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Research Paper

POLYMERASE CHAIN REACTION DETECTION OF EHRLICHIA CANIS THE CAUSATIVE AGENT OF CANINE MONOCYTIC EHRLICHIOSIS IN KENYA

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Ehrlichieae are important, emerging tick-borne gram negative, obligate intracellular bacteria from the *Anaplasmataceae* family that infect canines and are also zoonotic. There is limited information in scientific reports available indicating the species of ehrlichia causing disease, and its prevalence in Kenya. Dogs treated at the Small Animal Clinic, Faculty of Veterinary Medicine, University of Nairobi were recruited into the study after being diagnosed with ehrlichiosis on the basis of presenting clinical signs suggestive of infection and having inclusion bodies in monocytes. Polymerase Chain Reaction using genus and species-specific primers was done on DNA extracted from blood collected from the dogs. The PCR revealed 58.9% (113/192) positive for the genera Anaplasma and Ehrlichia using ECC and ECB primers. From these 42.5% (48/113) were positive for species-specific primers for Ehrlichia canis HE3 and ECANS5. The most common clinical signs in the dogs infected by *E.canis* were congestion of mucous membranes (53%), inappetance (49%), panting (36%), loose hair (31%), lethargy (29%), vomiting (28%), wasting (27%) and ocular discharges 27%. Increase in the mean concentration of alanine aminotransferase 59.04 U/L as compared to the reference range of 0-40 U/L. The clinical manifestations and anaemia observed in this study, though not specific, can be considered to be the case definition for *Ehrlichia canis* infection in dogs in this region. To the authors' knowledge, this is the first report on molecular identification of *Ehrlichia canis* in Kenya.

Keywords: Dogs. Ehrlichieae. PCR. Kenya

INTRODUCTION

Ehrlichieae are important, tick-borne gram negative, obligate intracellular bacteria from the Anaplasmataceae family that infect canines and are also zoonotic (Greig *et al*. 1996; Woldehiwet, 2006; and Stuen, 2007). In the host cells, monocytes and granulocytes, the bacteria reside in inclusion bodies, the morulae. Dogs maybe

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infected by several Anaplasmataceae agents (Dawnson *et al*. 1996; Breitschwerdt *et al*., 1998; Goldman *et al*., 1998; Murphy *et al*., 1998; Kordick *et al*., 1999; and Unver *et al*., 2001). *Ehrlichia canis* infects monocytes, and causes canine monocytic ehrlichiosis.

Polymerase Chain Reaction (PCR) is a method widely used for the diagnosis of infectious diseases (Anderson *et al.,* 1991) and has been widely used in the laboratory diagnosis of canine monocytic ehrlichiosis, especially during the acute phase of the disease before antibodies are detectable (Wen *et al*., 1997). Alexandre *et al*. (2009) reported detection by a nested PCR of *Ehrlichia canis* in dogs that were seronegative by indirect IFA test. In this study the method is used to identify the ehrlichia organisms present in Kenya. Seroprevalence studies have suggested the presence of *Ehrlichia canis* or related species infecting dogs throughout Africa (Brouqui *et al*., 1991; Bostros *et al*., 1995; Pretorius and Kelly 1998; and Kelly *et al*., 2004) and indeed the infection has been confirmed to be present by serology and culture. Studies by Ndip *et al*. (2005) in Cameroonian canines reported the presence of *Ehrlichia canis* by PCR. In Kenya confirmatory diagnosis of ehrlichial infection has been done by indirect fluorescent antibody test, cell culture and serology (Kaminjolo *et al*., 1976; Price, 1980; and Alexander *et al*., 1994). Though, these tests have mainly targeted *Ehrlichia canis* molecular typing of the etiological agent for ehrlichial infections in dogs in Kenya has not been done. No scientific reports are available describing by molecular identification of the species of Ehrlichia causing infection in Kenya.

MATERIALS AND METHODS

Sample Collection

Giemsa stained blood smears prepared with blood from ear tips were examined for ehrlichia parasites. Blood for hematology was collected, after aseptic preparation, by venapuncture in plastic tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) and hematological assays performed on the same day using a semi-automatic impedance cell counter (Compteur Analyseur d'Hematologie MS4, Melet Schoesing Laboratoires, 9 Chaussee Jules Cesar 95520 OSNY France). The following parameters were determined: packed cell volume, hemoglobin concentration, total erythrocyte count, mean corpuscular hemoglobin concentration, total and differential white blood count and total platelet count.

Blood was collected by jugular venapuncture in plain plastic tubes and serum samples separated by centrifugation two hours after the blood was drawn. Serum was analysed for albumins, alkaline phosphatase, alanine amonotransferase, blood urea, protein and creatinine.

DNA Extraction

EDTA anticoagulated blood was collected for PCR and stored at -20 ^{o}C until used for DNA extraction. DNA was extracted from 200 µl of each blood specimen. A commercially available QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols. In brief, 200 µl of the blood samples were mixed with 20 µl of protease and 200 µl of lysis buffer and incubated at 56 ^{o}C for 10 minutes. Then, 200 µl of ethanol were added to the sample and the mixture applied to a QIAamp Mini spin column and centrifuged at 8000 rpm for 1 minute. Two washing steps were then done with buffers 1 and 2. Finally, 150 µl of elution buffer were added to the column, and the DNA eluted in 1.5 ml

Eppendorf tubes after centrifugation and stored at -20 °C until use.

Polymerase Chain Reaction

The extracted DNA from each sample was used in PCR amplifications with primers that amplify a portion of the 16S rRNA gene. Primer ECC (5'-AGAACGAACGCTGGCGG- CAAGCC-3') and ECB (5'-CGTATTACCGCGGCTGCTGGC-3') which amplify all Ehrlichia species (Dawson *et al*., 1996; and Murphy *et al*., 1998) were used. These primers amplify a 478 base-pair fragment of the Ehrlichia 16S rRNA (Dawson *et al*., 1994)

Reaction (25 µl) contained 7.5 µl of template DNA in 12.5 µl Premix containing AmpliTaq polymerase (Qiagen), 1.25 µl of each primer and 2.5 µl distilled water. The thermocycle profile was as described below:

Initial denaturation at 94 °C for 5 minutes, 40 cycles of denaturation at 95 °C for 1 minute, annealing at 60 °C for 1 minute, and extension at 72 °C for 1 minute and the final extension was performed at 72 °C for 5 minutes.

The PCR reactions that resulted in positive amplification of a segment of the *Ehrlichia* species 16S rRNA were taken through a second PCR amplification using the species specific primers for *Ehrlichia canis* HE3 (5'-TATAGGTACCGTCA TTATCTTCCCTAT-3') and ECANS5 (5'-CAATTA TTTATAGCCTCTGGCTATAGGA-3') and the template was the PCR product from the first reaction. The reaction (25 µl) contained 1.0 µl of template DNA in 12.5 µl Premix containing AmpliTaq polymerase (Qiagen), 2.5 µl of each primer and 6.5 µl distilled water. The thermocycle profile was as described below:

Initial denaturation at 94 °C for 10 minutes, 40 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute, and extension at 72 °C for 1 minute and the final extension was performed at 72 °C for 4 minutes.

All PCR products were electrophoresed through 1.3% agarose gels in Tris-boric acid-EDTA buffer, and the DNA fragments visualized by ethiduim bromide staining under UV fluorescence.

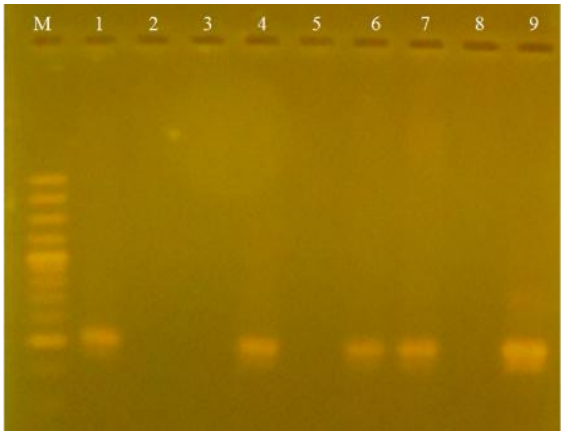
Statistical Analyse

The results of the clinical examinations were coded as "I" (meaning the clinical observation was present) and "0" (meaning the clinical observation was no present). All the data were stored in Microsoft Office Excel 2007 (Microsoft Corporation, 2007). The data were imported into SPSS and GENSTAT for Windows Edition 2 (VSN International). Anova was used to check for difference in ehrlichias

RESULTS

Out of the 192 samples analysed by PCR 113 (58.9%) were positive for the genera Anaplasma and Ehrlichia. From these samples, a 478 base-

Figure 1: PCR Products (Lane 1, 4, 6, 7, and 9) Amplified from DNA Purified from Dog EDTA-Blood Samples of Dogs Diagnosed with Ehrlichiosis Using Primers ECC and ECB that Amplify All the Anaplasma and Ehrlichia genera



pair product was amplified using ECC and ECB primers (Figure 1). Forty eight (48) samples representing 42.5% of the positive for the genera yielded a 389 base pair product for the primers HE3 and ECANS5 that are species specific for *Ehrlichia canis* (Figures 2 and 3).

The most common clinical signs in the dogs infected by *E.canis* were congestion of mucous membranes (53%), inappetance (49%), panting (36%), loose hair (31%), lethargy (29%), vomiting (28%), wasting (27%) and ocular discharges 27% (Table 1 and Figure 5). Only a small percentage

Figure 2: Nested PCR Products (Lane 1 and 2) Amplified from DNA Purified from Dog EDTA-Blood Samples of Dogs Diagnosed with Ehrlichiosis Using Primers that Amplify Ehrlichia canis

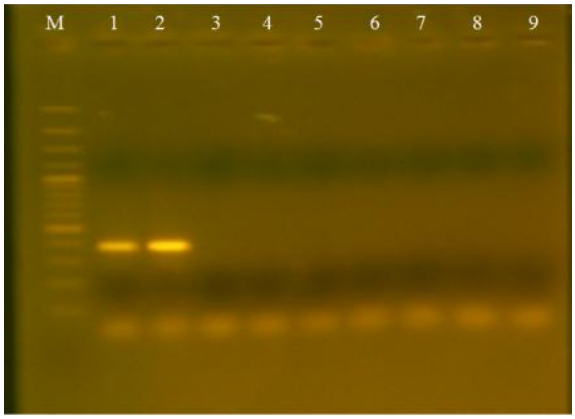


Figure 3: Nested PCR Products (Lane 6, 7, 8 and 9) Amplified from DNA Purified from Dog EDTA-Blood Samples of Dogs Diagnosed with Ehrlichiosis Using Primers that Amplify Ehrlichia canis

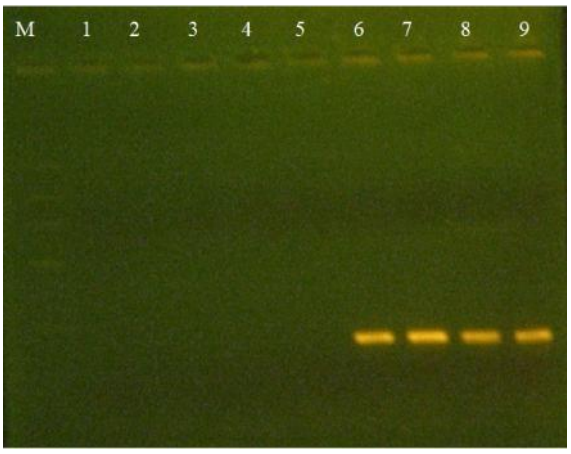


Table 1: Clinical Signs Observed in Dogs Confirmed by PCR to be Infected with Ehrlichia canis

Clinical Signs Upon Presentation of the Erhlichia Positive Dogs Clinical Sign	**Percentage**
Congestion	53 (18/34)
Inappetance	49 (16/33)
Panting	36 (10/28)
Loose hair	31 (10/32)
Lethargy	29 (9/31)
Vomiting	28 (7/25)
Wasting	27 (9/33)
Ocular discharge	27 (8/24)
Diarrhoea	25 (6/24)
Pallor	18 (5/28)
Ticks	8 (2/26)
Fleas	8 (2/26)

Figure 4: Nested PCR Products (Lane 1, 2, 3, 4, 7 and 8) Amplified from DNA Purified from Dog EDTA-Blood Samples of Dogs Diagnosed with Ehrlichiosis Using Primers that Amplify Ehrlichia canis

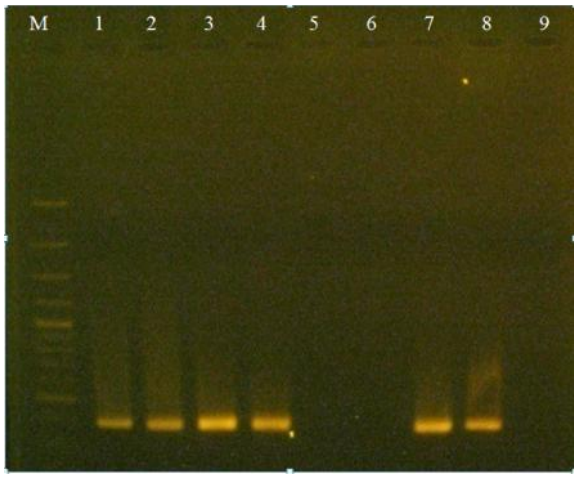


Table 2: Values of Haematological Parameters of Naturally Infected Dogs with E.canis

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
WBC (cells/µL)	13392.38	5127.76	5000	28520	6000-17000
Lymphocytes (cells/µL)	3106.45	1804.49	770	7682.01	1000-4800
Monocytes	414.7	181.94	80	689.4	150-1350
Granulocytes (cells/µL)	10108.95	4551.25	4150	24698.32	3000-11000
RBC ($X10^6$cells/µL)	5.57	1.93	0.58	9.5	5.5-8.5
Hematocrit (%)	38.1	13.06	4.2	63.1	37-55
Hemoglobin (g/dL)	13.06	3.17	4.6	19.4	12.0-18.0
Thrombocytes (/µL)	182895	116888	30000	421000	200000-500000

Table 3: Values of Blood Biochemistry Parameters of Naturally Infected Dogs with E.canis

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
Blood Urea Nitrogen (mg/dL)	20.38	14.96	9	59.7	10.0-20.0
Creatinine (mg/dL)	0.99	0.35	0.4	1.8	0.6-1.2
Total Protein ((gm/dL)	7.64	2.33	4.7	12.9	5.0-8.0
Albumin (gm/dL)	3.11	1.86	1.3	8.6	2.8-4
Globulin (gm/dL)	4.37	1.48	2.3	7.2	2,7-4.4
Albumin/Globulin Ratio	1.06	1	0.3	4	0.59-1.11
Calcium (mg/dL)	9.43	2.46	5.5	15.4	8.8-10.3
Phosphorus ((mg/dL)	5.49	3.84	1.7	14.5	2.5-5
Alkaline Phosphatase (U/L)	109.93	107.03	9	388	30-150
Alanine Aminotransferase (U/L)	59.04	59.36	0	173.5	0-40

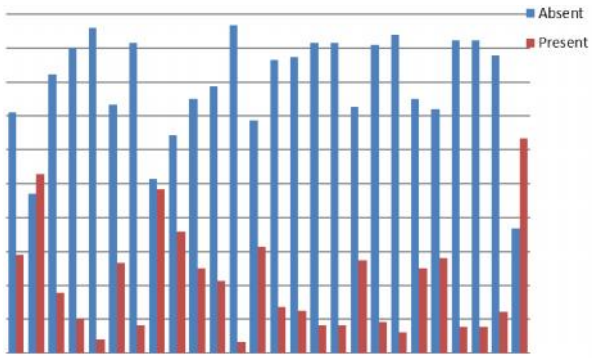
of dogs had external parasites found on them. There were fleas in 8% and ticks 8% (Table 1).

The red blood cells, haematocrit and haemoglobin values were all within the reference range but on the lower side. The thrombocytes were 182,895 cells per µL which is below the reference range (Table 2). Most biochemical values were found to fall within the reference values. Slight increases were noted for blood urea nitrogen and phosphorus. Slight increase above the reference values were noted for blood urea nitrogen 20.38 mg/dL and phosphorus 5.49 mg/dLas compared to reference ranges of 10.0-20.0 mg/dL and 2.5-5.0 mg/dL respectively. However, there was a large increase in the value of alanine aminotransferase 59.04 U/L as compared to the reference range of 0-40 U/L (Table 3).

DISCUSSION

As the presenting clinical signs in ehrlichial infections tend to be non-specific, a diagnosis of canine monocytic ehrlichiosis is generally made when a dog in an endemic area presents with clinical signs and haematological findings suspect

Figure 5: A Bar Graph Showing the Clinical Signs Observed in Dogs Diagnosed with E.canis



of the infection. Moreover, it is difficult to reach a definitive diagnosis based only on clinical and haematological abnormalities as natural infections may present with a variety of clinical signs that vary between different geographical regions (Asgarali *et al*., 2012). It is therefore, necessary for confirmation that indeed the aetiological agent is present in the geographical region.

Molecular techniques like Polymerase Chain Reaction (PCR) using parasite specific primers provide a better diagnostic tool in terms of both sensitivity and specificity are used in the laboratory diagnosis of canine monocytic ehrlichiosis, especially during the acute phase of the disease before antibodies are detectable (Wen *et al*., 1997; and Carlos *et al*., 2007). Alexandre *et al*. (2009) reported detection by a nested PCR of *Ehrlichia canis* in dogs that were seronegative by indirect IFA test, an indication of the sensitivity of this method in detecting the infection. Several studies have also shown PCR to be an effective and extremely sensitive method for the detection of *Ehrlichia* and *Anaplasma* species in dog blood and tissues (Iqbal and Rikihisa, 1994; Iqbal *et al*., 1994; Dawson *et al.*, 1996; Engvall *et al*., 1996; and McBride *et al*., 1996). In this study nested PCR amplification with *Ehrlichia* species primers yielded DNA fragments and the second PCR reactions with primers specific for *Ehrlichia canis*, yielded PCR products providing the first molecular evidence that this infectious agent is involved in canine disease in Kenya. The gene sequences for the isolated fragments have been lodged with the GeneBank with the accession numbers KT2279762-KT279795. Evidence of *Ehrlichia canis* infection in Kenya was based on microscopic observation of the inclusion bodies in Giemsa-stained blood smears, serology and in-vitro cultivation. With the molecular isolation of the DNA, *Ehrlichia canis* is confirmed as a causative agent of canine ehrlichiosis in Kenya though, the presence of other species of *Ehrlichia* or *Anaplasma* cannot be ruled out.

In the present study nested PCR assay targeting the 16S rRNA gene detected *Ehrlichia canis* the causative agent of the classical canine ehrlichiosis in 42.5% (48/113) of the positive results in this study is an indication this species may be the main cause of ehrlichiosis in Kenya. The finding is comparable to that of Unver *et al*. (2001) where PCR analysis using *Ehrlichia canis* species speciûc primers revealed that 17 of the 55 dog blood samples (31%) were positive and that of Singh *et al*. (2014) who reported detection of 41.59% using nested PCR assay. Though, the high prevalence in this study may be due to the fact the work was carried out on dogs presented to the Small Animal Clinic not only with clinical manifestations consistent with canine monocytic ehrlichiosis but that had been established to have inclusion bodies in monocytes on blood smears.

The most consistent clinical manifestations in these dogs with *Ehrlichia canis* infection were lymphadenopathy, congestion of mucous membranes, inappetance, panting, loose hair, lethargy, vomiting, wasting, ocular discharge and

diarrhoea. Congestion of mucous membranes a clinical manifestation that has not generally been reported in literature but recently noted in a retrospective study of ehrlichial infection (Kitaa *et al*., 2014), is one of the clinical signs that was observed in this study. Congestion of mucous membranes was also observed by Price (1980) in naturally infected dogs. However, experimental studies involving *Ehrlichia canis* in dogs have not made any reference to this clinical manifestation. Though, profound haematological changes have been found to occur in canine monocytic ehrlichiosis (Harrus *et al.*, 1999; and Waner, 2008) the present study only noted reduction in the values of haematological parameters. One of the haematological parameters observed in canine monocytic ehrlichiosis, anaemia, usually normocytic, normochromic and non-regenerative, suggests a restricted or absent bone marrow response (Gaunt *et al.*, 2010). In the present study the low red blood cells, haematocrit and haemoglobin levels observed confirms that anaemia is an important finding in *Ehrlichia canis* infection in dogs. The clinical manifestations and anaemia observed in this study, though not specific, can be considered to be the case definition for *Ehrlichia canis* infection in dogs this region.

In conclusion, to the authors' knowledge, this is the first report on molecular identification of *Ehrlichia canis* in Kenya.

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