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Epidemiological investigation of canine vector borne diseases through polymerase chain reaction in Koraput district of Odisha

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Abstract
Canine vector-borne diseases constitute an important group of illnesses affecting dogs, that includes haemoparasites (e.g., *Babesia vogeli*, *Leshmania infantum* and *Trypanosoma cruzi*), bacteria (e.g., *Anaplasma platys* and *Ehrlichia canis*), and helminths (e.g., *Dirofilaria immitis* and *Dipylidium caninum*) that are transmitted by a diverse range of arthropod vectors. Canine vector-borne diseases are endemic throughout India. However, their identity to a species level remains anecdotal. The present study was undertaken to know the prevalence of canine vector borne diseases like Anaplasmosis, Borreliosis, Ehrlichiosis and Dirofilariosis through polymerase chain reaction and record the risk factors associated with prevalent vector-borne diseases in Koraput District of Odisha. Ninety six blood samples collected randomly from dogs were subjected to PCR for detection of antigen. Ninteen dogs were found positive for Ehrlichia when subjected to PCR. It was again subjected to PCR with species specific primers and detected *Ehrlichia canis* in all the 19 samples. All positive PCR products were sent to a Molecular laboratory for sequencing to confirm the validity of PCR amplifications. Sequences were compared with Gen Bank by BLAST analysis. It showed 88-93% similarity with many *E. canis* species in other geographical regions worldwide. The present study revealed prevalence of Ehrlichiosis in Koraput district. Therefore appropriate treatment and management practices should be adopted to reduce the occurrence of the disease.

Keywords: Canine vector borne diseases, *Anaplasma phagocytophilum*, *borrelia burgdorferi*, *dirofilaria immitis*, *Ehrlichia canis*, PCR

1. Introduction
Canine vector-borne diseases (CVBDs) is one of an important group of illnesses affecting dogs that encompasses protozoa (*Babesia*, *Leshmania* and *Trypanosoma spp.*), bacteria (*Anaplasma* and *Ehrlichia spp.*), and helminths (*Dirofilaria* and *Dipylidium spp.*) that are transmitted by a diverse range of arthropod vectors. India has a wide range of climatic zones, which make it suitable for multiplication of vectors and pathogens of veterinary importance. Transmission and geographical distribution are closely linked to regional temperature, rainfall and humidity [1]. CVBDs are highly endemic in tropical and subtropical regions and have increasingly been recognised, not only in traditionally endemic areas but also in temperate regions [2]. This may be attributed to several factors including dog population dynamics, and environmental and climatic changes. Despite advances in diagnosis during recent years, most cases were diagnosed in the field by clinical signs, a technique that is limited by its low sensitivity and specificity. Thus, a more comprehensive review of the prevalence of these diseases is still required. A greater understanding of these diseases is needed in order to better inform veterinarians and pet owners about the risks, prevalence, treatment and control of CVBD. Valuable trained dogs have been deployed in Koraput district to perform various activities as it is one of the naxal affected hilly district of Odisha. This provokes us to conduct this study. The following study was done to assess the incidence of vector-borne disease(s) among canine population of Koraput district of Odisha. This is the first kind in hilly district of Odisha.

Materials and Methods
Ethical approval
The study was conducted as per the guidelines of Institute and Animal Ethical Committee. The blood samples were collected from pets after explaining the benefits of study to the owners.

Area of study
The present study was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar.

Experimental design and Screening of animals
Pets of different breed, sex and age group presented to Veterinary Dispensaries (VD) of Koraput district were included in the present study. Koraput district lies between 18.8606° N latitude and 82.5510° E longitude.

Analysis of Prevalence
The prevalence of CVBDs was calculated among the total number of cases screened. The prevalence was further analysed in relation to age, gender, breed, purchase history, contact with other dogs, and history of tick infestation. These demographic informations were recorded to know whether they were associated with the likelihood of dogs exhibiting CVBDs.

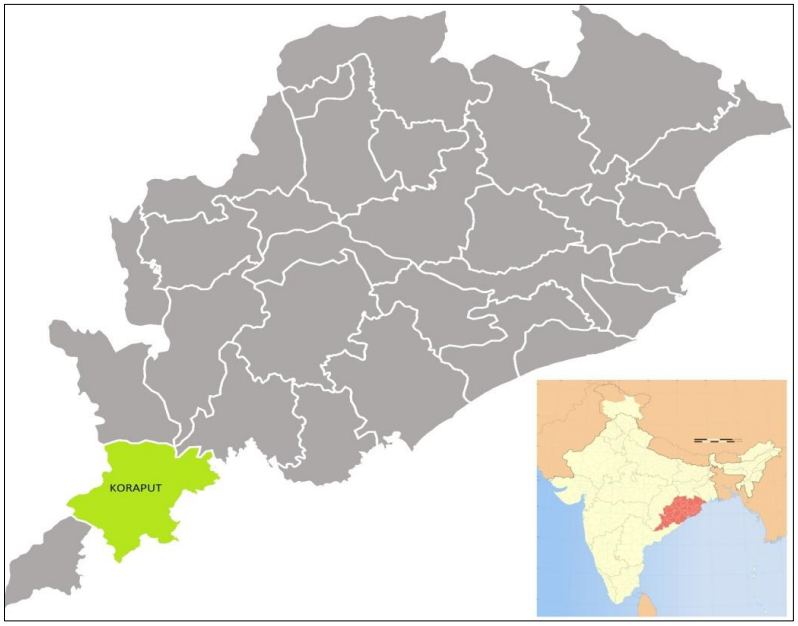

Fig 1: Map showing sample district of Odisha for study

Laboratory diagnosis
Antigen detection through PCR was included in the present study as diagnostic method. 2ml of blood sample from each dog was collected from recurrent tarsal vein, in EDTA vial. Blood in EDTA vials were used for estimation of antigen detection through PCR.

Diagnosis through PCR
Blood samples were subjected to PCR [3,4]. The genomic DNA was extracted from EDTA blood sample by QIAamp^{R} DNA blood Mini Kit, Qiagen Ltd., Germany, as per manufacturer's instructions and stored at -20^{0}C for subsequent use.

Table 1: Primers used for PCR to detect Ehrlichia antigen

Primer	Primer	Positive band	Reference
ECp28-F(f)	5'ATGAATTGCAAAAAAATTCTTATA3'	843 bp	McBride *et al*., 1999 [3]
ECp28-R(r)	5'TTAGAAGTTAAATCTTCCTCC3'		
ECAN5(f)	5'GAACGAACGCTGGCGGCAAGC3'	398 bp	Murphy *et al*., 1998 [4]
HE3(r)	5'TATAGGTACCGTCATTATCTTCCCTAT3'		

PCR was performed for 25 µl of total reaction volume containing 12.1µl of DEPC (Diethyl pyro-carbonate) water, 1.5µl of $MgCl_2$ (25mM), 2.0µl of dNTP, 2.5µl of 10xTaq buffer, 0.1 µl of 5U/ l TaqDNA polymerase and 2µl of each primer and 2µl of template DNA and the final volume was made up to 25µl. Primer sets used in this study were listed in table 1. For the amplification of Ehrlichia genus, ECp28-F and ECp28-R primers were used to amplify 843-bp fragment of the p^{28} gene. *Ehrlichia canis* species amplification was done using ECAN5 (F) and HE3 (R) primers derived from 16S rRNA gene. Solution was kept in the thermal cycler. The reaction for Ehrlichia genus was done under the following conditions: 5 min at 95^{0}C, 30 sec at 95^{0}C, 1 min at 55^{0}C, 2 min at 72^{0}C and 5 min at 72^{0}C. The PCR cycling conditions *i.e.*, denaturation, annealing and extension were repeated for 30 cycles followed by final extension. The reaction for *Ehrlichia canis* was done under the following conditions: 1 min at 94 ^{0}C, 1 min at 94 ^{0}C, 2 min at 65^{0}C, 2 min at 72^{0}C and 5 min at 72 ^{0}C. The denaturation, annealing and elongation were repeated for 30 cycles in a PCR thermal cycle.

Sequencing
All positive PCR products were sent to a Molecular laboratory (Applied Biosystems) for sequencing, to confirm the validity of PCR amplifications. Sequences were compared with Gen Bank by BLAST analysis.

Statistical analysis
Data obtained during the course of study were statistically

analyzed to ascertain the significance of 'p' values through t-test .The analysis was performed by using data analysis of Microsoft windows Excel package.

Results

The PCR results revealed 19 samples were positive for Ehrlichia genus in primers of p28 gene which produced amplicon at 843 bp is shown in figure 2. Again these samples were subjected to PCR with species specific primers (HE3 and ECAN5) for *Ehrlichia canis* and were found positive for *Ehrlichia canis* infection by the amplification of a 398 bp fragment of 16S rRNA which is shown in figure 3.

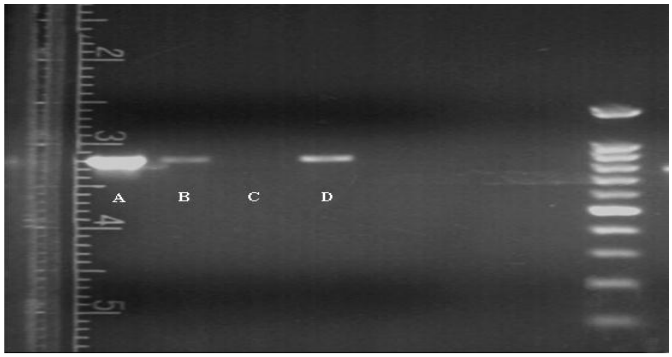

Fig 2: PCR bands after gel electrophoresis indicting presence of *Ehrlichia spp*. in tested blood samples (NB: A= Positive control;B and D=Positive sample at 843 bp (*Ehrlichia spp*.) and C= Negative sample)

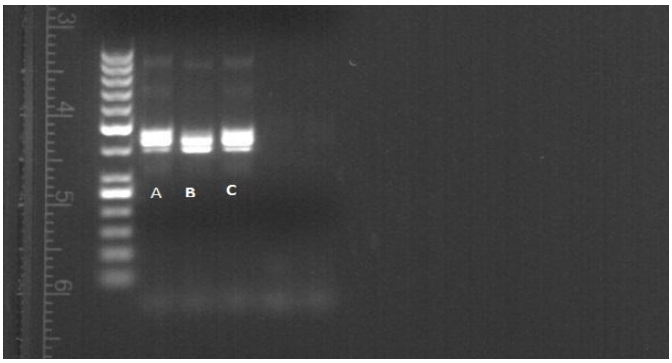

Fig 3: PCR bands after gel electrophoresis indicting presence of *Ehrlichia canis* in tested blood samples NB: A= Positive control; B and C=Positive sample at 398 bp (*Ehrlichia* canis)

Sequences of positive PCR amplifications belonging to DNA of *E. canis* were compared with Gen Bank databases using BLAST software and showed 88-93% similarity with many *E. canis* species in other geographical regions worldwide.

Out of the 19(19.79%) dogs positive for *Ehrlichia spp*., 12 (63.16%) were males and rest 7 (36.84%) were females. Ehrlichiosis was more frequent in male dogs in the present study. Breed-wise prevalence was found to be 12 (63.16%) and 7 (36.84%) in large and small breeds respectively. Higher prevalence of Ehrlichia infection was recorded in ≥1 year age group (57.89%, n=11) dogs in comparision to <1 year age groups (42.11%, n=8). Ehrlichia infection was higher in locally purchased dogs (78.95%, n=15) as compared to dogs purchased from outside the Koraput district (21.05%, n=4). Out of the 19 positive cases of Ehrlichia, 15 (78.95%) were having the history of tick infestation. Twelve (63.16%) positive cases of Ehrlichia were living in contact with stray dogs. Risk factors associated with Ehrlichiosis is shown in table 2.

Table 2: Risk factors associated with Ehrlichiosis (n=19)

Variables		No of positive dogs	Prevalence (%) of infected dogs
Gender	Male	12	63.16
	Female	7	36.84
Age	<1 year	8	42.11
	≥1 year	11	57.89
Breed	Large	12	63.16
	Small	7	36.84
Purchase history	Local	15	78.95
	Outside	4	21.05
Ticks infection	Present	15	78.95
	Absent	4	21.05
Contact with stray dogs	Yes	12	63.16
	No	7	36.84

Discussion

The PCR results revealed that about 19.79% (19) cases were positive to *Ehrlichia canis*. This finding has a resemblance with the previous findings who found 20 and 30% dogs as positive for Ehrlichia infection through PCR [5,6,7]. However, 40 and 50% of canine Ehrlichiosis through PCR were found in Chennai and Punjab respectively which is much higher than the present study [8,9]. Prevalence rate of 1.3% has been recorded from the South of the United States [10]. No positive cases of Dirofilaria were recorded in this study. It might be due to regular prophylaxis with endecticidal drug like ivermectin [11]. Also no positive cases of Anaplasmosis and Borreliosis were recorded during the study. The prevalence of Ehrlichiosis is more in large breeds (63.16%) with highest occurrence in German shepherd (6 cases) dogs. This apparent higher susceptibility of the German shepherd dog to ehrlichiosis has also been previously reported [9,12,13]. This susceptibility may be due to a defective cell-mediated immune response within this breed of dog [9]. The highest prevalence of Ehrlichiosis was noticed in males 12 (63.16%) than females 7 (36.84%). Similarly, higher prevalence of Ehrlichiosis in males (50.6%) was also recorded by various workers [14,15]. In contrast higher prevalence of Ehrlichiosis in female dogs was also recorded [13]. In age-wise prevalence study, there was higher incidence in 1 year and above aged (57.89%) dogs. Similar result with higher incidence in older dogs was previously recorded [16]. Higher incidence in older dogs may be due to greater contact time with ticks [17]. On contrary, there are also reports that suggest there is no significance between the incidence of Ehrlichiosis and sex as well as with the age of the dog [18,5]. However, dogs which were purchased locally and dogs living in contact with stray dogs and those that were parasitized by ticks revealed a higher risk of infection.

Conclusion

Incidence of Ehrlichiosis was recorded to a tune of 19.79% in Koraput district of Odisha. No other vector-borne diseases were detected in that area. Molecular diagnosis through PCR showed presence of *Ehrlichia canis.* Occurrence of Ehrlichiosis was more in large breed male dogs of age ≥1 year. Dogs with history of tick infestation were at higher risk of infection.

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