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Pathogenic and endosymbiont apicomplexans in *Ctenocephalides felis* (Siphonaptera: Pulicidae) from cats in Jerusalem, Israel

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ABSTRACT

This study was conducted to determine the prevalence of pathogenic and endosymbiont apicomplexans in the cat flea, *Ctenocephalides felis* (Bouché) infesting 185 stray cats in Jerusalem, Israel using PCR assay and sequencing approach.

Two pathogens, *Hepatozoon felis* and *Babesia vogeli* and an endosymbiont *Steinina ctenocephali* were detected in 1.9%, 0.2% and 5.8% of 685 *C. felis* evaluated respectively. There was a significant association ($p < 0.05$) between the prevalence of *H. felis* and the sex of cats hosting the fleas as well as the season of sampling but not for age or health status of the cats or sex of the fleas tested. Prevalence of *S. ctenocephali* was significantly ($p < 0.001$) associated with season, being higher in the warm season. This report represents the first molecular detection of *S. ctenocephali* in *C. felis*. Further studies to determine the potential role of *C. felis* in the epidemiology of *H. felis* and *B. vogeli* are warranted.

1. Introduction

The cat flea *Ctenocephalides felis* (Bouché 1835) is an important ectoparasite infesting animals and humans. This species has a cosmopolitan distribution and is a vector of several human and animal pathogens such as *Rickettsia felis, Bartonella henselae, Bartonella clarridgeiae* and *Acanthocheilonema reconditum* [1–5]. They also serve as intermediate host of the cestode *Dipylidium caninum* [6–8]. In addition to carrying pathogens, almost 20 different endosymbionts have been found to be associated with the cat flea, including bacteria, protozoa and helminths [9–12]. The role of cat fleas in carrying and potentially transmitting apicomplexan hemoparasites has not been studied in detail and deserves further attention.

The purpose of this study was to explore and identify the presence of DNA of piroplasms, *Hepatozoon* spp. and endosymbionts in *C. felis* collected from stray cats in Jerusalem, Israel by PCR and to establish relationships between the presence of these organisms and other factors including the sex and age of the cat host, sex of fleas and season of the year.

2. Materials and methods

2.1. Flea collection

Fleas infesting stray cats living in residential areas of Jerusalem, Israel, were collected from March 2011 to February 2012. Cats were examined for fleas by combing their fur for a period of 5 min [13] using a fine-toothed comb (Lochdan™; Regev Stainless Steel Industries Ltd, Ma'alot, Israel). The fleas from each cat were placed in labeled vials containing 70% ethyl alcohol and transported to the laboratory at the Hebrew University where they were identified according to species and sex using standard taxonomic keys [14]. Sorted fleas were kept in coded vials and stored at −20 °C until they were tested.

2.2. DNA extraction

DNA extraction was done for individual fleas after surface washing twice in sterile phosphate buffered saline (PBS). Each flea was then manually crushed with a sterile plastic pestle inside a micro tube containing 50uL PBS. DNA extraction from individual flea was performed

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using the Illustra Tissue Mini Spin kit, (GE Healthcare, Little Chalfont, UK) according to manufacturer's instructions. One negative PBS control was performed in parallel with the extraction of every set of 24 samples. The quality and quantity of DNA were assessed using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

2.3. Screening for piroplasms by conventional PCR assays

An initial molecular screening of all the samples was done using the general piroplasmid primers, Piroplasmid F (5′-CCA GCA GCC GCG GTA ATT C-3′) and Piroplasmid R (5′-CTT TCG CAG TAG TTY GTC TTT AAC AAA TCT-3′) targeting a 360 bp of the 18S ribosomal RNA (18S rRNA) gene of the piroplasmida [15]. The PCR program consisted of an initial denaturation at 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, annealing at 64 °C for 30 s, extension at 72 °C for 30 s with a final extension at 72 °C for 5 mins.

2.4. Conventional PCR assays for Babesia spp. and Hepatozoon spp

All samples that were positive in the initial screening reactions were further subjected to *Babesia* spp. and *Hepatozoon* spp. PCR reactions using primers Piro A and B, and Hep18S F and R respectively as previously described [16–18]. Positive controls of DNA extracted from the blood of naturally infected dogs with *B. vogeli* (Bab 36799) and *Hepatozoon canis* (HEP 7423), as well as negative DNA controls from colony-bred dogs negative by PCR for vector borne pathogens were run with each corresponding PCR reaction.

Non-template control (NTC) reactions were run using the same procedures and reagents described above but without DNA added to the PCR reaction to rule out contaminations. PCR was performed using the Syntezza PCR-Ready High Specificity kit (Syntezza Bioscience, Israel). Amplification was performed using a programmable conventional thermocycler (Biometra, Goettingen, Germany).

PCR products were electrophoresed on 1.5% agarose gels stained with ethidium bromide and evaluated under UV light for the size of amplified fragments by comparison to a 100 bp DNA molecular weight marker.

2.5. Sequencing

Positive PCR products were purified using a PCR purification kit (Exo-SAP, NEB; New England Biolabs, Inc., Ipswich, MA). Sequencing was performed in both directions (forward and reverse) at the Center for Genomic Technologies, Hebrew University of Jerusalem, Israel.

DNA sequences obtained were evaluated by the MEGA 6 software [19] and compared for similarity to sequences deposited in GenBank, using the BLAST program hosted by the National Center for Biotechnology Information (NCBI), National Institutes of Health, Bethesda, MD (www.ncbi.nlm.nih.gov/BLAST).

2.6. Statistical analyses

Data generated in the study were analyzed by the $\chi 2$ test using the statistical software package R [20]. P values < 0.05 were considered significant.

3. Results

3.1. Cats and cat fleas

Three hundred and twenty-eight stray cats living in residential areas of Jerusalem, Israel, were screened for the presence of fleas. The sampled cats included 155 (47.3%) males and 173 (52.7%) females. Young (kittens) and juvenile cats ≤12 months old constituted 52.7% of the sampled population. The highest number of cats, 51 (15.5%) were sampled in March (Fig. 1), followed by 40 (12.2%) and 36 (11%) in June and August 2011, respectively, while the least number of cats, 7 (2.1%), were sampled in October 2011.

One hundred and eighty-five (56.4%) of the cats were infested by fleas and 143 (43.6%) were free from flea infestation at time of sampling. The majority (n = 162; 87.6%) of the flea- infested cats appeared apparently healthy while a small number (n = 23; 12.4%) manifested one or more signs of illness (Table 1). The commonly encountered clinical signs were conjunctivitis (7), emaciation (7), alopecia (6) and stomatitis/gingivitis (6). Buccal ulcers were seen in three cats while diarrhea, abscesses, corneal ulcer, pyometra and rhinitis were recorded in two cats, each.

All the 685 fleas collected from the cats were identified as *C. felis* (Bouché 1835). Flea infestation ranged from 1 to 17 fleas per cat with a mean of 3.7 fleas/cat. The male to female proportion of fleas collected was 1: 4.

The highest number of fleas, 259 (37.8%) were collected in summer (June-August), followed by 245 (35.8%) in autumn (September-November), 102 (14.9%) in spring (March −May) and 79 (11.5%) in winter (December-February).

The flea index defined as the mean number of fleas per infected host [21] was lowest, 1.2 in the cold rainy winter months but increased gradually during the hot dry summer months reaching a peak, 6.5 in autumn (Fig. 1). A maximum of five fleas per cat host were randomly selected for this study, thus, a total of 467 [male = 91 (19.5%), female = 376 (80.5%)] fleas were screened for pathogen carriage by PCR.

3.2. Prevalence of piroplasm, Hepatozoon spp. and gregarines in C. felis by conventional PCR

DNA of *H. felis* and *B. vogeli* were detected in 1.9% (9 out of 467) and 0.2% (1 out of 467) *C. felis* fleas, respectively, using the general Piroplasmid screening PCR primers and DNA sequencing and confirmed by PCR using the Piro A and B, and Hep18S F and R primers followed by sequencing. All the fleas positive for *H. felis* were collected from apparently healthy cats. Male cats (48%) hosted significantly ($p = .027$) more *H. felis* positive fleas than females (52%). However, no significant relationship ($p > 0.05$) was found between *H. felis* positivity and the cat age group (< 12 months or adult), season of flea sampling or flea sex (Table 1). The only flea positive for *B. vogeli* was removed from an apparently healthy 4 year old female cat (Table 2).

Steinina ctenocephali DNA was detected by the Piroplasmid primers in 5.8% (27/467) of the *C. felis* fleas sampled. The prevalence of *S. ctenocephali* in *C. felis* was significantly associated with the season of flea sampling ($p = .001$) with more positive fleas in the summer and autumn. The health status, age and sex of the cats did not influence ($p > 0.05$) the detection of *S. ctenocephali* DNA in the hosted fleas (Table 1).

Nucleotide sequences for the 18S rRNA gene of *S. ctenocephali, H. felis* and *B. vogeli* detected in this study have been deposited in GenBank under the accession nos. MG722712–MG722714, MG722715–MG722718 and MG722719, respectively.

None of the *C. felis* fleas that tested positive for *H. felis* DNA in this study was positive for endosymbionts or pathogenic piroplasms. Similarly, the gregarine positive fleas were all negative for both *H. felis* and *B. vogeli* DNA.

4. Discussion

Apicomplexans are a diverse group of protozoa which infect vertebrates and invertebrates and include parasites of medical and veterinary importance such as the piroplasms [22,23]. We detected DNA of three different apicomplexan species, belonging to different genera, in the cat fleas included in this study.

Hepatozoon felis is a common blood parasite of the domestic cat which has been reported in several parts of the world. It is