Infection of *Ehrlichia ewingii* in Domestic Dogs, Horse, and Human in Taiwan

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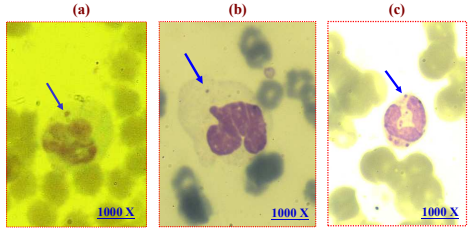
Abstract

Ehrlichia (E.) is a vector-borne, gram-negative bacterium. It is transmitted by *ticks,* and belongs to a obligately parasitized intracellular granulocyte. In 1992, *E. ewingii* was first recognized as a separate species in U.S.A. It is the causal organism of *granulocytic ehrlichiosis* in canines. The clinical signs and symptoms of this disease in animals, and patients include: fever, headache, myalgia, leukopenia, thrombocytopenia, and anemia. However, this disease was not found in many countries of the world. The aim of this study was to detect whether *E. ewingii* exist in domestic dogs, horses, and people in Taiwan. Wright-giemsa stain blood smears was used to confirm the existence of *E. ewingii*. Genomic DNA extracted from the whole blood was collected from dogs, other animals, and patients and 16s rRNA gene (416 bps in length) of *E. ewingii* was used as the positive marker for PCR examination. Nested PCR was used to screen *E. ewingii,* and the infection levels of *E. ewingii* were quantified by real-time PCR. Our data suggested that *E. ewingii* in Taiwan was different to the previously reported in other countries in terms of genomic sequence, host range, and clinical symptoms in infected animals.

Results



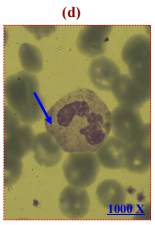


Figure 1. Wright-giemsa stain blood smears of the infected animals, and patients.

(a). *Ehrlichia ewingii* inclusion bodies in rat neutrophils.
(b). *Ehrlichia ewingii* inclusion bodies in dog eosinophils.
(c). *Ehrlichia ewingii* inclusion bodies in horse neutrophils.
(d). *Ehrlichia ewingii* inclusion bodies in patient neutrophils.

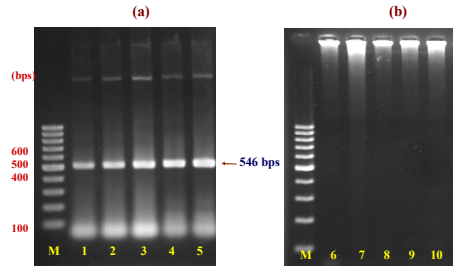


Figure 2. *Ehrlichia* genus in dogs (detected by using nested PCR).
Target amplified DNA fragment: 546 bps.
(a). Lane 1, 2, 3, 4, 5 are *Ehrlichia* positive samples.
(b). Lane 6, 7, 8, 9, 10 are *Ehrlichia* negative samples.
25 out of 250 dogs (10%) with confirmed cases of infection.

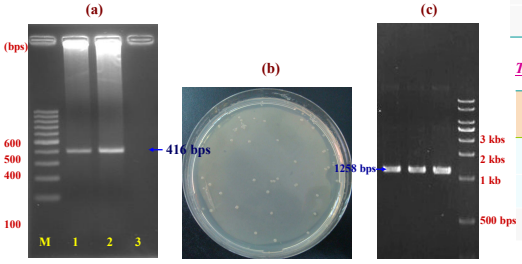


Figure 3.
(a). Nested PCR for *E. ewingii* screening; Target amplified DNA fragment: 416 bps; Four rats were confirmed *E. ewingii* positive; Lane 1, 2 are positive samples; Lane 3 is negative control.
(b). Transformation *E. ewingii* PCR product to competent *E. coli* cell.
(c). *E. ewingii* of plasmid DNA.

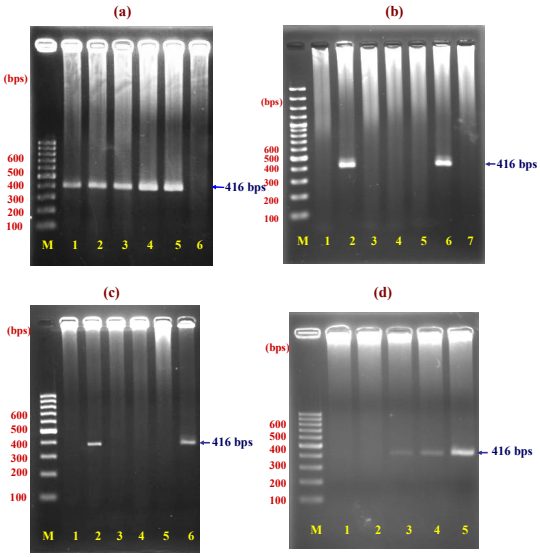


Figure 4. Detection of *E. ewingii* using nested PCR: Target amplified DNA fragment: 416 bps.
(a). *E. ewingii* in dogs; Four dogs were confirmed *E. ewingii* positive; Lane 1, 2, 3, 4 are positive samples; Lane 5 is positive control; Lane 6 is negative control.
(b). *E. ewingii* in cat; One cat was confirmed *E. ewingii* positive; Lane 2 is positive sample; Lane 1, 3, 4, 5 are negative samples; Lane 6 is positive control; Lane 7 is negative control.
(c). *E. ewingii* in horse: Lane 1 is negative control; Lane 2 is positive sample; Lane 3, 4, 5 are negative samples; Lane 6 is positive control.
(d). *E. ewingii* in patients; Two patients were confirmed *E. ewingii* positive; Lane 1 is negative control; Lane 2 is negative sample; Lane 3, 4 are positive samples; Lane 5 is positive control.
- Positive control: rat of *E. ewingii* plasmid DNA; Negative control: deionized distilled water (DDW); M is 100 bps DNA Ladder.
Nested PCR conditions: Annealing at 55^{0}C/2 mins; extension at 72^{0}C/1 min; 35 cycles.
- Samples (dogs, cats, and patients) were collected from National Chung Hsing Univesity, Veterinary Medical Teaching Hospital; Mouse were collected from China Medical University, Chih Yang Huang's Laboratory.

Table 1. Positive samples, and positive rates of nested PCR for the detection of *E. ewingii* in rat, dog, cat, horse, and patient.

	Rat	Dog	Cat	Horse	Patient
Positive samples (number)	4	4	1	1	2
Total (tested samples)	14	326	20	72	8
Positive rates (%)	28.57	1.27	5	1.39	25

Table 2. Types, and number of occurrence of clinical signs, physical examination of *E. ewingii* infections in dog, and cat.

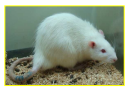
	Dog	Cat
Hypothermia	1	1
Vomiting	0	1
Diarrhea	0	1
Anemia	3	1
Thrombocytosis	0	1

Table 3. Monthly number of positive *E. ewingii* infections samples in 2011.

Time	Rat	Dog	Cat	Horse	Patient	Total
March	3	2	0	0	0	5
April	0	2	1	0	0	3
August	0	0	0	1	2	3
Total infected samples	4	4	1	1	2	12

First observation period: March 25 to April 8, 2011; Results: *E. ewingii* presented in eight out of twelve samples (66.67 %) .
Second observation period : August, 2011; Results: *E. ewingii* presented in three out of twelve samples (25.00 %).
Thus, most of the remaining samples were *E. ewingii* PCR-positive during the transfer season of the year.

Group 1: Female rats (7 rats)
Comparison: male rats (7 rats)



Group 2: Cancer of nude mice (15 mice)
Comparison: normal of mice (5 mice)

Figure 5: Comparion of normal, and poor immune of mouse (rats, and mice).
Result: four female rats with confirmed cases of infection in group 1; all of samples are negative in group 2.

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Nucleotide   63    148   227   279 280  297   389 391
U.S.A.  ...TTAGA... TACTG... GCTTAC... GG-AAC...CA-GA... GTAAAGCTCTTT
        ...|| ||... || ||... || |||... || |||...|| ||... || | |||||||
Taiwan  ...TT-GA... TA-TG... GCCTAC... GGTTAC...CAGGA... GTCA-GCTCTTT
Nucleotide   32    115   194   346 347  264   358 360
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Figure 6a. Comparison of *E. ewingii* DNA sequences in Taiwan, and U.S.A. (GenBank DQ365880-USA, 2006). 98% sequence homology, and 8 nucleotides differences were observed (361/369).

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Nucleotide   30 32 34   40    95   108   116   195   247248 265
Brazil  ...TA-TAGCTAGTTATT..GCTAT..ATAGG..TACTG..GCTTA..GG-A..CA-GA...
        ...|| | | ||||| ||..|| ||..|| ||..|| ||..|| ||..||   ..|| ||...
Taiwan  ...TATTTGATAGTTGTT..GCCAT..ATGGG..TA-TG..GCCTA..GGTT..CAGGA...
Nucleotide   29 31 33   39    94   107   115   194   246 247  264
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Figure 6b. Comparison of *E. ewingii* DNA sequences in Taiwan, and Brazil (GenBank HQ908082-Brazil, 2011). 97% sequence homology, and 11 nucleotides differences were observed (342/353).

Table 4. Real-time PCR quantification normalized of DNA concentration for *E. ewingii*.

Crossing point (CP) (mean)	DNA concentration (ng/ml)	Dilution folds	Result
	10^4		Not done
7.505	10^3	3	+
11.725	10^2	2	+
19.425	10	1	+
20.64	1	0	+
>25.00	0.1 ~ 0		–

→ 1 ng/ml (0.001 ng/µl)≦DNA concentration ≦1000 ng/ml (1ng/µl) can detect *E. ewingii*.

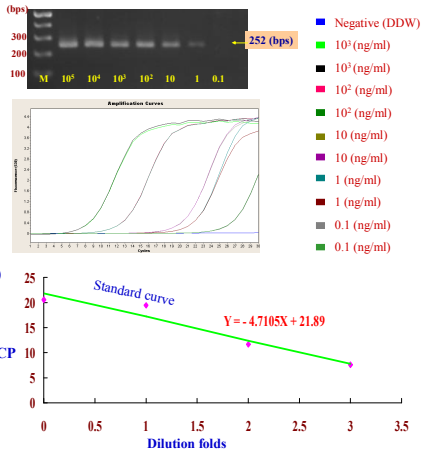

Figure 7. Determine DNA concentration of *E. ewingii* by nested PCR, and real-time PCR.
(a). Nested PCR for detection of different concentration of positive control.
(b). The quantification curves of positive control to detect *E. ewingii.*
(c). The standard curve of real-time PCR to detect *E. ewingii*.

Table 5. Determine infection levels of *E. ewingii* in dog, cat, rat, and patien by real-time PCR.

Sample	Crossing point (CP)			Dilution folds	DNA concentration (ng/ml)
	Sample	Repl. of sample	Mean		
Rat (♀)	20.98	20.93	20.955	0.1985	15.7943
Dog	19.94	20.06	20	0.4012	25.1884
Cat	20.82	20.85	20.835	0.2240	16.7494
Horse	23.01	22.79	22.9	-0.2144	6.1038
Patient	>25.00	>25.00	>25.00	-0.6602	<2.1868

Conclusion:

Our study demonstrated that *E. ewingii* could be detected in rat, dog, cat, and people in Taiwan. Our data also suggest that *E. ewingii* infection may only cause minor clinical symptoms, and it became serious when it concurrently infected with other pathogens.