	TCCCATACACCACATTTCCCG		
Hepatozoon-F	GGTAATTCTAGAGCTAATACATGAGC	574 bp	(22)
Hepatozoon-R	ACAATAAAGTAAAAAACAYTTCAAAG		
Ehrlichia spp.-F	CTAGAGGTCGAAAGAGGATAG	555 bp	
Ehrlichia spp.-R	GTGCTGATTTGACATCATCC		
Babesia-18S-F	CCGTGCTAATTGTAGGGCTAATACA	551 bp	(22)
Babesia-18S-R	GCTTGAAACACTCTARTTTTCTCAAAG		
Theileria-F	CTTCAGCACCTTGAGAGAAAT	477 bp	(23)
Theileria-R	TCDATCCCCRWCACGATGCRBAC		
AIV-F	ATGAGYCTTCTAACCGAGG	299 bp	
AIVR	CGTCTACGCTGCAGTCCT		
PIV5-F	GATCATTCCGCTTAATCCC	449 bp	
PIV5-R	CTTTCCAACATCCCCTACC		
CPV-F	GTAAGCTTCCAGGAGACTTT	600 bp	
CPV-R	GTAAGCTTCGTCGTGTTCTT		
CDV-F	ATAGATGTCTTGACACCGCTCTT	587 bp	
CDV-R	GTACATACCTTGGCTTTGGAACT		
EMCV-F	TCTGTTGAATGTCGTGAAGGA	286 bp	
EMCV-R	AGGCCCCAGATCAGATCC		
CHV-F	GGTAGACCCTCCTCGTAGGTAT	357 bp	
CHV-R	GGGGCAGCTAAAACTAATCCCA		

(NOTE: *, primers without indicated references were designed and kept in our lab.)

Table 2: Detection of *Ehrlichia spp.* infection in ticks and tissues collected from Malayan pangolin

Pangolin ID (Gender)	NO. of ticks of each pangolin	NO. of ticks for detection	NO. of ticks positive for *Ehrlichia spp.*	*Ehrlichia spp.* in pangolin lung	*Ehrlichia spp.* in pangolin spleen	*Ehrlichia spp.* in pangolin blood
1 (♂)	12	9 (7♂, 2♀)	5	-	-	/
2 (♂)	1	1 (1♂)	0	+	/	/
3 (♂)	13	7 (3♂, 4♀)	3	/	/	/
4 (♂)	2	2 (2♂)	1	-	+	/
5 (♂)	16	7 (5♂, 2♀)	4	/	/	/
6 (♂)	1	1 (1♂)	0	/	/	/
7 (♂)	9	2 (1♂, 1♀)	0	/	/	/
8 (♂)	18	15 (11♂, 4♀)	8	+	/	/
9 (♂)	8	6 (4♂, 2♀)	5	+	/	/
10 (♂)	0	0	0	/	/	/
11 (♂)	21	17 (14♂, 3♀)	13	+	+	+
12 (♂)	6	5 (5♂)	4	+	/	/
13 (♂)	0	0	0	/	+	+
14 (♂)	38	34 (12♂, 22♀)	30	/	/	/
15 (♂)	5	2 (1♂, 1♀)	0	/	/	/
16 (♂)	31	28 (15♂, 13♀)	13	/	+	/
17 (♂)	5	4 (4♂)	0	/	/	-
18 (♂)	26	21 (17♂, 4♀)	11	/	+	/
19 (♂)	10	9 (6♂, 3♀)	2	/	/	-
20 (♂)	16	14 (8♂, 6♀)	5	/	/	/
21 (♂)	65	40 (29♂, 11♀)	23	/	+	+

(NOTE: NO, numbers; ♂, male; ♀, female; +, positive; -, negative; /, not tested.)

Figures

Figure 1

Ticks carried by the confiscated Malayan Pangolin. A: Tick on Malayan pangolin surface. B: Dorsa view (left panel) and ventral view (right panel) of male (lower panel) and female (lower panel) ticks collected from confiscated Malayan pangolins.

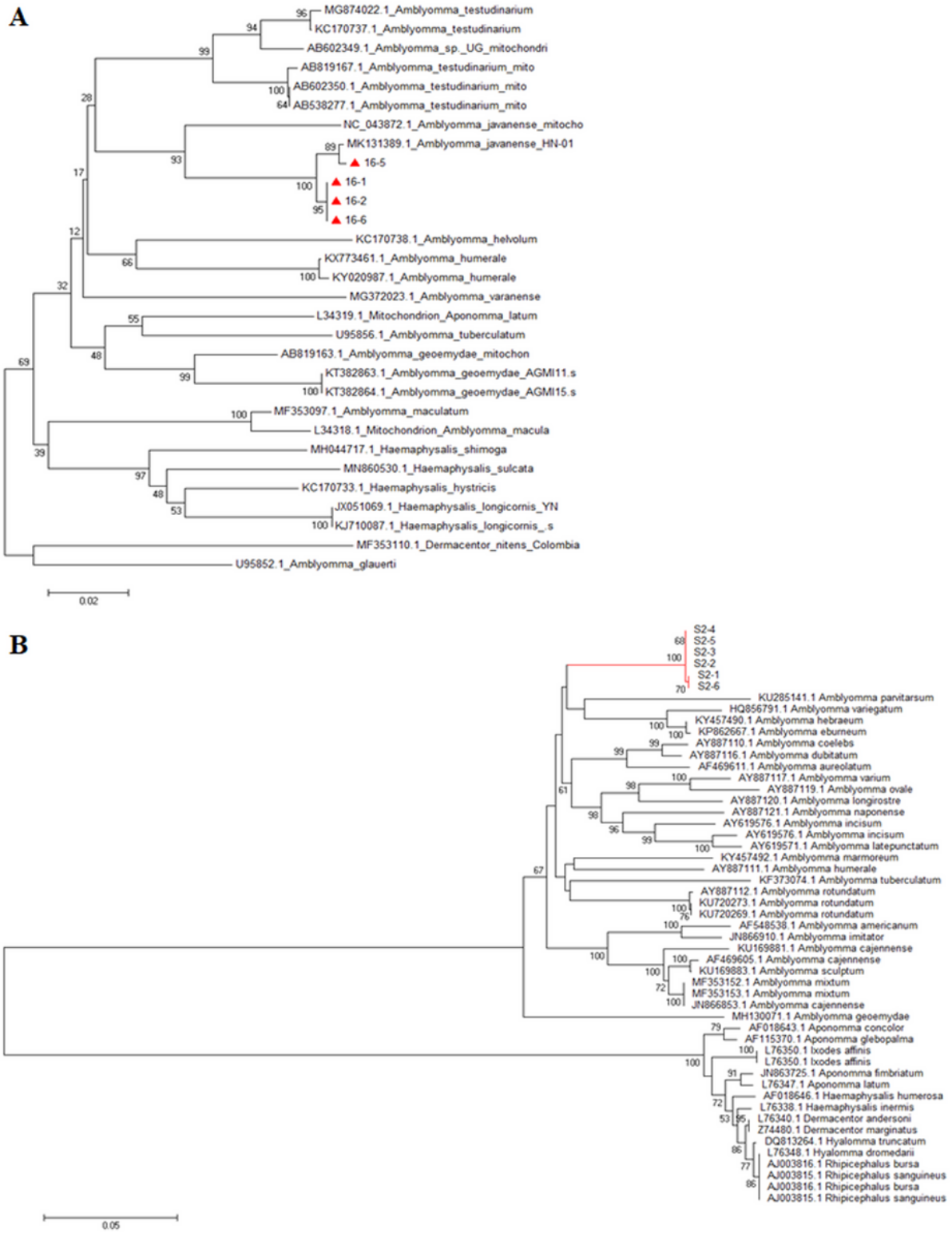

Figure 2

Phylogenetic tree based on the 16S rRNA (A) and ITS2 (B) of ticks. Analyses were conducted using MEGA 6.0 with the Maximum Likelihood algorithm. Bootstrap values were calculated with 1,000 replicates. The number on each branch indicates bootstrap value. Red triangles and red lines: sequences of ticks obtained in this study.

Figure 3

Gross pathological lesions of dead pangolins after autopsy. A: Pale, even purple, superficial mucosa around the nose and mouth. B: Congestion and hemorrhage observed in the lung. C: Myocardial edema, pericardium effusion and ventricular congestion in the heart. D: Kidney congestion.

Figure 4

The histopathological examination of pangolin tissue slide using hematoxylin-eosin (HE) staining. A: Heart, myocardial cells were necrotic, and the myoplasm at the necrosis was dissolved into vacuoles, some of which were lipid droplet vacuoles. B: Liver, sinus hepaticus was dilatate with blood stasis. C: Spleen, splenic cord widened and lymphocytes multiplied. D: Lung, alveoli collapse, inflammatory cell infiltration and capillaries congestion. E: Kidney, the renal tubules were transparent, with capillaries congested and cystic spaces dilated in the glomeruli. F: Lymph nodes, medullary blood vessels were dilated and congested, and there were many macrophages in the medullary cord. G: Salivary glands, epithelial cells of mucosa necrosis and submucosa congestion. H: Bladder, mucosa folds inward when empty. A-E, 400 X; F-H, 200X.

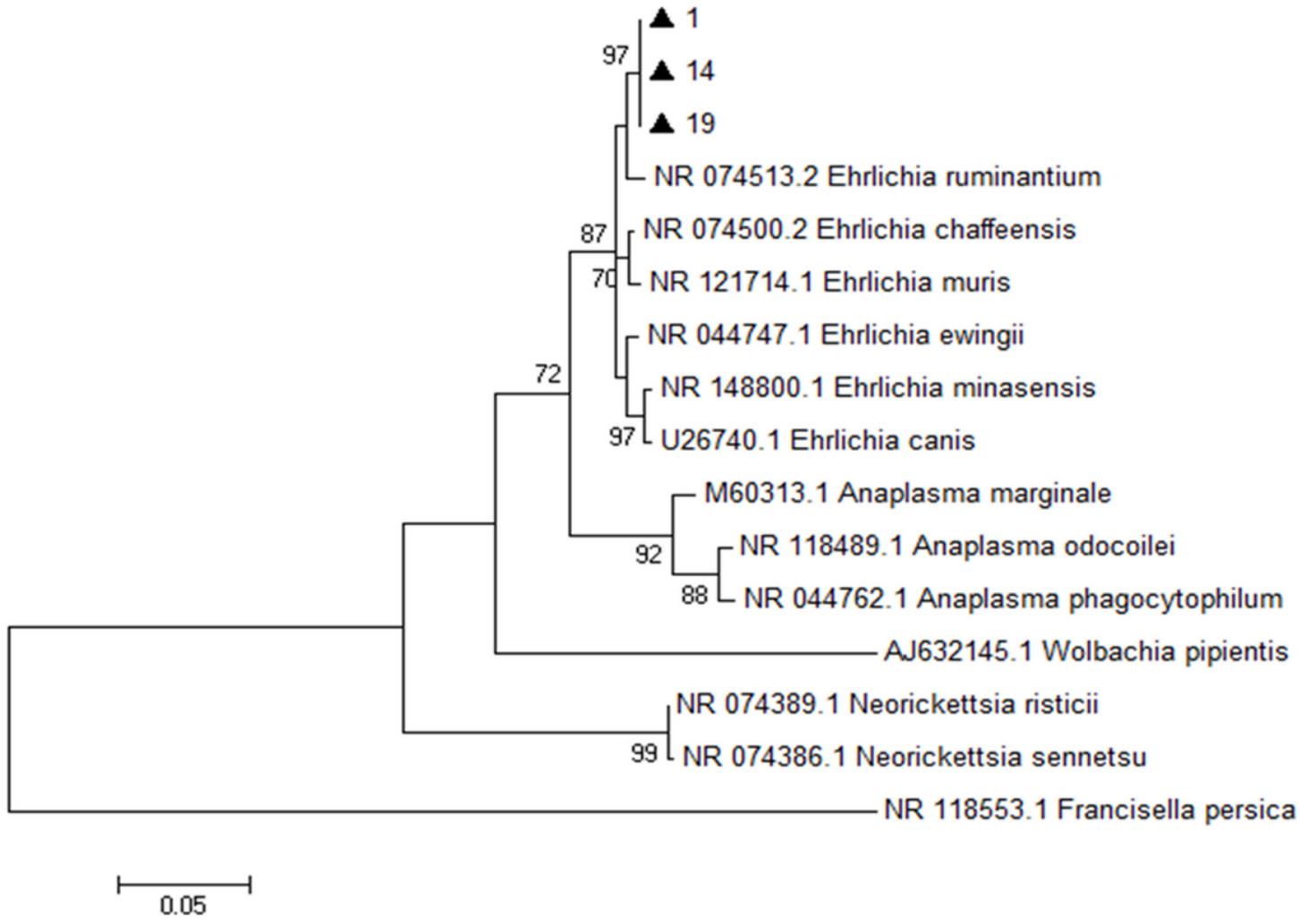

Figure 5

Phylogenetic tree based on the 16S rRNA of pathogens found in ticks from pangolins. Analyses were conducted by using MEGA software version 6.0 with the Maximum Likelihood algorithm. Bootstrap values were calculated with 1,000 replicates. The number on each branch indicates bootstrap values. Black triangles: sequences of Ehrlichia spp. obtained in this study.

Supplementary Files

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- supplement10.docx