Original Research Article

Molecular detection of *Coxiella burnetii* in stray dogs in Klang Valley, Malaysia

Abstract

Aims: *Coxiella burnetii* is a tick-borne pathogen that may cause clinical disease in a number of animals including dogs. *C. burnetii* has garnered attention as a zoonotic agent. With the increasing contact between dogs and humans through ownership of pets and the growing stray dog population, it is alarming how there is still a paucity of information concerning this pathogen in stray dogs in Klang Valley, Malaysia.

Methods: In order to determine the rate of infection of Q fever, blood and ticks were collected from stray dogs from animal shelters and dog pounds and screened for *htp*AB-associated repetitive element of *Coxiella burnetii* using nested PCR. Chi-square test was used to establish the association or differences among/between proportions of the categories (age, breed and sex) at the significance level of α=0.05.

Results: The method was initially applied on blood samples from which 62 (33%) of dogs were detected out of 188 total population then ticks as it is one of the transmission means but, non was found positive. However, none of the sampled ticks were found to be positive. Comparison based on age showed young dogs to have a high prevalence of 35.7% (95%CI, 0.07-0.64) than adults (32.8%, 95%CI, 0.26-0.40). According to breed, the local breed had a higher infection rate (33.2%, 95%CI, 0.26-0.40) than pedigree dogs (25.0%, 95%CI, -0.55-1.05) and based on sex, male dogs had a higher infection rate (34.3%, 95%CI, 0.21-0.42) than their female counterparts (31.3%, 95%CI, 0.25-0.44). However, there was no significant difference $P > 0.05$ between all the three factors observed for *Coxiella burnetii.*

Conclusion: This study found *Coxiella burnetii* present in Klang Valley, therefore necessitate action against stray dogs population to avoid shedding or contamination of the organism in environments and transmission to naive animals and humans.

Keywords: blood*, Coxiella burnetii,* nested PCR, Klang Valley, stray dogs and ticks*.*

Introduction

Coxiella burnetii is an obligate intracellular bacterium that parasitizes eukaryotic cells and is the aetiological agent of Q fever [1]. It is an infectious zoonotic agent that has several modes of transmission and has the potential to cause significant disease in both animals and humans. It is extremely contagious and even a few organisms can lead to infection in man [2]. *C. burnetii* is hardy and can survive in the external environment and resist disruptions from physical and chemical substances [3]. There are a wide range of reservoirs of *C. burnetii* that include many wild and domestic mammals, birds and ticks [3]. Rodents, birds and rabbits play an important role as reservoirs [2, 4]. However, cattle, sheep and goats are the common reservoirs that pass infection to humans. Cats and less frequently dogs have also been implicated in human infections [5]. Therefore, extra care is recommended during parturition because, reactivation of infection may occur and placentas become heavily infected during pregnancy [2]. Consequently, veterinarians, animal handlers and owners are expected to proceed with precausion when handling possible reservoirs of the agent.

In Malaysia, there are few reports of Q fever in humans [6, 7], ruminants and farm workers [8] and pet dogs [9]. Despite increase in pet ownership in Malaysia coupled with the transmission mode of the disease, it is alarming how there is still a paucity of scientific information regarding the agent of Q fever in Malaysia. Nested PCR appeared to be highly sensitive in *C. burnetii* diagnosis [10] and was suggested to be more superior than the

Immuno fluoresence Assay (IFA) serological test for the primary diagnosis of Q fever because greater sensitivity achieved and being less time consuming [11]. In light of the aforementioned research information, a cross sectional study was designed focusing on stray dogs due to their closeness with human/animal environments, constant contact with ticks and lack of documented history of diagnosis and treatment of Q fever infection.

Methods

Aim, design and area of the study

In order to detect *C. burnetii*, blood and ticks collected from stray dogs from animal shelters and dog pounds were screened for *htp*AB-associated repetitive element of *Coxiella burnetii* using nested PCR. Fisher exact test was used to establish the association or differences among/between proportions of the categories (age, breed and sex) at the significance level of α=0.05. A total of 188 blood and 438 ticks samples were collected from shelters and dog pounds from different locations in Klang Valley for detection of *C. burnetii*. Of 188 sampled population, 159 dogs were sampled from Dewan Bandaraya Kuala Lumpur (DBKL), dog pounds and 29 from Society for Prevention of Cruelty in Animals (SPCA), animal shelters (Ampang and Paws Subang Jaya . Of the 438 ticks collected; 212 were males,171 females and 55 nymphs. On average, 3-9 ticks were collected per dog and placed into properly labeled and sterile microcentrifuge tubes for further processing. Age, breed and sex of the dogs from which blood and ticks samples were collected were recorded.

This study was carried out from 2013- 2014 on stray dogs in and around Klang Valley. Klang Valley is an area in Peninsular Malaysia that comprises of Kuala Lumpur and its outer

reaches and neighbouring cities and towns in the state of Selangor. It is situated within 3^0 6' Northern latitude and 101^0 39' Eastern latitude. A heartland of industry and commerce covering important areas including Kuala Lumpur International Airport [12].

DNA extraction from blood and ticks

DNA from canine blood was extracted from 200 µl of whole blood using QIAamp animal Dneasy blood and tissue kit (QIAGEN® GmbH, Hilden, Germany). The protocol was followed based on manufacturer's instruction and the extracted canine DNA was stored at -20^0C until further analysis. Ticks were initially identified under stereomicroscope using Kohls taxonomic classification guideline [13] and Soulsby morphological keys [14]. After identification, the ticks were washed twice with 95% ethanol followed by a final washing step with sterile distilled water. They were then crushed with certified RNase, DNase and DNA free pestle and transferred to 1.5 ml microcentrifuge tubes mixed and some were cut into pieces with a scalpel blade on glass slides then transferred to 2 ml microcentrifuge tubes. DNA was then extracted from the ticks using blackPREP Tick DNA / RNA Kit, Analytik Jena AG (Life Science, Konrad-Zuse-Strasse, Jena, Germany) and QIAamp animal Dneasy blood and tissue kit (QIAGEN® GmbH, Hilden, Germany) using manufacturer's instruction and stored at -20^0C until further use.

Amplication of *Coxiella burnetii* DNA

Screening PCR for detection of *C. burnetii* in canine blood was then carried out. The first PCR amplification was performed using primers; IS111F1 and IS111R1 (Fournier and Raoult, 2003) which flank a 484-500 bp fragment of the *htp*AB-associated repetitive element

100 (Table 1). A nested PCR was carried out with primers IS111F2 and IS111R2 flanking a 260-
101 300 bp fragment. The amplification was performed in a reagent mixture of 4.0 µl of 5x Green
102 Go Taq buffer, 1.2 µl of 25mM Mgcl, 0.8 µl of 10mM dNTP, 1.0 µl of 20 pmol of both
103 forward and reverse primers, 0.3 µl of 5U/µl of Taq polymerase and 5.0 µl of DNA template
104 set up to 20 µl by adding 6.7 µl of sterile distilled water. The amplification protocol for both
105 screening and nested PCR were the same except that the first product was diluted ten times
106 and used as template for nested PCR. The positive control was *C. burnetii* Nine Mile strain
107 DNA isolated from cell culture obtained from Japan. The first PCR cycling conditions
108 consisted of an initial denaturation step at 95ºC for 2 min followed by 40 cycles at 95ºC for
109 30 s, 52ºC for 30 s, 72ºC for 1 min and a final extension step at 72ºC for 5 min. Cycling
110 conditions for the nested PCR was carried out using the same parameters except that the
111 number of cycles was reduced to 35 and an annealing temperature was set to 48ºC. The
112 resulting amplified products were electrophoresed on a 2.0% agarose gel at 100 V for 30 min,
113 stained for 30-40min in ethidium bromide and subsequently visualized under a UV
114 transilluminator.
115
116 In order to determine whether tick DNA was successfully extracted, PCR was conducted to
117 amplify the 28S rRNA gene of ticks. The primers (Inokuma *et al.*, 2003) (Table 1). PCR
118 amplification was performed in a final volume of 20 µl containing 5.0 µl of DNA template,
119 0.8 µl of 10 mM of each dNTP, 25 mM of Mgcl, 1µl of 20pmol of each primer, 0.3µl of
120 5U/µl of GoTaq DNA polymerase in 5X Green GoTaq reaction buffer (Promega Corporation,
121 Woods Hollow, Madison, WI, USA). The cycling conditions consisted of an initial
122 denaturation step at 95ºC for 2 min followed by 35 cycles at 95ºC for 30 s, 55ºC for 30 s,
123 72ºC for 30 s and a final extension step at 72ºC for 5 min. The resulting amplified products

were electrophoresed on a 2.0% agarose gel at 100 V for 30 min, stained for 30-40 min in ethidium bromide and subsequently visualized under a UV transilluminator.

PCR was performed with tick DNA using the same reaction volume, IS111F1 and IS111R1 primers for the first PCR and IS111F2 and IS111R2 set for nested PCR as described for screening of *C. burnetii* in canine blood

Table 1: Sequences and target regions of oligonucleotide primers used for detection of *Coxiella burnetii* in canine blood and tick DNA

Primer target regions	**Primer sequences 5'-3'**	**Amplicon sizes (bp)**	**References**
***C.burnetii* -*htp*AB; associated repetitive element**	IS111F1-TACTGGGTGTTGATATTGC IS111R1-CCGTTTCATCCGCGGTG IS111F2-GTAAAGTGATCTACACGA IS111R2-TTAACAGCGCTTGAACGT	485-500 260-300	[15]
Ticks -28S rRNA	28SF-GACTCTAGTCTGACTCTGTG 28SR-GCCACAAGCCAGTTATCCC	484-500	[16]

For sequencing, PCR products prepared in total volumes of 75 µl were initially confirmed for successful amplification. After the verification, the products were then purified using Wizard® SV Gel and PCR clean-up system (Promega, USA) according to the manufacturer's instructions before sending for standard sequencing to NHKBioscience (Korean). Sequences were aligned and blasted to compare with other sequences stored in the GenBank.

Data analysis

The data collected during this study were recorded and stored in Microsoft Excel for windows. Frequency tables were used to calculate the prevalence of *C. burnetii* based on age, breed and sex of the stray dogs at the estimated confidence interval of 95%. The data were then subjected to statistical analysis using statistical package IBM SPSS statistics (version 20.0, SPSS, Inc., Chicago, IL, USA). Fisher exact test was used to establish the association or differences among/between proportions of the categories at a significance level of α=0.05.

The sampled population was categorized according to age; young and adult, according to breed; local and pedigree and according to sex; male and female. Stratification of the dogs based on district or region was not possible because some of the information on these variables were not clearly recorded or could not be obtained.

Results

Descriptive statistics and prevalence of *Coxiella burnetii* in stray dogs

Of the total 188 dogs sampled, 105 were males while 83 were females (Table 2). In terms of sources, a total 159 dogs were sampled from Dewan Bandaraya Kuala Lumpur (DBKL) dog pound and 29 dogs were sampled from Animal shelters (SPCA, Ampang and Paws, Subang Jaya). *C. burnetii* was detected in 62 / 188 (33.0%) of the dogs sampled with GenBank, accession number KU215930. Comparison based on age showed young dogs had a relatively higher prevalence of 35.7% than adults (32.8%). According to breed, the local

164 breed had a relatively higher infection rate (33.2%) and based on sex, male dogs had a
165 relatively higher infection rate (34.3%) than their female counterparts (31.3%). However, it
166 should be noted that differences between all the three factors age, breed and sex of dogs for
167 the presence of *C. burnetii* were not statistically significant $P > 0.05$ (Table 3).

168
169
170 **Table 2. Proportion of the total dogs sampled and that of tick infested dogs that had**
171 ***Coxiella burnetii* infection according to age, breed and sex**
172

Variables	Category	Sample Proportion n=188 (%)	Tick positive dogs n= 141 (%)	Coxiella positive tick infested dogs. n=141(%)
Age	Young	14 (7.4)	10 (7.1)	1 (0.1)
	Adult	174 (92.6)	131 (92.9)	42 (29.9)
Breed	Local	184 (97.9)	139 (98.6)	43 (30.0)
	Pedigree/mixed	4 (2.1)	1 (1.4)	0 (0.0)
Sex	Male	105 (55.9)	82 (58.2)	26 (18.0)
	Female	83 (44.1)	59 (41.8)	17 (12.0)

173
174
175
176 **Table 3: Prevalence of *Coxiella burnetii* amongst the total dog population sampled**
177 **according to age, breed and sex**
178

Variables	Category	Prevalence (%)	Standard dev. ±	95% CI	P-value
Age	Young	35.7	0.50	0.07- 0.64	0.777
	Adult	32.8	0.47	0.26- 0.40	-
Sex	Male	34.3	0.47	0.21-0.42	0.755
	Female	31.3	0.48	0.25- 0.44	-

Breed	Local	33.2	0.47	0.26-0.40	1.000
	Pedigree	25.0	0.50	-0.55-1.05	-

Discussion

This study to the best of our knowledge is the first to report *C. burnetii* in canine blood in Malaysia with a prevalence of 33.0% (62/188) in stray dogs in Klang Valley. There are previous reports of Q fever in humans [6, 17] as well as a recent report in ruminants and farm workers [8] in Malaysia, but no reports thus far on stray dogs. A most recent report detected a Coxiella-like bacteria (89%) in ticks recovered from wild boars, single porcupine and goats (Khoo et al., 2016). Another high prevalence (59.0%) of the pathogens in ticks was also reported from pet dogs visiting University Veterinary Hospital (UVH), University Putra Malaysia (UPM) [9]. This poses a risk of sharing of infection between dogs and owners [9]. The implication of our findings is that stray dogs may play a greater role in dissemination of the pathogen and may serve as a source of infection to humans and other naive dogs. The recent report of the pathogen detection in pets [9], together with the present study on stray dogs reiterates the existence and persistence of *C. burnetii* in dogs in Klang Valley, Malaysia.

Since the data used in this study is limited, information on prevalence of *C. burnetii* in the study area in terms of the spatial and temporal distribution of the pathogen cannot be explored as some variables were too scanty and most samples were obtained from Dewan Bandaraya Kuala Lumpur (DBKL), thus the limitations of this study.

The primers used for amplification of the *htp*AB-associated repetitive element was reported sensitive and specific for detection of *Coxiella* DNA [15]. It should be noted that the first PCR with the outer primers; IS111F1 and IS111R1 could not amplify the *C. burnetii* DNA in blood, however when a nested PCR was carried out with the inner primers, IS111F2 and IS111R2, positive results were obtained from the canine blood. Despite the reported sensitivity of the primers, nested PCR on all the tick DNA was negative for *C. burnetii* and screening test was carried after running the success of tick DNA extraction. This nested approach is adequate for detection because it is highly conserved with a unique component common to a variety of *C. burnetii* strains, thus able to amplify a small bacterial load in blood and milk [9].

Adult 57/62 (91.9%) stray dogs in Klang Valley had a relatively higher infection ratio compared to the younger dogs 5/62 (8.1%) as shown in Table 2. This may result from increase in the risk of abortion, still birth or death of puppies during pregnancy or after whelping related to *C. burnetii* infection in some bitches [5]. However, adult dogs were over represented and the difference was not statistically significant $P > 0.05$. Comparison within breed showed the local breed had a relatively higher infection rate (33.2%) than pedigree (25%) even though the difference was not statistically significant. The greater part of the local canine population in the country consists of stray dogs and owned [18] local breeds, thus explaining the results. According to sex, male dogs appeared to have a high prevalence (34.3%) compared to the females (31.3%), but the difference was not statistically significant (Table 3). As the difference between all variables was not statistically significant, it can be assumed that age, sex and breed do not contribute or affect *C. burnetii* infection rates.

Conclusion

In conclusion *C. burnetii,* the aetiological agent of Q-fever was found to be present in blood samples obtained from stray dogs in Klang Valley, Malaysia. Ticks collected from dogs were all negative suggestive that ticks may not necessarily be the source of infection of *C. burnetii* to the stray dogs sampled in the study. Rather, the dogs may likely be the source of infection to ticks during blood meal up-take. The relatively high prevalence rate of *C. burnetii* in dogs sampled is worrisome in light of the public health risk and therefore warrants further investigation and control as most of the infected dogs were sampled from Dewan Bandaraya Kuala Lumpur and are mainly dogs that are caught roaming around the city.

Abbreviations

IFA= Immuno Fluorescence Assay

UPM= Universiti Putra Malaysia

UVH= Universiti Veterinary Hospital

DBKL= Dewan Bandaraya Kuala Lumpur

SPCA= Society for Prevention of Cruelty in Animals

Declarations

Ethics approval : This research was conducted following approval from the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM), which was an adopted version of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. (The research grant No. 01-01-09-0662RU. Informed

consent and approval was obtained from the International care and use committee, UPM (approval code RD74/2013)).

Consent of publication: Not applicable.

Availability of data and material: GenBank, accession number is KU215930.

Competing interests: All authors approved and declared that they have no competing interest.

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