Original Article

Molecular Prevalence of Zoonotic Pathogens in Pet and Stray Dogs in Southern Taiwan

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Abstract

The prevalence of important zoonotic diseases including Lyme disease, Q fever, heartworm disease, leptospirosis and toxoplasmosis in pet and stray dogs in southern Taiwan (Kaohsiung City and Pingtung County) was assessed using nested, semi-nested or traditional polymerase chain reaction. The correlations between the prevalence and regional demiographic data were also analyzed. Blood samples were randomly collected from pet and stray dogs monthly from September 2009 to August 2011. The PCR sensitivity for *Borrelia burgdorferi* was 10 pg, 100 fg for *Coxiella burnetii*, 1 pg for *Dirofilaria immitis*, 100 pg for *Leptospira* spp. and 10 fg for *Toxoplasma gondii,* respectively, in 0.8~1.5 μg total DNA from the blood sample. No cross-reaction was observed between the nucleic acids of interest and DNA extracted from *Ehrlichia canis, Babesia canis* and *Babesia gibsoni* for the selected PCR primers. The 5 zoonotic pathogens and their prevalence rates in dogs were *B. burgdorferi* 0.07% (1/1440), *C. burnetii* 4.79% (69/1440), *D. immitis* 7.08% (102/1440), *Leptospira* spp. 0.07 % (1/1440) and *T. gondii* 0.14% (2/1440), which highlights the importance of *C. burnetii* and *D. immitis* among dogs in southern Taiwan. Further analysis between the prevalence and associated epidemiological factors demonstrated that prevalence of *C. burnetii* is correlated with the season, age and source (pet or stray) but not breed and gender. The prevalence of *D. immitis* was relevant to age and source but not with season, breed or gender. Overall, this study reveals hygienic implications as seen with the prevalence of Q fever and heartworm disease and raises public health awareness in southern Taiwan as well as the neighboring areas and countries.

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

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Introduction

Stray dogs have become increasingly important in bacterial and parasitic zoonotic pathogens (Chen et al., 2012; Jimenez-Coello et al., 2010; Chou et al., 2014). In some Asian countries, including Taiwan, stray dogs are a social and hygienic threat as the number of pet dogs has been significantly increased during the year 2009 to 2014 in Taiwan. Certainly Asian countries where stray dogs are more common have been reported to have higher prevalence rate of some zoonotic pathogens than other parts of the world (Victoriano et al., 2009; Nguyen et al., 2012; Yan et al., 2012; Chou et al., 2014), suggesting geographical differences exists. Our previous study indicated that over 5% prevalence of *Dirofilaria immitis*, *Borrelia burgdorferi*, *Leptospira* spp., and *Coxiella burnetii* are found in 720 stray dogs in the middle region of Taiwan (Chou et al., 2014), however, since stray dogs are usually regional residents, it is not clear whether the prevalence of these pathogens also representative of other parts of the same country. Therefore, we intended to study the prevalence of reported zoonotic pathogens in the most hot and humid part of Taiwan and compare the results to previous studies.

The zoonotic pathogens commonly associated with stray dogs include *Borrelia burgdorferi*, *Coxiella burnetii Dirofilaria immitis*, *Leptospira* spp. and *Toxoplasma gondii* (Nguyen et al., 2012; Yan et al., 2012; Lai et al., 2005; Chou et al., 2014). *Borrelia burgdorferi,* which was first recovered in early 1980s from the tick, *Ixodes dammini* (Chang et al., 1998; Chao et al., 2013; Burgdorfer et al., 1982) is the causative agent of Lyme disease. *B. burgdorferi* is a spirochete possessing a helical shape and multiple endoflagella with distinct morphological, structural, ecologic, and genomic characteristics. Infection with *B. burgdorferi* sensu lato is capable of causing dermatological, neurological, and musculoskeletal disorders (Aguero-Rosenfeld et al., 2005). However, symptoms associated with the infection have been reported to show geographical differences. For instance, arthritis was shown be more frequent in North America whereas lymphocytoma, acrodermatitis chronica atrophicans and encephalomyelitis were mainly found in Europe (Aguero-Rosenfeld et al., 2005). Qualitative PCR is usually sufficient for laboratory diagnosis of *B. burgdorferi* sensu lato infection (Aguero-Rosenfeld et al., 2005). *Coxiella burnetii* is the causative agent of Q fever, a zoonotic disease distributed worldwide (Arricau-Bouvery and Rodolakis, 2005) and classified as a Category B agent by the Centers for Disease Control. *C. burnetii* is a pleomorphic and obligate intracellular Gram-negative bacterium capable of infecting a wide range of animals including dogs, ruminants, birds, rodents and reptiles, through inhalation, and in contact with contaminated urine, feces, milk (Brouqui and Raoult, 2001; Honarmand, 2012). Livestock with *C. burnetii* infection may induce pneumonia and lead to abortion, stillbirth, and delivery of weak lambs, calves or kids (Honarmand, 2012; Arricau-Bouvery and Rodolakis, 2005). Therefore, Q fever is considered a highly zoonotic disease for animals and for farmers, laboratory workers and veterinarians (Honarmand, 2012; de Rooij et al., 2012). *C. burnetii* infection in humans usually shows no symptoms or a mild disease with spontaneous recovery so many Q fever infections are not diagnosed (Maurin and Raoult, 1999; de Rooij et al., 2012). Acute Q fever can develop into chronic Q fever, in which endocarditis or vascular infection with a high case fatality may occur (de Rooij et al., 2012).

Domesticated and wild dogs are also reservoirs for *Dirofilaria species.* The filarial nematode *Dirofilaria immitis* causes cardiopulmonary dirofilariasis that is potentially fatal to dogs and cats (Simon et al., 2012). It is also correlated with pulmonary nodules and causes pulmonary dirofilariasis in humans (Rossi et al., 2010). *D. immitis* worms were present in tissues including hepatic, intraocular and mesenteric adipose tissues (Theis, 2005; Avellis et al., 2011, Simon et al., 2012). Canine dirofilariasis caused by *D. immitis* has been reported in East, South and South East Asia so its importance should be underlined. In some regions of Malaysia, prevalence of canine *D. immitis* reached 70% (Simon et al., 2012) whereas in Japan, high prevalence in dogs (46.8%) was reported since 1980s to early 2000s (Fujinami et al., 1983). The prevalence was reduced to 23% during 2009-2011 (Oi et al, 2014). In South Korea, the prevalence of *D. immitis* was 40% (339/848) in a study (Song et al., 2003). In Taiwan *D. immitis* seroprevalence was 13.4% and dogs ≥ 6 years presented higher seroprevalence of 23.7% (Fan et al., 2001). In the tropical or sub-tropical countries, leptospirosis caused by different serotypes of *Leptospira interrogans* is also widely-distributed, which prevalence could be underestimated (Victoriano et al., 2009). The pathogen *Leptospira* spp. has at least 18 species by DNA relatedness (Levett, 2001; Victoriano et al., 2009). The severity of leptospirosis in humans is highly variable, ranging from asymptomatic to subclinical infection and high mortality (Levett, 2001; Victoriano et al., 2009). Fever, jaundice and renal failure have been most commonly found in humans with acute leptospira infection in Taiwan (Yang, 2007). Rodents, wild and domestic animals, including dogs, pigs and cattle can act as reservoir hosts for one or more serotypes of *Leptospira, and c*linically normal dogs can be chronic carriers shedding the bacterium in their urine (Brown and Prescott, 2008; Rojas et al., 2010; Victoriano et al., 2009). Pets and humans may be infected through contaminated water, soil or mud while participating in outdoor activities (Brown and Prescott, 2008). It is important to note that leptospires can survive longer in warm and humid conditions and thus the pathogens represent higher risk in warm and wet regions (Levett, 2001) such as Taiwan.

T. gondii is a protozoan parasite which infects almost one third of the world population as well as other warm-blooded vertebrates. Toxoplasmosis is a National Notifiable Communicable Disease in the 4th category since 2007 (Hill et al., 2005; Montoya and Liesenfeld, 2004; Chiang et al., 2012). In animals, toxoplasma has been described for over 350 species, including mammals and avian. The seroprevalence of toxoplasma in wild fields is usually high, up to 100% in some cases. The environmental contaminations are associated with the shedding of oocysts from intermediate wild hosts to stray or pet dogs and cats

close to farms (Robert-Gangneux and Darde, 2012). It was assumed that about 25~30% of human population in the world is infected by *T. gondii* while prevalence rate of the zoonotic protozoa in humans was 10~30% in South East Asia (Montoya and Liesenfeld, 2004; Robert-Gangneux and Darde, 2012). Although the main transmission routes of *T. gondii* to humans are via the ingestion of soil, water, or food contaminated with oocysts in the environment or through the ingestion of tissue cysts in infected meat, previous studies have shown that risk factors for infection were still inexplicable in 14 to 49% of cases (Robert-Gangneux and Darde, 2012). Thus, the prevalence rates of T. *gondii* in pet and stray dogs should be noticed. *T. gondii* infection may cause severe complication in immunocompromised people, pregnant women and newborns (Dubey and Jones, 2008; Montoya and Liesenfeld, 2004; Robert-Gangneux and Darde, 2012). *T. gondii* can be horizontally transmitted to humans either from the ingestion of cysts in meat or via the ingestion of soil, water, or food contaminated with sporulated oocysts. Serological assays are the main method for the examination of chronic infection in animals (Robert-Gangneux and Darde, 2012). We have previously shown that stray dogs in central Taiwan from 2009 to 2011 displayed 3.89% prevalence of *T. gondii* (Chou et al., 2014) and the prevalence of *T. gondii* has shown geographical difference between countries and even in a single country (Sukthana, 2006; Robert-Gangneux and Darde, 2012; Chiang et al., 2012). Hence, further investigation of the regional prevalence of *T. gondii* in stray dogs is warranted.

We have previously established and used molecular detective techniques to examine viral, bacterial and parasitic DNA (Liu et al., 2003; Chiu et al., 2002; Yin et al., 2003; Chou et al., 2014) for successful epidemic identification of pathogens in Taiwan. The aims of this study are to further investigate the prevalence of these five important zoonotic pathogens and to assess the correlations between the prevalence and the demiographic difference between domestic and stray dogs in southern Taiwan. The epidemiological data on the five zoonotic pathogens in this study should uncover molecular similarities and differences helpful for hygienic prevention and control of these diseases stray and pet dogs in Taiwan and facilitate the protection for humans.

Materials and Methods

Sample collection and preparation: A total of 1440 blood samples were collected from pet (720 samples) and stray dogs (720 samples) in southern Taiwan from August 2009 to July 2011. Two shelters located at Kaoshiung City and Pingtung County in southern Taiwan (Fig 1) were the sources of stray dogs. The population of stray dogs in these two shelters in 2009 and 2011 was 6105 and 15142, respectively, while the estimated number of pet dog in 2009 and 2011 was 175864 and 199652, respectively (from the website of animal protection information: http://animal.coa.gov.tw/html3/index.php). A total of sixty blood samples (30 from stray dogs randomly collected from the shelters and 30 from pet dogs randomly collected from 3 animal hospitals) were taken every month in the 2-year period. The specimens were stored at 4°C until analysis. Gender, breed (mixed or purebred) and collection date were recorded. The DNA of each blood sample was extracted with a commercial kit (Biokit, Miaoli County, Taiwan) according to the manufacturer's instructions. The extracted and purified DNA was stored at -20°C for specific polymerase chain reaction (PCR) analysis of *B. burgdorferi*, *C. burnetii*, *D. immitis*, *Leptospira* spp. and *T. gondii.*

PCR assays for B. burgdorferi, C. burnetii. D. immitis, Leptospira spp. and T. gondii: DNA of the blood samples collected from pet or stray dogs was assessed by traditional PCR (for *B. burgdorferi, D. immitis* and *Leptospira* spp.), nested PCR (for *C. burnetii*) or semi-nested PCR (for *T. gondii*) as previously described (Chou et al., 2014). The primers used for DNA detection of the five zoonotic pathogens are shown in Table 1 (Demaerschalck et al., 1995; Zhang et al., 1998; Mar et al., 2002; Kawabata et al., 2001; Hurtado et al., 2001). The PCR products were electrophorezed on 1.5% agarose gel at 100 V for 40 min to determine the size of products.

Sensitivity and specificity of PCR: The PCR sensitivity for 5 zoonotic pathogens was determined by preparation of serial concentrations (100, 10, 1 ng/μL, 100, 10, 1 pg/μL, 100, 10 fg/μL) of the target genes. The sensitivity for the target gene *B. burgdorferi was* 10 pg, 100 fg for *C. burnetii,* 1 pg *for D. immitis*, 100 pg for *Leptospira* spp. and 10 fg for *T. gondii* respectively, in 0.8~1.5 μg total DNA from the blood sample (Fig 2A~2E). The specificity of PCR primers for the 5 zoonotic pathogens were compared with other 8 pathogens, no cross-reaction was observed, indicating high specificity of the primers used for our target pathogens (Fig 3A~3E).

DNA cloning and sequencing: Perfectprep Gel Cleanup Kit (Eppendorf, Berkhausenweg, Germany) was used to purify the DNA bands of interest. DNA inserts of 5 zoonotic pathogens were cloned into pGEMT easy vector (Promega, Madison, WI, USA). Ligation and transformation were carried out according to the manufacturer's instructions. The ligation products were transformed into BL21 (DE3) plysS competent cells (Novagen, Germany). Fifty μL competent cell suspension was carefully added to 2 μL of ligation reaction in a sterile tube. The processes of heat-shocking and re-proliferation of bacterial competent cells were performed as shown previously (Chou et al., 2014). After 16-18 hr of 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 for linearization. Electrophoresis on 1.5% agarose gel at 100 V for 40 min was performed to separate the mixture and to examine whether the DNA inserts were contained in the plasmids. The plasmid DNA sequences were validated by T7 and SP6 primers on an automatic analyzer in Tri-I Biotech (New Taipei City, Taiwan).

Statistical analysis: Numbers and percentages were used to represent categorical variables in each group.

Chi-square test for independence performed to compare the categorical data. The analyses are two-tailed and P value ≤ 0.05 was set for statistical significance. Odds ratios were used to analyze association between prevalence rates and specific parameters when appropriate. All statistical analyses were conducted by STATA software.

Table 1 Sequences of PCR primers

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

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Results

Prevalence of target pathogens: Table 2 represents the percentages of positive samples (positive specimen numbers/total specimen numbers) between August 2009 and July 2011. The prevalence of *B. burgdorferi*, *C. burnetii, D. immitis*, *Leptospira* spp. and *T. gondii,* in dogs were 0.07 % (1/1440), 4.79 % (69/1440), 7.08 % (102/1440), 0.07 % (1/1440), and 0.14 % (2/1440), respectively (Table 2). The prevalence of *C. burnetii* in pet dogs and stray dogs was 2.78% (20/720) and 6.81% (49/720), respectively while the prevalence of *D. immitis* in pet dogs and stray dogs was 4.58% (33/720) and 9.58% (69/720) (Table 4 and 5). The one *B. burgdorferi* positive sample and two *T. gondii* positive samples were both found in stray dogs (Table 3 and 7) and the only *Leptospira* spp. positive specimen was found in pet dogs (Table 6).

The associations between prevalence rates and the age, gender, breed and source: The prevalence rate of *C. burnetii* was significantly associated with age, sampling season and being pet dogs or stray dogs (p<0.05, p<0.001 and p<0.001, respectively, Table 4). The odds of *C. burnetii* prevalence for pet dogs were approximately 0.39 lower than those for stray dogs. The odds of *C. burnetii* prevalence for pure dogs were about 0.64 lower than those for mixed dogs. The prevalence rates of 5 zoonotic pathogens were not associated with the gender and breed (mix or purebred) of dogs. The prevalence rate of *D. immitis* was significantly associated with age regardless of being pet or stray dogs (p<0.05 and p<0.001, respectively, Table 5). The odds of *D. immitis* prevalence for pet dogs showed about 0.45 lower than those for stray dogs.

Comparison of DNA sequencing results: The sequencing results of the DNA fragments of *B. burgdorferi*, *C. burnetii, D. immitis*, *Leptospira* spp., and *T. gondii,* were compared with NCBI (National Center for Biotechnology Information) databases using sequence pair distances analysis on DNASTAR® software. Consequently, DNA sequences of the only *B. burgdorferi* positive specimen has 97~98% identities to the strains of USA, Germany and Sweden (Fig 4A and 4B). The sequences of *C. burnetii* positive specimens have 97~100% identities in comparison with the strains of Japan, Korea and USA (Fig 4C and 4D). DNA sequences of *D. immitis* positive samples showed 95~97% and 89~94% identities to the strains of Taiwan/China and Iran, respectively (Fig 4E and 4F). The sequences of The *Leptospira* spp. positive specimen showed 99.5% identities compared with the strains of China, Brazil, USA and Japan (Fig 4G and 4H) while the two *T. gondii* positive specimens show 98.9% identities in comparison with the strains of USA, Germany, Brazil and China (Fig 4I and 4J).

Discussion

Taiwan is a tropical country where zoonotic diseases are legitimate threats. Complete investigation on the prevalence of multiple zoonotic pathogens is important, but few were done in the past. The current

work represents one of the few such studies in Taiwan in addition to our previous report (Chou et al., 2014).

In the present study, the spirochetes *B. burgdorferi* were nearly not found from 2009 to 2011 in southern Taiwan. This is in contrast to 5.42% in central Taiwan in the same time period (Chou et al., 2014) and also different from a 4.7% unpublished data in northern Taiwan (Chiu et al., unpublished data). Spirochetes of *B. burgdorferi* sensu stricto have been isolated from 6 species of rodent hosts in Taiwan (Shih et al., 1998). The transmission, pathogenic isolation and identification of human borreliosis have been shown in Taiwan. A 16.6% positive rate of *Borrelia Burgdorferi*-like spirochetes was found in captured rodents (53 of 320) (Shih and Chao, 1998). Regional findings on prevalence and molecular identification of *Burgdorferi* spirochetes in ticks collected from rodents suggest that *Borrelia* spirochetes have an enzoonotic cycle between *Ixodes granulatus* ticks and rodent hosts in Taiwan and Southeast Asia (Chao et al., 2013). *Borrelia burgdorferi* sensu stricto was further isolated and molecularly identified from skin biopsies of patients and thus significance of genetic diversity of Borrelia spirochetes among patients was also highlighted in Taiwan (Chao et al., 2011). The fact that the spirochetes *B.burgdorferi* were nearly not found from 2009 to 2011 in southern Taiwan, suggested that the vector tick species of *Ixodes sp.* could not widely spread among stray dogs in southern Taiwan. This phenomenon may be associated with the climate factors that have been shown in Europe to partially affect the vector biology and tick-borne disease transmission (Gray et al., 2009) since it has been shown that central Taiwan had less drop of annual total rain days and less dry spells (data measured at Taichung city) compared with other regions of Taiwan including southern Taiwan (data measured at Hengchun town) from 2000 to 2010, according to a scientific climate report (Hsu et al., 2011). Moreover, southern Taiwan experienced a clear decrease of annual total rain days and more frequent extreme dry spells (data measured at Hengchun town) than central Taiwan (data measured at Taichung city) in Taiwan from 2000 to 2010 (Hsu et al., 2011). These different climate changes between central and southern Taiwan could partly affect the tick biology and tick-borne disease transmission in the two regions of Taiwan. Host diversity and community composition have been shown to be able to reduce the incidence of Lyme disease (LoGiudice et al., 2003) and these factors might also affect the prevalence of *B. burgdorferi* in pet and stray dogs in southern Taiwan. It is also possible that different genospecies of *B. burgdorferi* sensu lato are distributed unevenly to exhibit distinct ecologic features (Wang et al., 1999).

Table 2 The prevalence of *B. burgdorferi*, *C. burnetii, D. immitis*, *Leptospira* spp. and *T. gondii,* in dogs

	B. burgdorferi	*C. burnetii*	*D. immitis*	*Leptospira spp*	*T. gondii*
Positive sample number / total sample number	1/1440	69/1440	102/1440	1/1440	2/1440
Positive rate	0.07%	4.79%	7.08%	0.07%	0.14%

Q fever has been indicated as one of the most common rickettsioses in humans in Taiwan from April 2004 to April 2008. Sixty-eight cases were serologically validated as acute Q fever in 223 suspected cases in southern Taiwan (Lai et al., 2009). Meanwhile, the seroprevalence of *C. burnetii* was 4.2% in both in-patient (15/357) and physical examination participant (11/259) groups (Ko et al., 2000). Our previous report had shown that the prevalence rate of *C. burnetii* in stray dogs in central Taiwan between 2009 and 2011 is 5.83% (Chou et al., 2014). The current study further found that the prevalence rate of *C. burnetii* for stray dogs (6.81%, 49/720) is over 2-fold higher than that for pet dogs (2.78%, 20/720) (Table 4). From central to southern Taiwan, the epidemiological data of Q fever in our studies revealed the presence of *C. burnetii* in dogs. This might imply somewhat hygienic connections with serological prevalence of Q fever in humans in southern Taiwan in previous reports due to possible indoor and outdoor contact between dogs and humans. Q fever was also reported in southern France and Australia (Honarmand, 2012; Arricau-Bouvery and Rodolakis, 2005). It should be noted that in East Asia high *C. burnetii* seropervalence (41.7%, 15/36) in stray cats in Japan was reported while in pet cats *C. burnetii* seropervalence was 14.2% (44/310) and 8.6% (10/116) in Japan and Korea, respectively (Komiya et al., 2003). Also in Japan, seropervalence of *C. burnetii* up to 13.5% in veterinarians was reported while the counterparts were 3.6% and 5.1% in blood donors and medical workers, respectively. The data uncovered in these studies implicated the hygienic risk and importance of Q fever, especially for veterinarians (Abe et al., 2001). The results in both our recent and current studies were highly associated with the season, implicating that spread and prevalence of Q fever could be associated with the dog breeding season. It should be particularly noted that both data represent over 5% prevalence rate of Q fever in stray dogs in southern and central Taiwan, signifying its public health implications in both regions. Based on the previous and current findings on Q fever prevalence, the outdoor spread for *C. burnetii* should not be neglected. The farm owners should prevent stray dogs and pet dogs from closing livestock on their farms. Dogs and cats should be particularly kept away from aborted fetuses as well as placentas and away from aborted animals. The public veterinarians should also monitor the conditions of the stray dogs in the regional animal shelters and inform the authorization of the suspected cases. The current findings in combination to the previous data shown in Japan, Australia and Korea suggest that Q fever is still a zoonotic concern for the public, particularly for veterinarians and pet owners in the Asian Pacific countries.

Table 3 The associations between infection rates and the age, gender, breed and source for *B. burgdorferi.*

	No. of examined dogs	No. of infected		X^2	p
		Dogs	Rate(%)		
Sex					
Female	804	0	0.00%	1.26502944	0.26070123
Male	636	1	0.16%		
Age(years)					
less 2	501	0	0.00%	2.56613848	0.27718525
2 - 6	535	0	0.00%		
6 up	404	1	0.25%		
Breed					
Pure	734	1	0.14%	0.96252128	0.32655246
Mixed	706	0	0.00%		
Season					
Spring	360	1	0.28%	3.00208478	0.39130386
Summer	360	0	0.00%		
Fall	360	0	0.00%		
Winter	360	0	0.00%		
Pet or stray					
Pet dogs	720	0	0.00%	1.00069493	0.31714241
Stray dogs	720	1	0.14%		
Total	1440	1	0.07%		

*p<0.05, ***p<0.001

Table 4 The associations between infection rates and the age, gender, breed and source for *C. burnetii.*

	No.of examined dogs	No. of infected		X^2	p	Odds ratio
		Dogs	Rate(%)			
Sex						
Female	804	40	4.98%	0.13429870	0.71401598	1.095866
Male	636	29	4.56%			
Age(years)						
less 2	501	29	5.79%	6.62332504	0.03645552*	-
2 - 6	535	30	5.61%			
6 up	404	10	2.48%			
Breed						
Pure	734	28	3.81%	3.13212746	0.07676350	0.643267
Mixed	706	41	5.81%			
Season						
Spring	360	29	8.06%	28.54152792	0.00000280***	-
Summer	360	5	1.39%			
Fall	360	8	2.22%			
Winter	360	27	7.50%			
Pet or stray						
Pet dogs	720	20	2.78%	12.80182666	0.00034628***	0.391254
Stray dogs	720	49	6.81%			
Total	1440	69	4.79%			

*p<0.05, ***p<0.001

The prevalence of heartworm disease in dogs in Taiwan was over 10% around 15 years ago. In the past 3 years, the prevalence rate of *D. immitis* has been decreased to below 8% (Chou et al., 2014). A similar annual reduction of heartworm prevalence was reported in South America (Vezzani et al., 2011). Possible reasons have been proposed that infected dogs die, older dogs have better medical care or these dogs may spend more time indoor (Vezzani et al., 2011). The prevalence rate of *D. immitis* in stray dogs in northern 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 prevalence in the stray dogs in central Taiwan displayed slightly higher infection rate of 7.22% (Chou et al., 2014). In the current study we found that the prevalence rate of heartworm disease in stray dogs in southern Taiwan reached 9.58% (69/720) (Table 5), which is highest among the 3 regions in Taiwan. As the species of mosquitoes *Aedes albopictus* has been shown as the vector of *D. immitis* in Taiwan (Lai et al., 2001), it is highly possible that the high temperature and humid marine tropical climate in southern Taiwan favor mosquito breeding in the environment.

Table 5 The associations between infection rates and the age, gender, breed and source for *D. immitis*..

	No. of examined dogs	No. of infected		X^2	p	Odds ratio
		Dogs	Rate(%)			
Sex						
Female	804	55	6.84%	1.58428995	0.20814384	0.920234
Male	636	47	7.39%			
Age(years)						
less 2	501	24	4.79%	6.91884537	0.03144791*	-
2 - 6	535	41	7.66%			
6 up	404	37	9.16%			
Breed						
Pure	734	52	7.08%	0.00000293	0.99863377	1.000352
Mixed	706	50	7.08%			
Season						
Spring	360	22	6.11%	1.22395146	0.74726568	-
Summer	360	24	6.67%			
Fall	360	29	8.06%			
Winter	360	27	7.50%			
Pet or stray						
Pet dogs	720	33	4.58%	13.67449222	0.00021739***	0.453199
Stray dogs	720	69	9.58%			
Total	1440	102	7.08%			

*p<0.05, ***p<0.001

Table 6 The associations between infection rates and the age, gender, breed and source for *Leptospira spp.*

	No. of examined dogs	No. of infected		X^2	p
		Dogs	Rate(%)		
Sex					
Female	804	0	0.00%	1.26502944	0.26070123
Male	636	1	0.16%		
Age(years)					
less 2	501	1	0.20%	1.87555397	0.39149717
2 - 6	535	0	0.00%		
6 up	404	0	0.00%		
Breed					
Pure	734	1	0.14%	0.96252128	0.32655246
Mixed	706	0	0.00%		
Season					
Spring	360	0	0.00%	3.00208478	0.39130386
Summer	360	0	0.00%		
Fall	360	1	0.28%		
Winter	360	0	0.00%		
Pet or stray					
Pet dogs	720	1	0.14%	1.00069493	0.31714241
Stray dogs	720	0	0.00%		
Total	1440	1	0.07%		

*p<0.05, ***p<0.001

Table 7 The associations between infection rates and the age, gender, breed and source for *T. gondii.*

	No.of examined dogs	No. of infected		X^2	p
		Dogs	Rate(%)		
Sex					
Female	804	2	0.25%	1.58428995	0.20814384
Male	636	0	0.00%		
Age(years)					
less 2	501	1	0.20%	0.78400904	0.67570106
2 - 6	535	1	0.19%		
6 up	404	0	0.00%		
Breed					
Pure	734	0	0.00%	2.08221207	0.14902408
Mixed	706	2	0.28%		
Season					
Spring	360	2	0.56%	6.00834492	0.11120493
Summer	360	0	0.00%		
Fall	360	0	0.00%		
Winter	360	0	0.00%		
Pet or stray					
Pet dogs	720	0	0.00%	2.00278164	0.15701084
Stray dogs	720	2	0.28%		
Total	1440	2	0.14%		

*p<0.05, ***p<0.001

Leptospirosis is a worldwide re-emerging bacterial zoonosis. In Asia Pacific areas, although the incidence of leptospirosis is not well-documented, investigation has shown that among 28 countries, Thailand, the Philippines and Taiwan are ones with highest incidence (Pappas et al., 2008). In the Asia Pacific, particularly in developing countries, it has been further indicated that leptospirosis is highly prevalent and mostly a water-borne disease (Victoriano et al., 2009). Given the fact, studies have also indicated that leptospirosis is an underestimated disease in Taiwan (Yang, 2007). Increased incidence of lepospirosis after flood damage due to typhoons may have implication in the higher incidence in Taiwan and other countries in Southeast Asia like China, the Philippines and north areas of Indonesia, Malaysia, Singapore, Thailand and Vietnam (Fuh et al., 2011). In Taiwan from 2004 to 2008, 5 of 6 serelogically positive cases were confirmed just after a typhoon (Chiu et al., 2009). Moreover, in 2009 the outbreak of human leptospirosis in Taiwan occurred due to severe floods caused by the Morakot typhoon (Fuh et al., 2011). On the other hand, a recent new trend in human leptospirosis outbreaks has been found to be relevant with recreational activities contacting contaminated water or soil (Pappas et al., 2008; Brown and Prescott, 2008). Rodents and domestic animals such as dogs, pigs and cattle are the main reservoir hosts but the pathogen has been isolated from nearly all mammalian species (Victoriano et al., 2009). We have previously revealed that the prevalence of *Leptospira* spp. reached a noticeably average rate of 7.22% in 720 spray dogs from 2009 to 2011 in central Taiwan while higher prevalence was particularly found after typhoons or during high rainfall seasons (Chou et al., 2014). However, in the current work in southern Taiwan between 2009 and 2011, *Leptospira* spp. prevalence was not found in 720 blood samples of stray dogs and only 1 blood sample of pet dogs was *Leptospira* spp. positive, suggesting possible regional difference in the distribution of *Leptospira* spp. in stray dogs in Taiwan. Nonetheless, an earlier report in northern Taiwan showed that seropositive rate of *Leptospira* spp. infection reached 45.6% (110/241) (Lai et al., 2005), suggesting that the monitoring and control of leptospirosis in stray dogs should still be emphasized.

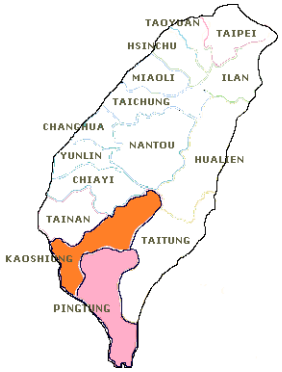

Figure 1 Sampling regions (Kaohsiung City and Pingtung County) of southern Taiwan.

We have previously showed that the PCR positive rate for *T. gondii* in stray dogs is 3.89% (28/720) in central Taiwan (Chou et al., 2014). However, the prevalence rate of *T. gondii* for stray and pet dogs is 0.14% (2/1440, both in stray dogs) in southern Taiwan. In stray dogs in northern Taiwan, the *T. gondii* infection rates were reported to be between 3.61% and 4.9% (Liu et al. and Chiu et al., unpublished results). According to these data, it seems that *T. gondii* has distinct infection rates in southern Taiwan. Differential distribution in different regions in a country have been reported elsewhere (Sukthana, 2006; Robert-Gangneux and Darde, 2012; Chiang et al., 2012). In the neighboring country, 21.6% (132/611) of *T. gondii* seroprevalence in pet dogs was revealed in southwest China, whereas the positive rate of *T. gondii* specific antibodies reached to 40.3% (93/231) in stray dogs in eastern China (Duan et al., 2012; Yan et al., 2012). Despite geographical differences, similarity in age distribution was discovered in this study in comparison to others. While the infection rate of *T. gondii* from 1995 to 1996 in Taiwan showed older and mixed-breed privately-owned dogs have higher seropositivity than younger and pure-breed counterparts (Lin 1998), our results also showed similar trend in stray dogs. In addition, the DNA sequences of *T. gondii* analyzed have 98.9% identities in comparison with the strains of USA, Germany, Brazil and China, which is very similar with our previous findings in central Taiwan (Chou et al., 2014), but different from what is observed in north Taiwan (Chiu et al., unpublished data) in which 98.5~100% identities was found with the strains reported in China.

Taken together, in this epidemiological study we showed the molecular prevalence rates of *B. burgdorferi*, *C. burnetii, D. immitis*, *Leptospira* spp. and *T. gondii* in 1440 stray and pet dogs from 2009 to 2011 in southern Taiwan. It is noteworthy that in stray dogs the prevalence rate of *C. burnetii* was over 6% and that of *D. immitis* was over 9%. Furthermore, the prevalence rate of *C. burnetii* in stray and pet dogs was associated with the season. Based on the findings revealed in the current study, the control and prevention of Q fever and heartworm disease in stray and pet dogs in southern Taiwan should be strengthened and routine surveillance of the zoonoses is necessary for the welfare of public health.

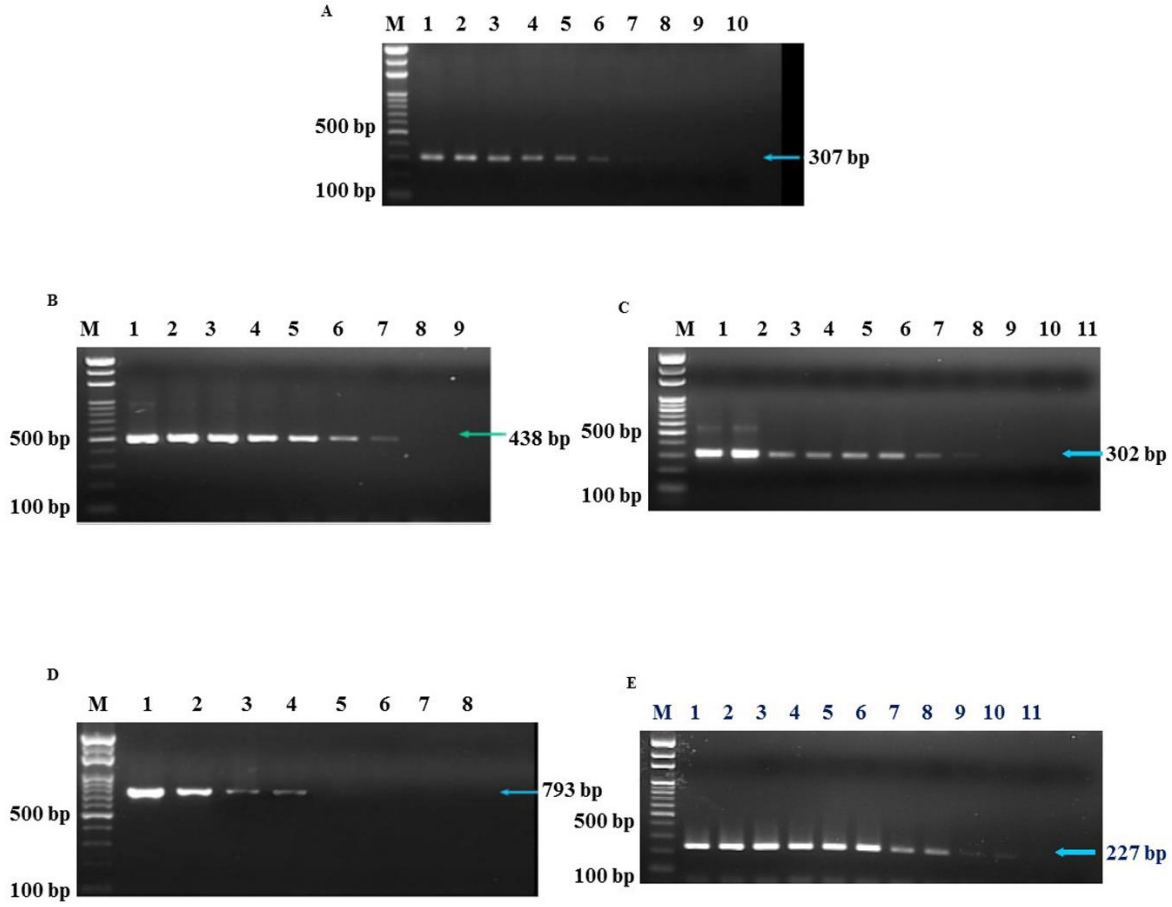

Figure 2 A: The sensitivity of *B. burgdorferi* PCR. B: The sensitivity of *C. burnetii* nested PCR. The sensitivity of PCR for (C) *D. immitis* and (D) *Leptospira* spp. E: The sensitivity of *T. gondii* semi-nested PCR. Lane M and Lane 1~10 in subfigure A and C were as follows: M: DNA ladder; 1: 1 µg/µL; 2: 100 ng/µL; 3: 10 ng/µL; 4: 1 ng/µL ; 5: 100 pg/µL ; 6: 10 pg/µL ; 7: 1 pg/µL ; 8: 100 fg/µL ; 9: 10 fg/µL ; 10: negative control. Lane M and Lane 1~10 in subfigure B, D and E were represented as below: M: DNA ladder; 1: 100 ng/µL; 2: 10 ng/µL; 3: 1 ng/µL; 4: 100 pg/µL ; 5: 10 pg/µL ; 6: 1 pg/µL ; 7: 100 fg/µL ; 8: 10 fg/µL ; 9: 1 fg/µL ; 10: negative control.

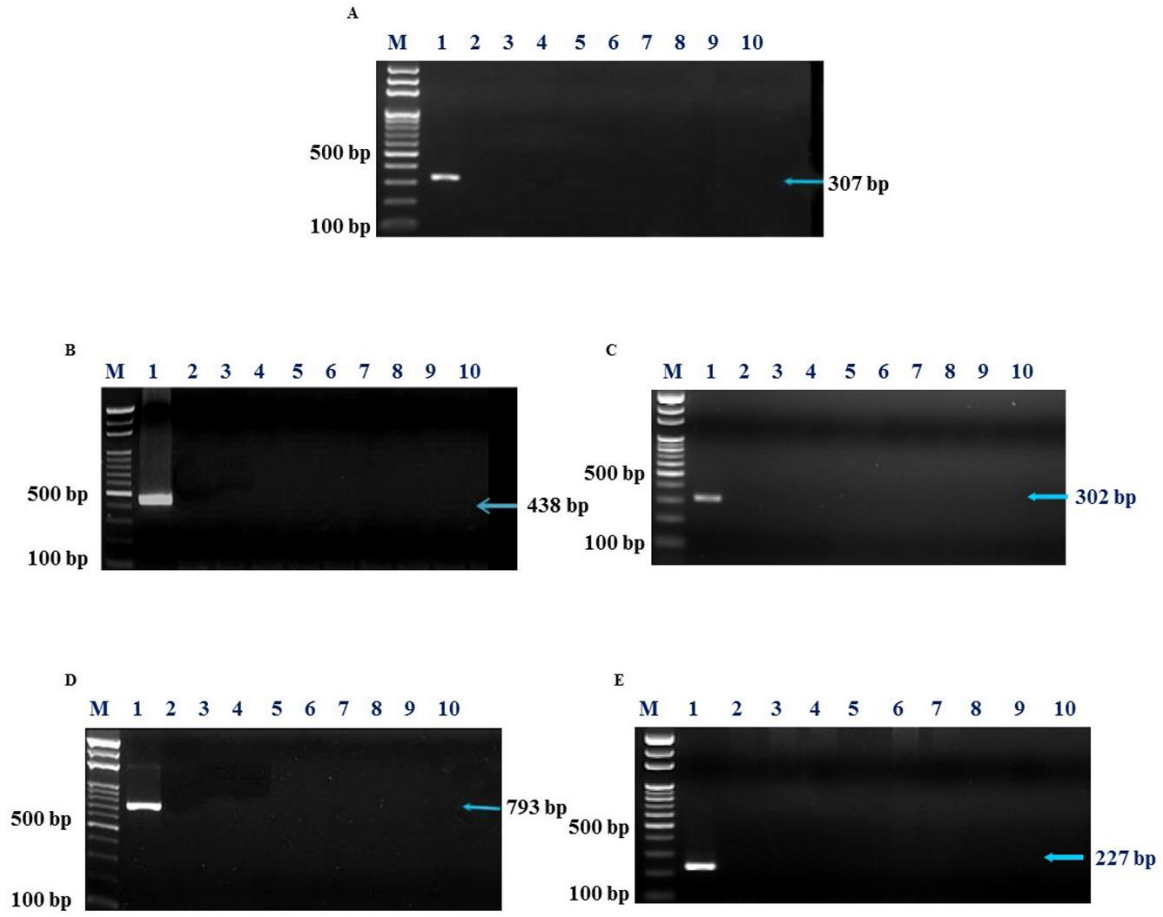

Figure 3 No cross-reaction was found for the PCR primers used in the detection of specific zoonotic pathogens (A) *B. burgdorferi,* (B) *C. burnetii,* (C) *D. immitis*, (D) *Leptospira* spp and (E) *T. gondii* in the specificity tests. The tested primers of target zoonotic disease were loaded onto Lane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

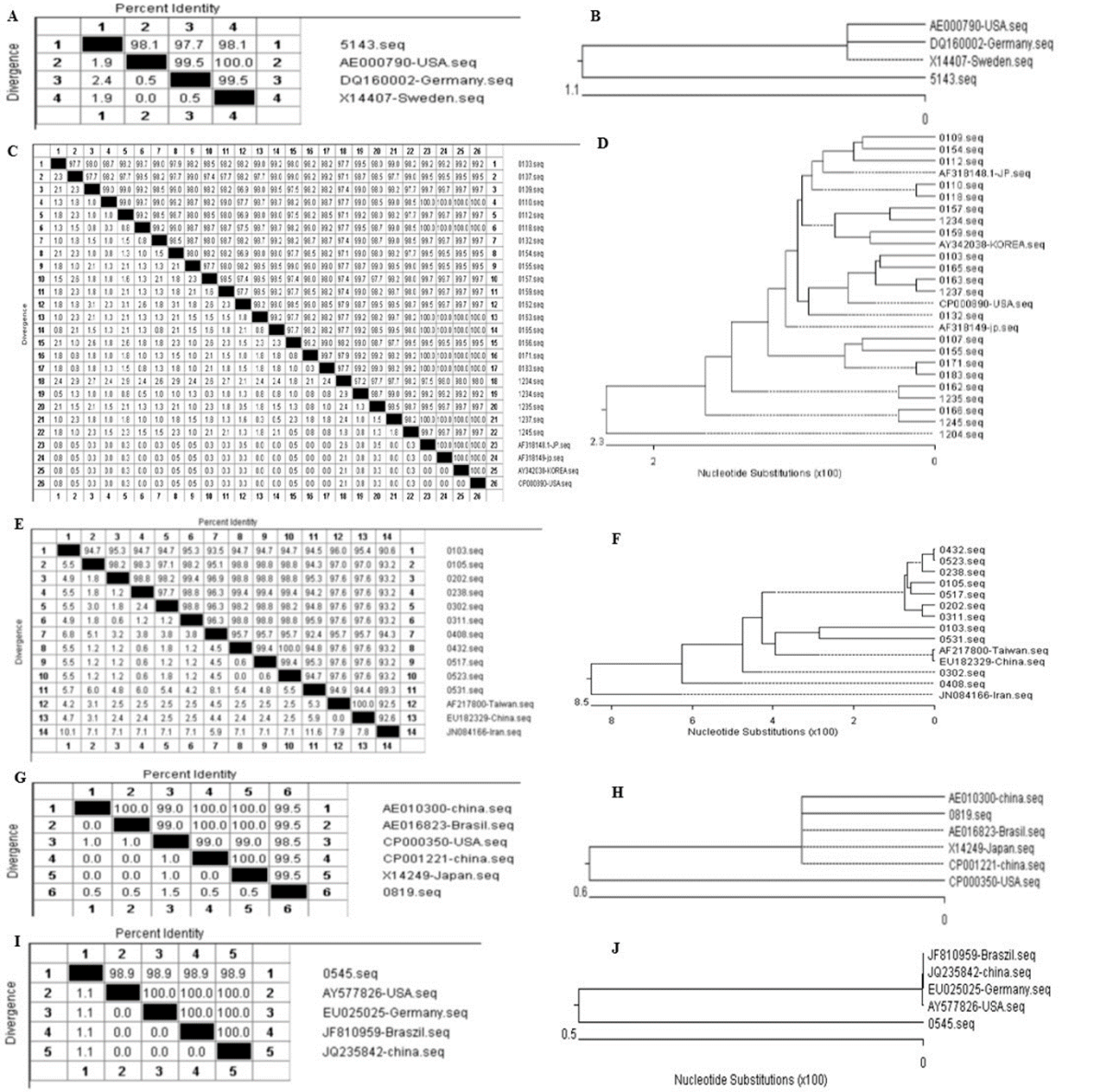

Figure 4 Analytic results of sequence pair distances exhibit that *B. burgdorferi* DNA sequences of the only positive sample have 97~98% identities in comparison with the strains of USA, Germany and Sweden strains (A and B). *C. burnetii* DNA sequences display 97~100% identities in comparison with the strains of Japan, Korea and USA (C and D). The sequences of *D. immitis* have 95~97% and 89~94% identities compared with the strains of Taiwan/China and Iran, respectively (E and F). DNA sequences of the merely *Leptospira* spp. positive specimen exhibit 99.5% identities compared with the strains of China, Brazil, USA and Japan (G and H) as sequences of the two *T. gondii* positive samples have 98.9% identities in comparison with the strains of USA, Germany, Brazil and China (I and J).

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บทคัดย่อ

ความชุกของเชื้อโรคติดต่อจากสัตว์สู่คนตรวจโดยเทคนิคทางอณูชีวโมเลกุลในสุนัขเลี้ยงและสุนัขจรจัดในตอนใต้ของประเทศไต้หวัน

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ความชุกของโรคติดต่อจากสัตว์สู่คนที่สำคัญที่ร่วมไปถึง โรคลัยม์ โรคไข้คิว โรคหนอนพยาธิหัวใจ โรคเล็ปโตสไปโรซิส และโรคท๊อกโซพลาสโมซิสในสุนัขเลี้ยงและสุนัขจรจัด ที่อยู่บริเวณตอนใต้ของไต้หวัน (เขตอำเภอเมืองเกาสง และเขตอำเภอผิงตงในบริเวณรอบนอก) ทดสอบโดยใช้วิธีเนสเต็ต เซมิเนสเต็ต หรือ วิธีโพลีเมอเรสเชนรีเอกชั่น ความสัมพันธ์ระหว่าง ความชุกและข้อมูลเชิงประชากรในภูมิภาคได้ถูกนำมาวิเคราะห์ครั้งนี้ ตัวอย่างเลือดได้สุ่มเก็บจากสุนัขเลี้ยงและสุนัขพเนจรในช่วงเดือน กันยายน ค. ศ. 2009 ถึง เดือนสิงหาคม ค. ศ. 2011 จำนวนดีเอ็นเอทั้งหมดที่สกัดได้จากตัวอย่างเลือดอยู่ในช่วงระหว่าง 0.8 ถึง 1.5 ไมโครกรัมและ ความไวของปฏิกิริยาของพีซีอาร์อยู่ที่ 10 เฟมโตกรัมและ 100 พิโคกรัม สำหรับพีซีอาร์ไพร์เมอร์พบว่าไม่เกิดปฏิกิริยาข้ามระหว่างกรดนิวคลีอิกและดีเอ็นเอที่สกัดมาจากจากเชื้อโรค *Ehrlichia canis Babesia canis และ Babesia gibsoni* โรคติดต่อจากสัตว์สู่คนทั้งห้าโรคและความชุกของโรคในสุนัขมีดังต่อไปนี้ได้แก่: *Borrelia burgdorferi* ร้อยละ 0.07 (1/1440), เชื้อแบคทีเรีย *Coxiella burnetii* ร้อยละ 4.79 % (69/1440), หนอนพยาธิ *Dirofilaria immitis* ร้อยละ 7.08 (102/1440), เชื้อแบคทีเรีย *Leptospira* spp. ร้อยละ 0.07 (1/1440) และ เชื้อปารสิต *Toxoplasma gondii* ร้อยละ 0.14 (2/1440) ซึ่งโรคที่เกิดจากเชื้อแบคทีเรีย *C. burnetii* และ โรคหนอนพยาธิ *D. immitis* มีความสำคัญสูงที่สุดในสุนัขบริเวณตอนใต้ของไต้หวันนอกจากนี้การวิเคราะห์ระหว่างความชุกของโรคและปัจจัยความเกี่ยวเนื่องกับจำนวนประชากรได้ให้ผลที่ว่า ความชุกของโรค *C. burnetii* ความสัมพันธ์กับฤดูกาล, อายุและ แหล่งที่อยู่ (ที่อยู่แบบเป็นที่ หรือ ที่อยู่แบบไม่เป็นที่) แต่ไม่สัมพันธ์กับ พันธุ์ และ เพศ ส่วนความชุกของโรค *D. immitis* มีความเกี่ยวข้องกับ อายุและแหล่งที่อยู่ แต่ไม่เกี่ยวข้องกับ พันธุ์และเพศ ในการศึกษาครั้งนี้ความสะอาดด้านสารณสุขกับการแพร่ระบาดของโรคไข้คิวและโรคหนอนพยาธิมีความเกี่ยวข้องกัน และจึงควรมีการเพิ่มการป้องกันด้านสาธารณสุขในตอนใต้ของไต้หวันรวมไปถึงพื้นที่ใกล้เคียงของประเทศรวมไปถึงประเทศอื่นๆ

คำสำคัญ: สุนัขในไต้หวัน โรคหนอนพยาธิหัวใจ โรคไข้คิว โรคเล็ปโตสไปโรซิส โรคลัยม์ โรคท๊อกโซพลาสโมซิส

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