Original Article

Prevalence of Zoonotic Pathogens by Molecular Detection in Stray Dogs in Central Taiwan

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Abstract

This study investigated the prevalence of zoonotic pathogens in stray dogs including toxoplasmosis, heartworm disease, Lyme disease, leptospirosis and Q fever in central Taiwan (Taichung, Changhua and Yunlin County) using nested, semi-nested or traditional polymerase chain reaction. Associations between prevalence of the infections and demiographic data of stray dogs in central Taiwan were also analyzed. Blood samples were randomly collected from stray dogs every month from August 2009 to July 2011. Total DNA extracted from blood samples ranged from 0.8 to 1.5 μg as the sensitivity of PCR ranged from 10 fg to 100 pg. For the PCR primers no cross-reaction was found between the nucleic acids of interest and DNA extracted from *Ehrlichia cani, Babesia canis* and *Babesia gibsoni*. The prevalence rates of 5 zoonotic diseases in the stray dogs were as follow: *Toxoplasma gondii* 3.89 % (28/720), *Dirofilaria immitis* 7.22% (52/720), *Borrelia burgdorferi* 5.42% (39/720), *Leptospira* spp. 7.22% (52/720), and *Coxiella burnetii* 5.83% (42/720). The association analysis between the prevalence rates and epidemiological data of stray dogs as well as sampling season showed that the prevalence rates of *Toxoplasma gondii*, *Borrelia burgdorferi*, and *Leptospira* spp. were correlated with the age and season but not with breed and gender. The prevalence rate of *Dirofilaria immitis* species was associated with breed and the season but not with gender and age while the prevalence rate of *Coxiella burnetii* was linked with the season but not with breed, gender and age. Our study reveals the prevalence of toxoplasmosis, heartworm disease, Lyme disease, leptospirosis and Q fever in stray dogs in Taiwan and provides crucial information for prevention and control of zoonotic diseases in Taiwan and neighboring areas and countries.

Keywords: heartworm disease, Q fever, leptospirosis, Lyme disease, stray dogs in Taiwan, toxoplasmosis

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Introduction

Toxoplasmosis became one of the 4th category of National Notifiable Communicable Diseases in Taiwan in 2007 (Chiang et al., 2012). It is well-known that *Toxoplasma gondii* has 3 infective stages, which are rapidly dividing invasive tachyzoite, slowly dividing bradyzoite in tissue cysts and the environmental stage of sporozoite (Robert-Gangneux and Darde, 2012). *T. gondii* has been indicated as one of the most common protozoan parasites associated with infections in humans and in all warm-blooded animals (Hill et al., 2005; Montoya and Liesenfeld, 2004). Horizontal transmission of *T. gondii* to human results either from ingestion of tissue cysts in infected meat or from ingestion of soil, water, or food contaminated with sporulated oocysts derived from the environment. It has been indicated that serological assays are the main manner to detect chronic infection with *T.gondii* in animals (Robert-Gangneux and Darde, 2012). It should be noteworthy that the seroprevalence of *T. gondii* in pet dogs reached 21.6% (132/611) in southwest China between June 2011 and February 2012 while in eastern China the positive rate of specific *T. gondii* antibodies were up to 40.3% (93/231) in stray dogs (Duan et al., 2012; Yan et al., 2012). Taiwan is geographically close to China so it is necessary to realize the prevalence of *T. gondii* in dogs in Taiwan.

Dirofilaria immitis is a filarial nematode and the parasite causes canine and feline heartworm disease as well as human pulmonary dirofilariasis (Rossi et al., 2010). It is a serious and potentially fatal disease which first affects the blood vessels and lungs and then the right chamber of heart (Simon et al., 2012). Traditionally it was considered *D. immitis* as in association with pulmonary nodules in humans, but *D. immitis* worms have also been found in other tissues like cranial, hepatic, intraocular and mesenteric adipose tissues (Theis, 2005; Avellis et al., 2011, Simon et al., 2012). Previously in Taiwan, *Aedes albopictus* is the species of mosquitoes which is vector of *D. immitis* (Lai et al., 2001). An obvious decreasing annual prevalence of heartworm has been shown in South America and possible reasons have been proposed that infected dogs die, worms die, older dogs have better medical care or these dogs may spend more time indoor (Vezzani et al., 2011). Investigation into *D. immitis* between 1998 and 1999 in Taiwan showed a seroprevalence of 13.4%, in which the seroprevalence of ≥ 6–years-old dogs was 23.7% (Fan et al., 2001). On the other hand, Lyme disease is an important and common vector-borne disease in the USA and Europe (Githeko et al., 2000). The transmission and infection of human borreliosis have been confirmed in Taiwan (Chao et al., 2011). An earlier study found that 16.6% (53 of 320) captured rodents were infected with *Borrelia Burgdorferi*-like spirochetes (Shih and Chao, 1998). This spirochete, with a helical shape and multiple endoflagella, possesses several morphological, structural, ecologic, and genomic features. For laboratory diagnosis of *B. burgdorferi* sensu lato infection, a qualitative PCR is usually sufficient (Aguero-Rosenfeld et al., 2005). Recently, prevalence and molecular identification of *Burgdorferi* spirochetes in ticks collected from rodents have been revealed, implying an enzoonotic cycle between *Ixodes granulatus* ticks and rodent hosts for *Borrelia* spirochetes in Taiwan and Southeast Asia (Chao et al., 2013). Chao et al.(2011) further showed the first isolation and molecular identification of *Borrelia burgdorferi* sensu stricto from skin biopsies of patients in Taiwan but public health studies regarding *B. burgdorferi* in stray dogs in Taiwan is very limited.

Leptospirosis, also known as fall fever or mud fever, is a worldwide zoonotic disease caused by different serotypes of *Leptospira interrogans*. The spirochete leptospira has approximately 200 serotypes (Brown and Prescott, 2008). The disease spectrum caused by *Leptospira* species in humans is various and rangs from subclinical infection to a severe syndrome of multiorgan infection with high mortality as the first report by Adolf Weil on icteric leptospirosis with renal failure has been shown for over 100 years (Levett, 2001; Weil, 1886). Dogs can act as a reservoir host for one or more *Leptospira* serotypes (Brown and Prescott, 2008; Rojas et al., 2010). It has been shown that canine leptospirosis is correlated with serovars Canicola and Icterohaemorrhagiae but antibodies against a range of serovars such as Autumnalis, Bratislava, Grippotyphosa, Hardjo, Pomona, and Zanoni were also found in canine serum (Rojas et al., 2010). Since leptospirosis has high prevalence in warm and wet environments, this zoonotic disease is of hygienic importance in Taiwan.

Q fever is bacterial zoonosis caused by *Coxiella burnetii.* This obligate intracellular bacterium, which is able to live and multiply in the cellular phagolysosomes, can infect different animals including sheep, goats, cattle, dogs, cats, birds and rodents. However, the infection in livestock is often unnoticeable. Humans may get the disease by breathing in contaminated droplets from infected animals or drinking raw milk (Brouqui and Raoult, 2001; Honarmand, 2012). Thus, Q fever is an essential zoonotic disease for farmers, laboratory workers, sheep and dairy workers, and veterinarians (Honarmand, 2012). Although *C. burnetii* infection in humans shows no symptoms or a mild disease with spontaneous recovery is observed, it may still cause serious complications and even death in patients, in particular those with meningoencephalitis or myocarditis, and more often in patients with chronic infetions of endocarditis (Maurin and Raoult, 1999). It should be noticeable that *C. burnetii* is highly infectious and the pathogen was found nearly everywhere in according to reports from around world, except New Zealand (Honarmand, 2012). Currently, stray dogs are still a social and hygienic issue in some Asian countries, including Taiwan, while molecular techniques have been established to examine viral, bacterial and parasitic DNA by our research team (Liu et al., 2003a; Liu et al., 2003b; Chiu et al., 2002; Yin et al., 2003). Therefore, it is necessary to realize the prevalence of the five zoonotic pathogens in the stray dogs in Taiwan. The objectives of this study were to examine the molecular prevalence of the five important zoonotic pathogens and to analyze and discover associations between the prevalence of the infections and demiographic data of stray dogs in Taiwan. The epidemiological data on toxoplasmosis, heartworm

disease, Lyme disease, leptospirosis and Q fever revealed in this study will be important and helpful for the prevention and control of zoonotic diseases in Taiwan.

Materials and Methods

Blood collection: A total of 720 blood samples were randomly collected from stray dogs surrendered in three shelters respectively located in Taichung, Changhua and Yunlin County in central Taiwan (Fig 1) from August 2009 to July 2011. A representative population of stray dog in the three shelters between 2009 and 2011 ranged from 2685 to 12804 (from the website of animal protection information: http://animal.coa.gov.tw/html3/index.php). Thirty blood samples from different stray dogs were randomly taken from the shelters every month in a 2-year period. The specimens were stored at 4°C for laboratory analysis. Date of blood collection and gender of dogs were recorded. Breed data of dogs were classified into two groups, mixed dogs and purebred dogs, according to the features of stray dogs.

DNA extraction: DNA Extraction of the blood samples from stray dogs was conducted using a commercial DNA extraction and purification kit (Biokit, Miaoli County, Taiwan) according to manufacturer's instructions. The DNA extracted and purified from each blood sample was stored at -20°C for specific polymerase chain reaction (PCR) analysis of the 5 zoonotic pathogens.

Table 1 Sequences of PCR primers.

Pathogens	Oligonucleotide sequence (5′-3′)	Expected size (bp)
T. gondii	Tg-NP-1: 5′-GTGATAGTATCGAAAGGTAT-3′(internal)	
	Tg-NP-2: 5′-ACTCTCTCTCAAATGTTCCT-3′(internal)	
	NN1:5′-CCTTTGAATCCCAAGCAAAACATGAG-3′(external)	
	NN2: 5′-GCGAGCCAAGACATCCATTGCTGA-3′(external)	227
D. immitis	5′-CAAATTTTTTACTTACAAAATATTACATA-3′	
	5′-AACGTATCATTTAAATTTTGATTCATTCAT-3′	208
B. burgaorferi	5′-CTAGTGTTTTGCCATCTTCTTTGAAAA-3′	
	5′-AATAGGTCTAATAATAGCCTTAATAGC-3′	307
Leptospira spp.	L-flaB-F1: 5'-TCTCACCGTTCTCTAAAGTTCAAC-3'	
	L-flaB-R1: 5'-CTGAATTCGGTTTCATATTTGCC-3'	793
C. burnetii	OMP1: 5′-AGTAGAAGCTCCCAAGCATTG-3′	
	OMP2: 5′-TGCCTTGCTAGCTAACGATTG-3′	501
	OMP3: 5′-GAAGCGCAACAAGAAGAACA-3′	
	OMP4: 5′-TTGGAAGTTATCACGCAGTTG-3′	438

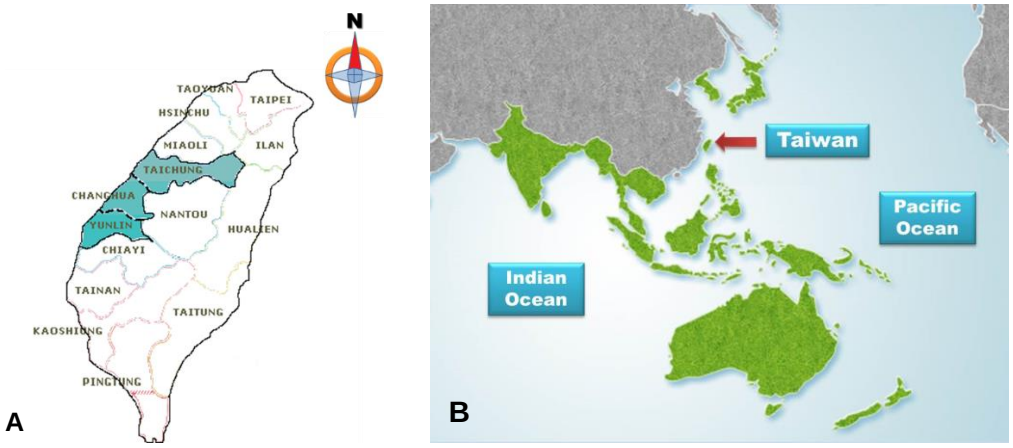

Figure 1 A: Sampling regions (Taichung, Changhua and Yunlin County) at central Taiwan. B: Regional map of Taiwan (From the website of Ministry of Foreign Affairs of Taiwan)

PCR for DNA detection of T. gondii, D. immitis, B. burgdorferi, Leptospira spp., and C. burnetii: Nested PCR (for *C. burnetii*), semi-nested PCR (for *T. gondii*) or traditional PCR (for *D. immitis*, *B. burgdorferi* and *Leptospira* spp.) was performed to detect DNA of the 5 zoonotic pathogens in the blood samples of stray dogs. Sequences of PCR primers are shown in Table 1. A region of 227 bp of the ITS1 of *T. gondii* DNA in blood samples was detected by semi-nested PCR. 25 µL reaction mixtures with 5 µL DNA extract of blood sample, 0.4 µM internal primers (Tg-NP-1 and Tg-NP2), 0.01 µM external primers (NN1 and NN2) (Hurtado et al., 2001), 5 µL 10X buffer, 10 mM dNTPs, 25 mM $MgCl_2$, and 0.5 U DNA polymerase (Promega, Madison, WI, USA) were preheated at 94°C for 3 min and 15-cycle PCR was conducted as follow: 94°C for 30 sec, 65°C for 45 sec and 72°C for 1 min. The PCR for *T. gondii* was followed by 35-cycle reaction: 94°C for 20 sec, 53°C for 30 sec and 72°C for 30 sec and the extension was completed at 72°C for 5 min. For the traditional PCR of *D. immitis,* reaction tubes containing 5 µL DNA extract of blood sample, 0.3 mM of each primer (Mar et al., 2002), 0.1 mM dNTPs, 3.5 mM $MgCl_2$, and 2.5 U DNA polymerase were preheated at 94°C for 3 min. The three stages of PCR were as follow: 94°C for 3 min, 63°C for 1 min and 72°C 1 min and the extension was accomplished at 72°C for 7 min. The traditional PCR for OspA gene of *B. burgdorferi* was carried out in reaction tubes containing 5 µL DNA extract of blood sample, 0.2 µM of each primer (Demaerschalck et al., 1995), 5 µL 10X buffer, 10 mM dNTPs, 25 mM $MgCl_2$, and 0.5 U DNA polymerase. The reaction tubes were preheated at 93°C for 5 min and followed by 35-cycle reaction: 93°C for 1 min, 65°C for 1 min and 72°C for 1 min. The extension was completed at 72°C for 5 min. For detection of *flaB* gene of *Leptospira* spp., traditional PCR reaction tubes with 5 µL DNA extract of blood sample, 0.2 µM of each primer (Kawabata et al., 2001), 5 µL 10X buffer, 10 mM dNTPs, 25 mM $MgCl_2$, and 0.5 U DNA polymerase were preheated at 95°C for 5 min and 35-cycle PCR was

Table 2 Prevalence of 5 zoonotic pathogens in stray dogs by monthly from August 2009 to July 2011.

Year	Month	*T. gondii*	*D. immitis*	*B. burgaorferi*	*Leptospira* spp.	*C. burnetii*
2009	Aug	0/30	1/30	1/30	0/30	0/30
	Sep	0/30	0/30	0/30	0/30	9/30
	Oct	25/30	17/30	4/30	1/30	1/30
	Nov	0/30	0/30	0/30	0/30	1/30
	Dec	0/30	0/30	0/30	0/30	10/30
2010	Jan	1/30	0/30	0/30	0/30	10/30
	Feb	0/30	0/30	0/30	0/30	0/30
	Mar	1/30	3/30	1/30	0/30	1/30
	Apr	1/30	3/30	0/30	0/30	0/30
	May	0/30	0/30	3/30	0/30	0/30
	Jun	0/30	1/30	1/30	0/30	9/30
	Jul	0/30	1/30	0/30	1/30	0/30
	Aug	0/30	9/30	0/30	28/30	0/30
	Sep	0/30	9/30	27/30	19/30	0/30
	Oct	0/30	0/30	2/30	2/30	1/30
	Nov	0/30	1/30	0/30	1/30	0/30
	Dec	0/30	0/30	0/30	0/30	0/30
2011	Jan	0/30	0/30	0/30	0/30	0/30
	Feb	0/30	1/30	0/30	0/30	0/30
	Mar	0/30	0/30	0/30	0/30	0/30
	Apr	0/30	0/30	0/30	0/30	0/30
	May	0/30	4/30	0/30	0/30	0/30
	Jun	0/30	1/30	0/30	0/30	0/30
	Jul	0/30	1/30	0/30	0/30	0/30
Prevalence rate		28/720 (3.89%)	52/72 (7.22%)	39/720 (5.42%)	52/720 (7.22%)	42/720 (5.83%)

conducted as follow: 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min. The extension was completed at 72°C for 5 min. The nested PCR for *C. burnetii* was carried out in reaction tubes containing 5 µL DNA extract of blood sample, 0.5 µM of each OMP1, OMP2, OMP3 and OMP4 primer designed for *com-1* gene of *C. burnetii* (Zhang et al., 1998), 5 µL 10X buffer, 10 mM dNTPs, 25 mM $MgCl_2$, and 0.5 U DNA polymerase. The first reaction was preheated at 94°C for 5 min and followed by 36-cycle reaction: 94°C for 60 sec, 54°C for 60 sec and 72°C for 60 sec. The first extension was completed at 72°C for 5 min. One µL of the first PCR product was added to the second PCR, which was preheated at 94°C for 5 min and followed by 36-cycle reaction: 94°C for 30 sec, 54°C for 20 sec and 72°C for 60 sec. The second extension was completed at 72°C for 5 min. The PCR products of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* were electrophorezed on 1.5% agarose gel at 100 V for 40 min to identify the size of products.

DNA cloning and sequencing: The target DNA bands were placed into eppendorf tubes and purified using Perfectprep Gel Cleanup Kit (Eppendorf, Berkhausenweg, Germany) according to the manufacturer's instructions. DNA inserts of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* were cloned into pGEMT easy vector (Promega). Ligation and transformation were performed according to the instructions of the manufacturer. The products of ligation reaction (plasmids) were then transformed into the competent cells BL21 (DE3) plysS (Novagen, Germany) in a sterile tube. Two µL of ligation reaction was transferred to the tube and then 50 µL competent cell suspension was carefully added. The mixture was gently flicked to fully mix up and then placed on ice for 20 minutes. The cells were then heat-shocked in a 42°C water bath for 45-50 seconds and placed on ice for 2 minutes, followed by addition of 950 µL sterile LB broth and 120 minute incubation, 150 rpm shaking at 37°C. After 16-18 h re-proliferation of bacterial cells, the plasmids were extracted and isolated from the cell suspension using alkaline lysis method. One µg of the plasmid DNA was used in linearization. The reaction mixture was run and separated on DNA 1.5% agarose gel at 100 V for 40 min to check whether the plasmids contained DNA inserts of 5 pathogens. DNA sequences of the plasmids were verified using T7 and SP6 primers on an automatic analyzer in Tri-I Biotech (New Taipei City, Taiwan).

Identity comparison of DNA sequencing results of the pathogens: Sequence pair distances analysis was conducted to compare the sequencing results of the DNA fragments of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* with NCBI (National Center for Biotechnology Information) databases of the strains of other countries or areas using DNASTAR® software. As a result, the DNA sequences of *T. gondii* positive specimens had 95~100% identities in comparison with the strains of USA, Germany, Brazil and China (Fig. 4A and 4B). The sequences of *D. immitis* positive samples had 96~97% and 93% identities compared with the strains of Taiwan/China and Iran, respectively (Fig 4C and 4D). The sequences of *B. burgdorferi* positive specimens had 98~100% identities compared with the strains of USA, Germany, Sweden and France (Fig 4E and 4F). The DNA sequences of *Leptospira* spp. positive specimens had 97~100% identities compared with the strains of China, Brazil, USA and Japan (Fig 4G and 4H), while the DNA sequences of *C. burnetii* positive specimens showed 99~100% identities compared with the strains of Japan and USA (Fig 4I and 4J).

Table 3 The associations between gender of stray dogs and the infection rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii.*

Gender	Numbers of dogs examined	Numbers and infection rates (%)				
		T. gondii	***D. immitis***	***B. burgdorferi***	***Leptospira* spp.**	***C. burnetii***
Male	322	13 (4.04)	24 (7.45)	18 (5.59)	22 (6.83)	18 (5.59)
Female	398	15 (3.77)	28 (7.04)	21 (5.28)	30 (7.54)	24 (6.03)
X^2		0.0343	0.0465	0.0342	0.1322	0.0628

X^2 chi-square test for independence. * : < 0.05, ** : P < 0.01 , *** : P < 0.001.

Table 4 The associations between breed of stray dogs and the infection rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii.*

Breed	Numbers of dogs examined	Numbers and infection rates (%)				
		T. gondii	***D. immitis***	***B. burgdorferi***	***Leptospira* spp.**	***C. burnetii***
Mixed	576	26 (4.51)	49 (8.51)	29 (5.03)	44 (7.64)	36 (6.25)
Purebred	144	2 (1.39)	3 (2.08)	10 (6.94)	8 (5.56)	6 (4.17)
X^2		3.0099	7.0941 **	0.8201	0.7462	0.9102

X^2:chi-square test for independence. * : < 0.05, ** : P < 0.01 , *** : P < 0.001.

Statistical analysis: Categorical variables in each analytic group were presented as numbers and percentages. Chi-square test for independence was performed to compare categorical data, as appropriate. All analyses were two-tailed and *P* value ≤ 0.05 indicated statistical significance. The statistical analyses were carried out using STATA software.

Results

Sensitivity and specificity of PCR for T. gondii, D. immitis, B. burgdorferi, Leptospira spp., and C. burnetii: The sensitivity of PCR for *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* was determined by preparing serial concentrations (100, 10, 1 ng/μL, 100, 10, 1 pg/μL, 100, 10 fg/μL) of the respective target gene of pathogen. The PCR sensitivity for the target gene of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* was approximately 10 fg, 1 pg, 10 pg, 100 pg and 100 fg, respectively, in the blood samples with 0.8~1.5 μg total DNA (Fig. 2A~2E). The specificity of PCR primers for *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* was analyzed. When the respective primer pair reacted with the other 8 DNA of pathogens, no cross-reaction was observed, indicating high specificity of the primers used in PCR detection of target pathogens in this study (Fig 3A-3E).

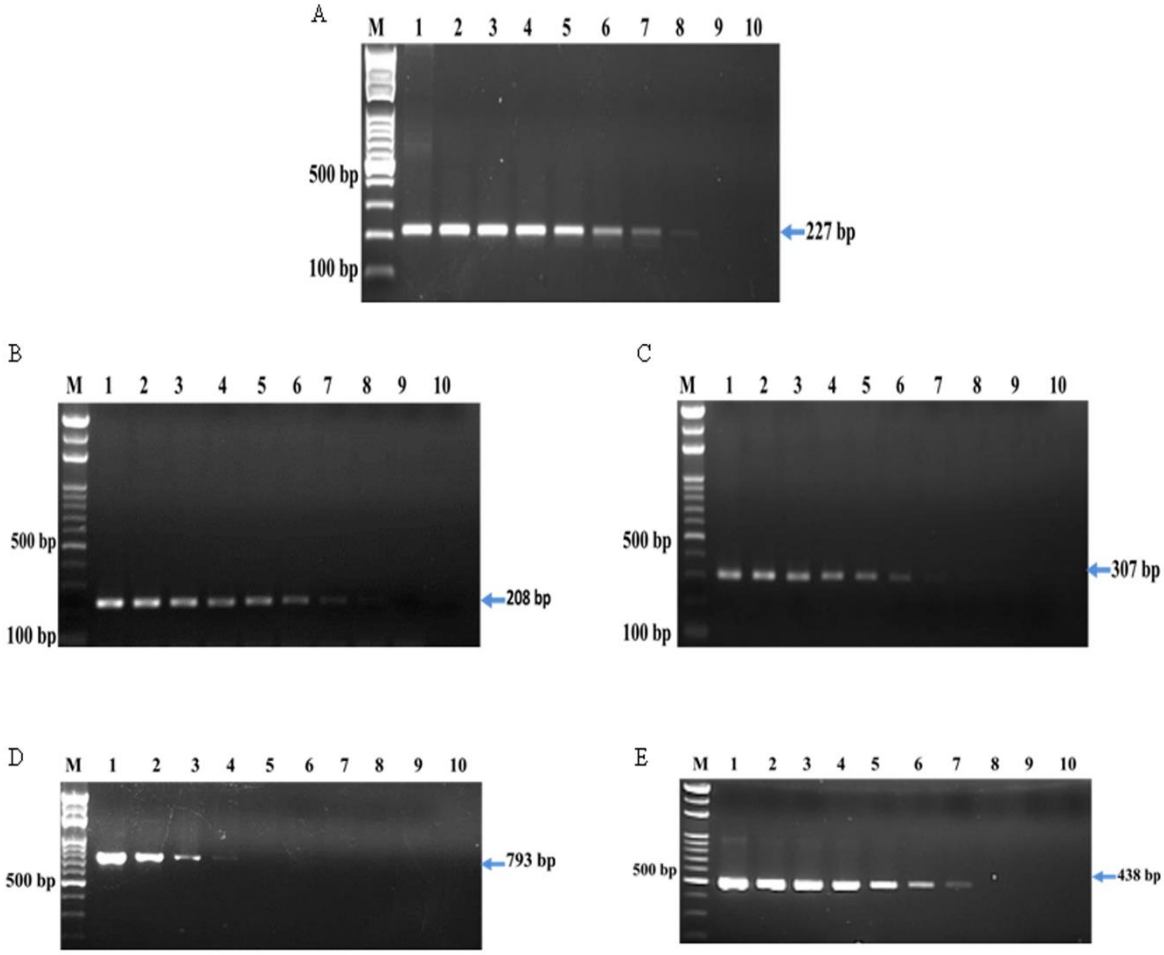

Figure 2 A: The sensitivity of semi-nested PCR for *T. gondii*. B: The sensitivity of PCR for *D. immitis*, (C) *B. burgdorferi* and (D) *Leptospira* spp. E: The sensitivity of nested PCR for *C. burnetii*. For subfigure A, D and E, Lane M: DNA ladder; Lane 1: 100 ng; Lane 2: 10 ng; Lane 3: 1 ng; Lane 4: 100 pg ; Lane 5: 10 pg ; Lane 6: 1 pg; Lane 7: 100 fg; Lane 8: 10 fg; Lane 9: 1 fg; Lane 10, negative control. For subfigure B and C, Lane M: DNA ladder; Lane 1: 1 μg; Lane 2: 100 ng; Lane 3: 10 ng; Lane 4: 1 ng; Lane 5: 100 pg; Lane 6: 10 pg; Lane 7: 1 pg; Lane 8: 100 fg; Lane 9: 10 fg; Lane 10, negative control. All the positive control DNA used in this study was from cloning plasmids of each pathogen, which has 100% identities in comparison with representative DNA sequences of each pathogen acquired from the Primer3 program (Rozen and Skaletsky, 2000).

Prevalence of DNA of T. gondii, D. immitis, B. burgdorferi, Leptospira spp., and C. burnetii: The percentages of positive samples (positive specimen numbers/total specimen numbers collected) by month from August 2009 to July 2011 are shown in Table 2. The prevalence rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* were 3.89% (28/720), 7.22% (52/720), 5.42% (39/720), 7.22% (52/720) and 5.83% (42/720), respectively.

Associations between infection rates and gender/breed of dogs: The prevalence rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* in association with the gender and breed (mixed or pure-bred) of dogs were analyzed. The infection rates of 5 zoonotic pathogens were not associated with gender of dogs (Table 3). The prevalence rate of *D. immitis* was significantly associated with the breed of dogs (P<0.01, Table 4).

Associations between infection rates and age of dogs: The prevalence rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* in association with the age of dogs were analyzed. As a result, the prevalence rates of *T. gondii, B. burgdorferi* and *Leptospira* spp. were significantly associated with the age of dogs (P<0.05 and P<0.01, Table 5).

Associations between infection rates and season: The prevalence rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* in association with the season were analyzed. As a result, the prevalence rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii* were significantly associated with season (P<0.001, Table 6).

Discussion

Toxoplasmosis is one of the most common zoonoses in the world and it represents various infection rates in different countries/areas. Over 350 host species, including mammals and birds for *Toxoplasma* have been previously reported. It has been indicated that there is no gold standard screening test for the largely diverse host species of of *Toxoplasma* (Robert-Gangneux and Darde, 2012). The seropervalence of *T. gondii* assessed by ELISA from 1995 to 1996 in Taiwan showed that older and mixed-breed privately-owned

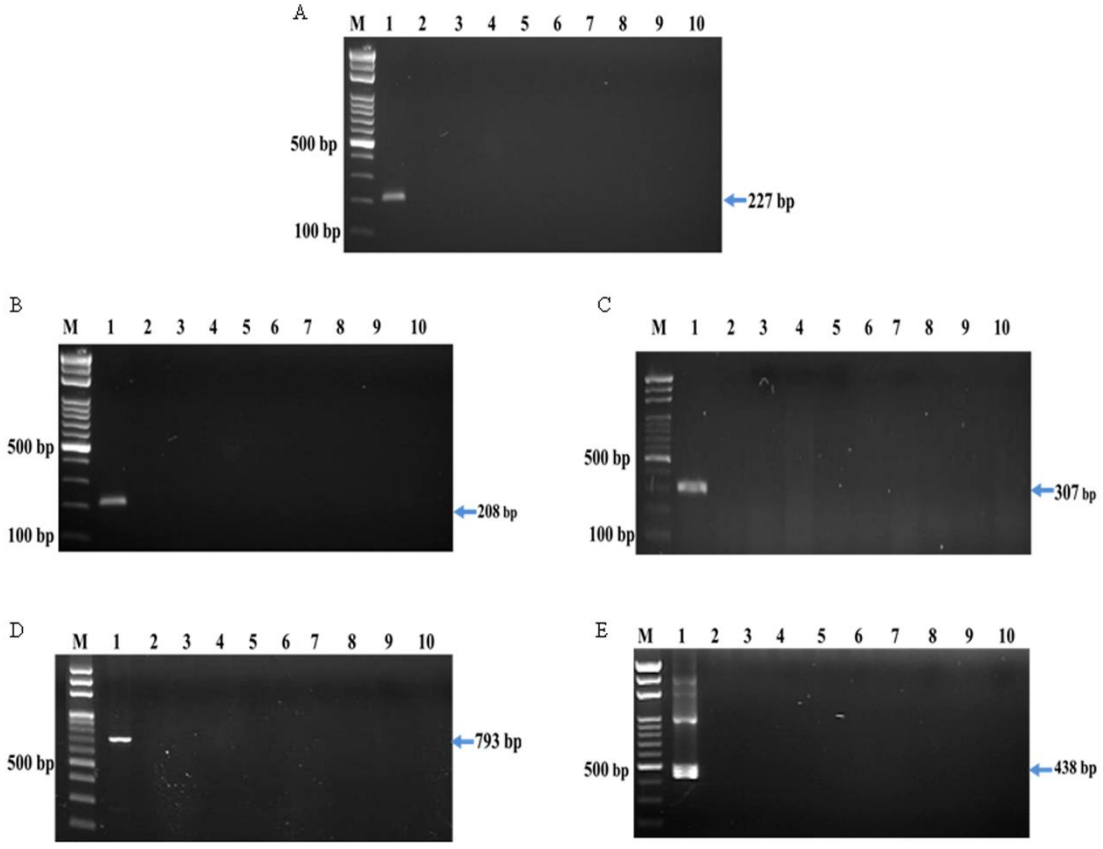

Figure 3 No cross-reaction was found for the primers used to detect the specific zoonosis (A) *T. gondii,* (B) *D. immitis* (C) *B. burgdorferi*, (D) *Leptospira* spp and (E) *C. burnetii* in the PCR specificity tests. The tested primers of specific zoonotic disease were loaded onto Lane 1. Extracted DNA from the rest 4 zoonotic pathogens in this study was loaded onto Lane 2~5, respectively. Extracted DNA of related pathogens was also tested as below: Lane 6 *Ehrlichia canis*; Lane 7: *Babesia canis*; Lane 8: *Babesia gibsoni*. Lane 9 was canine WBC DNA from a healthy dog and Lane 10 was negative control. All the positive control DNA used in this study was from cloning plasmids of each pathogen, which has 100% identities in comparison with representative DNA sequences of each pathogen acquired from the Primer3 program (Rozen and Skaletsky, 2000).

dogs had higher odds of seropositivity than younger and pure-bred counterparts, respectively (Lin, 1998). The results are partially similar with our data in stray dogs. In the current study the prevalence rate of *T. gondii* was also correlated with the age of dogs (Table 5). This could be due to the fact that older dogs spend longer time in the environment with exposure to *T. gondii* oocysts than young counterparts. The prevalence rate of the mixed-breed stray dogs showed higher, but statistically not significant, prevalence rate compared with that of the pure-bred dogs (Table 4). Moreover also, high prevalence of *T. gondii* was observed in Sep 2009. It could be due to the increased horizontal transmission via soil, water, or food contaminated with sporulated oocysts, which possibly resulted from flood damage caused by the Morakot typhoon from Aug to Sep in 2009.

A study revealed that *T. gondii* IgG positive rate in 1783 participants in 2007 was 9.3% and having a cat in the household was associated with anti-*T gondii* IgG seropositivity (Chiang et al., 2012), indicating the importance of toxoplasmosis in public health in Taiwan. Although for humans oocysts from infected cats are the main sources of infection, studies indicated that risk factors remained inexplicable in 14 to 49% of *T. gondii* infection cases in humans (Robert-Gangneux and Darde, 2012). Here we showed that the prevalence rate of *T. gondii* in spray dogs from 2009 to 2011 was 3.89%, implicating that toxoplasmosis in stray dogs might be a hygienic issue in central Taiwan. In contrast, *T. gondii* antibodies were detected in 40 (9.4%) of the 427 stray dogs in Bangkok, Thailand from Oct 2001 to Sep 2002 while higher *T. gondii* antibody positive rates were reported in dogs in Vietnam and Sri Lanka (Jittapalapong et al., 2007; Dubey et al., 2007a; Dubey et al., 2007b). These results suggest that the infection in stray dogs need to be accentuated to reduce *T. gondii* transmissions among animals and humans in many Asian countries. Moreover, in our study the DNA sequences of *T. gondii* positive specimens had 95~100% identities in comparison with the strains of USA,

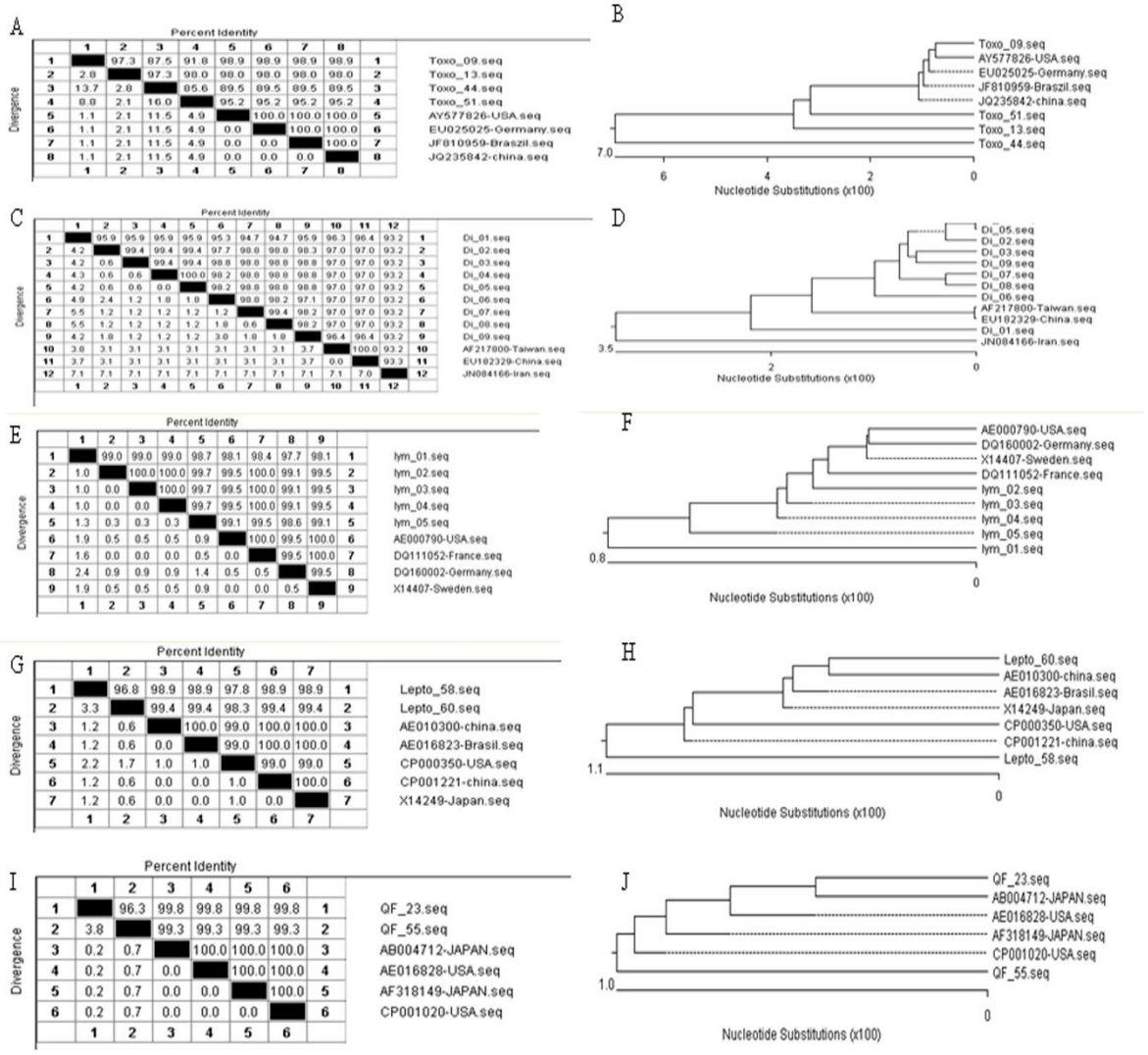

Figure 4 Data of sequence pair distances analysis show that DNA sequences of *T. gondii* have 95~100% identities in comparison with the strains of USA, Germany, Brazil and China (A and B) as *D. immitis* have 96~97% and 93% identities compared with the strains of Taiwan/China and Iran, respectively (C and D). *B. burgdorferi* DNA sequences have 98~100% identities compared with USA, Germany and Sweden strains (E and F). DNA sequences of *Leptospira* spp. have 97~100% identities compared with the strains of China, Brazil, USA and Japan (G and H) as *C. burnetii* DNA sequences show 99~100% identities in comparison with Japan and USA strains (I and J).

Germany, Brazil and China. This shows more regional diversity of similar *T. gondii* strains compared with the findings of our research group in north Taiwan (Chiu et al., unpublished data), in which *T. gondii* sequences had 98.5~100% identities in comparison with the strains of China. The reasons could be that north Taiwan is geographically closer to mainland China than central Taiwan.

Prevalence of *D. immitis* in countries in East Asia represents diversity. In Japan, studies on *D. immitis* in 1980s found high prevalence rates for *D. immitis* (46.8%) (Fujinami et al., 1983). Increased prevalence rates of *D. immitis* have been reported in South Korea and Taiwan in 1990s and early 2000s (Lee et al., 1996; Song et al. 2003; Wu and Fan, 2003). In Japan, although high prevalence of *D. immitis* (46%) was reported in 1999-2001, a decrease in prevalence of canine heartworms (23%) was shown in a recent study (Oi et al., 2014). Similarly, the prevalence of heartworm disease in dogs was over 10% around 15 years before in Taiwan in the previous report (Fan et al., 2001). In the recent 3 years, the prevalence rate of *D. immitis* has been reportedly reduced below 8%. The PCR prevalence rate of *D. immitis* in stray dogs in north Taiwan was 3.2% while one year later the rate was 5.63% in our unreported findings (Chiu et al. and Liu et al., unpublished data). The current study displays slightly higher prevalence rate of *D. immitis* in stray dogs in central Taiwan (7.22%). This is probably in association with higher temperature and mostly humid subtropical climate leading to more mosquitoes in the environment in central and south Taiwan. Firthermore Also, a rise of prevalence rate for *D. immitis* in Oct 2009 found in our study was possibly correlated with the aid of mosquito breeding after the Morakot typhoon in Aug 2009.

Lyme disease is caused by the spirochetes *B. burgdorferi* sensu lato, which can be categorized into at least 13 genospecies (Chao et al., 2013). It has been shown that in addition to wide distribution of Lyme disease in the USA and European countries, this zoonotic disease also occurs in far eastern Russia and some Asian countries (Wang et al., 1999). Furthermore, molecular analysis has indicted that *Ixodes granulatus* ticks are possible vectors for *B. burgdorferi* sensu lato in Taiwan (Chao et al., 2009a; Chao et al., 2009b) while 42.9%,

Table 5 The associations between the age of stray dogs and the infection rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii*.

Age of dogs	Numbers of dogs examined	Numbers and infection rates (%)				
		T. gondii	***D. immitis***	***B. burgdorferi***	***Leptospira* spp.**	***C. burnetii***
< 1y	148	9 (6.08)	10 (6.76)	3 (2.03)	2 (1.35)	6 (4.05)
1y-< 4y	257	8 (3.11)	13 (5.06)	14 (5.45)	16 (6.23)	20 (7.78)
4y-< 8y	210	3 (1.43)	20 (9.52)	11 (5.24)	23 (10.95)	13 (6.19)
> 8y	105	8 (7.62)	9 (8.57)	11 (10.48)	11 (10.48)	3 (2.86)
Total	720	28 (3.89)	52 (7.22)	39 (5.42)	52 (7.22)	42 (5.83)
X^2		9.6268*	3.7892	8.5791*	14.0138**	4.3717

X^2:chi-square test for independence. * : < 0.05, ** : P < 0.01 , *** : P < 0.001.

Table 6 The associations between sampling season and the infection rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp., and *C. burnetii*.

Season	Numbers and infection rates (%)				
	T. gondii	***D. immitis***	***B. burgdorferi***	***Leptospira* spp.**	***C. burnetii***
Spring (Feb-Apr)	2/180 (1.11)	7/180 (3.89)	1/180 (0.56)	0/180 (0)	1/180 (0.56)
Summer (May-Jul)	0/180 (0)	8/180 (4.44)	4/180 (2.22)	1/180 (0.56)	9/180 (5.0)
Autumn (Aug-Oct)	25/180 (13.89)	36/180 (20.0)	34/180 (18.89)	50/180 (27.78)	11/180 (6.11)
Winter (Nov-Jan)	1/180 (0.56)	1/180 (0.56)	0/180 (0)	1/180 (0.56)	21/180 (11.67)
X^2	67.2289***	65.994***	91.1862***	164.1758***	21.8868***

X^2:chi-square test for independence. * : < 0.05, ** : P < 0.01 , *** : P < 0.001.

36%, and 52.7% *Borrelia* infection has been respectively shown in nymph, male as well as female stages of *Ixodes granulatus* ticks (Chao et al., 2013). In our study we found that the PCR prevalence rate of *B. burgdorferi was* 5.42% from 2009 to 2011 in central Taiwan, suggesting that the vector tick species of *Ixodes granulatus* exists in the transmission cycle of Lyme disease in central Taiwan. It has been shown that in Europe climate factors partially affect the vector biology and disease transmission of zoonotic tick-borne diseases (Gray et al., 2009). In the life cycle of *Ixodes* ticks, adult ticks feed on animals in fall and early spring (Centers for Disease Control and Prevention, 2011). Investigation also showed that in seven states in America between 1993 and 2001 significant positive relations were found for the correlation of early summer disease incidence with the June moisture index (Subak, 2003). Moreover, it was shown that the occurrence of Lyme disease was higher than average when late spring/early summer precipitation (mainly moisture) was greater than average, probably due to enhanced tick activity and survival rate during wet conditions (McCabe and Bunnell, 2004). These previous studies suggest that the rise of *B. burgdorferi* prevalence in Sep 2010 in our study might be associated with the increased moisture in spring/summer of 2010. Further data of stray dogs collected from different regions of Taiwan are required to prove whether these influences on the infections of *B. burgdorferi* in Europe also occur in Taiwan. Despite possible underestimation by false negative results of *B. burgdorferi* and *T. gondii* for molecular detection from blood samples, the current study still found that the prevalence of *B. burgdorferi* and *T. gondii* was 5.42% and 3.89%, respectively. This implicates that the occurrence of these zoonotic pathogens in stray dogs should be noticed

Leptospirosis has been indicated as an underestimated zoonotic disease in Taiwan (Yang, 2007). This emerging worldwide bacterial disease infects humans and dogs. The outbreak of human leptospirosis occurred in Taiwan in 2009 due to floods caused by a typhoon. A total of 203 people were diagnosed with leptospirosis in 2009 after the Morakot typhoon (Fuh et al., 2011). In our study over 96% cases detected between August and October are coherent with previous investigation (Sykes et al., 2011; Brown and Prescott, 2008). It has been found that humans suffered leptospirosis after typhoon in Taiwan and contaminated water, moist soil and vegetation were the suspected sources of the pathogens (Chiu et al., 2009). Stray dogs, particularly old stray dogs, expose themselves to these suspected sources of *Leptospira* spp. at high frequencies. Therefore, the high prevalence of *Leptospira* spp. in the stray dogs in central Taiwan in Aug-Sep, 2010 could be relevant with the possible contaminations after high rainfalls and/or typhoon and we also found that the age of dog was a factor associated with the prevalence rate of *Leptospira* spp. in stray dogs. Rodents and domestic animals such as dogs, pigs and cattle are the main reservoir hosts of *Leptospira* (Victoriano et al., 2009). In the current study the PCR prevalence rate of *Leptospira* spp. reached 7.22% in 720 stray dogs from 2009 to 2011 in central Taiwan, which should be concerned and highlighted in both hygienic and veterinary aspects.

Animals infected with Q fever shed the bacterium *C. burnetii* in birth products, feces, urine and milk so the infected dogs may transmit *C. burnetii* to humans via these routes but not via ticks. The bacterial zoonotic disease occurs over the world and has been reported commonly in southern France and Australia (Honarmand, 2012). The serological evidence of *C. burnetii* in domestic dogs and public health implications have been reported in Australia (Cooper et al., 2011). Q fever has been indicated as one of the most common rickettsioses in humans in Taiwan as from April 2004 to April 2008, 68 cases were serologically validated as acute Q fever in 223 suspected cases in South Taiwan (Lai et al., 2009). The current study for the first time showed the molecular prevalence of Q fever in stray dogs as it accounted for 5.83% in central Taiwan between 2009 and 2011. Since humans often get Q fever via breathing in contaminated droplets released from infected animals (Honarmand, 2012), the findings of Q fever in stray dogs in the present study also implied potentially hygienic risks for humans in central Taiwan. The prevalence rate of Q fever was associated with the season of occurrence, implicating that spread and prevalence of Q fever might be linked with the dog breeding season.

Taken together, in this study we showed the molecular prevalence rates of *T. gondii, D. immitis*, *B. burgdorferi*, *Leptospira* spp. and *C. burnetii* in 720 stray dogs in central Taiwan from 2009 to 2011. It should be noticed that the prevalence rates of *D. immitis*, *B. burgdorferi*, *Leptospira* spp. and *C. burnetii* was over 5% and prevalence rates of all the 5 investigated zoonoses were associated with the season. Prevalence rates of *T. gondii, B. burgdorferi* and *Leptospira* spp. were also correlated with the age of dogs. According to the findings revealed in the current study, the control and prevention of zoonotic toxoplasmosis, heartworm disease, Lyme disease, leptospirosis and Q fever in stray dogs should be evoked and performed and the routine surveillance is still necessary for animal health or hygienic considerations.

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บทคัดย่อ

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การศึกษานี้ได้ทาการตรวจสอบความชุกของโรคที่สามารถติดต่อจากสัตว์สู่คนซึ่งพบบ่อยในสุนัขพันธุ์ทาง ได้แก่ โรคท็อกโซพลาส-โมซิส โรคหนอนพยาธิหัวใจ โรคลัยม์ โรคเล็ปโตสไปโรซิส และโรคไข้คิว ในบริเวณตอนกลางของไต้หวัน (มณฑลไท่จง มณฑลจางฮั่ว และมณฑลยุ่นลิ่น) โดยใช้วิธีเนสเต็ต เซมิเนสเต็ต หรือ วิธีโพลีเมอเรสเชนรีเอกชั่น และได้นำความสัมพันธ์ระหว่างความชุกของโรคและข้อมูลประชากรของสุนัขจรจัดในภาคกลางของไต้หวันมาวิเคราะห์ด้วย ทำการสุ่มตัวอย่างเลือดจากสุนัขจรจัดในทุกๆเดือนเริ่มจากเดือนสิงหาคม ๒๕๕๔ ถึงเดือนกรกฎาคม ๒๕๕๖ ดีเอ็นเอรวมที่สกัดจากตัวอย่างเลือดอยู่ระหว่าง 0.8-1.5 ไมโครกรัม ซึ่งความไวของปฏิกิริยาพีซีอาร์จะอยู่ที่ 10 เฟมโตกรัมต่อไมโคลิตร ถึง 100 พิโคกรัม สำหรับพีซีอาร์ไพร์เมอร์ พบว่าไม่เกิดปฏิกิริยาข้ามกับการตรวจพบกรดนิวคลีอิกของเชื้ออื่นที่ศึกษา และดีเอ็นเอที่ได้จากการสกัดมาจากเชื้อโรค *Ehrlichia cani, Babesia canis และ Babesia gibsoni* อัตราที่ให้ผลบวกของโรคทั้งห้าดังกล่าวในสุนัขจรจัดมีดังต่อไปนี้: เชื้อปรสิต *Toxoplasma gondii* ร้อยละ 3.89 (28/720) หนอนพยาธิ *Dirofilaria immitis* ร้อยละ 7.22 (52/720) *Borrelia burgdorferi* ร้อยละ 5.42 (39/720) เชื้อแบคทีเรีย *Leptospira* spp. ร้อยละ 7.22 (52/720) และเชื้อแบคทีเรีย *Coxiella burnetii* ร้อยละ 5.83 (42/720) การวิเคราะห์ความสัมพันธ์ระหว่างอัตราการติดเชื้อและข้อมูลระบาดวิทยาของสุนัขจรจัดที่เก็บได้ในแต่ละฤดูแสดงให้เห็นว่าอัตราการติดเชื้อของ *Toxoplasma gondii Borrelia burgdorferi* และ *Leptospira spp*. มีความสัมพันธ์กับอายุและฤดู แต่ไม่มีความสัมพันธ์ในสายพันธุ์และเพศ ส่วนอัตราการติดเชื้อจากพยาธิสายพันธุ์ *Dirofilaria immitis* มีความเกี่ยวข้องกับสายพันธุ์และฤดูแต่ไม่เกี่ยวข้องกับเพศและอายุ ในขณะที่การติดเชื้อจาก *Coxiella burnetii* ความเกี่ยวข้องกับฤดู แต่ไม่เชื่อมโยงกับพันธุ์ เพศ และ อายุ การศึกษาครั้งนี้ได้แสดงให้เห็นถึงความชุกของโรคท็อกโซพลาสโมซิส โรคหนอนพยาธิหัวใจ โรคลัยม์ โรคเล็ปโตสไปโรซิส และโรคไข้คิวในสุนัขจรจัดในไต้หวัน และการศึกษาในครั้งนี้ได้ให้ข้อมูลที่สาคัญสาหรับการป้องกันและการควบคุมโรคในสัตว์ในมณฑลดังกล่าวและบริเวณเขตพื้นที่ใกล้เคียงในไต้หวัน

คำสำคัญ: โรคหนอนพยาธิหัวใจ โรคไข้คิว โรคเล็ปโตสไปโรซิส โรคลัยม์ สุนัขจรจัดในไต้หวัน โรคท็อกโซพลาสโมซิส

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