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Molecular detection and first characterization of *Ehrlichia canis* from Gajapati in Odisha

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ABSTRACT

Ehrlichia canis (*E. canis*), a rickettsial pathogen transmitted by tick vectors, causes canine monocytic ehrlichiosis, a significant infectious disease in dogs worldwide. The Gajapati region in Odisha is characterized by warm and humid climate, providing a conducive environment for the proliferation of ticks. Despite this, there has been no documentation on the genetic diversity of *E. canis* in this region. So, this study was taken up to detect and molecularly characterize *E. canis* from the Gajapati district in Odisha, India. A total of 64 blood samples were collected from the dogs showing clinical signs consistent with ehrlichiosis. All the samples were preliminarily screened by Giemsa blood smear examination followed by Polymerase chain reaction (PCR) targeting the 16S rRNA of *E. canis*. Of the 64 samples tested, 15 (23.4%) were positive by Giemsa staining and PCR analysis revealed a higher prevalence, with 35 (54.7%) samples testing positive, suggesting its superior sensitivity. Two of the isolates namely GEC-1, and GEC-2 were sequenced, and submitted to GenBank. The phylogenetic analysis revealed that the two study isolates were clustered together at bottom and form a distinct clade indicating distinct lineage from other reported strains. The present study is the first study on molecular characterization of *E. canis* from this region to our knowledge and whole genome sequencing has to be done for better understanding of the divergence.

Introduction

Canine ehrlichiosis is an emerging vector borne rickettsial zoonotic disease of global distribution. In general, three Ehrlichia species (*Ehrlichia canis*, *E. ewingii*, and *E. chaffeensis*) are known to infect the dogs. Among them, *E. canis* is the well-known etiological pathogen affecting platelets, monocytes, and granulocytes (Aziz *et al*., 2022). The brown dog tick, *Rhipicephalus sanguineus* is the main vector responsible for disease transmission (Groves *et al*., 1975; Aguiar *et al*., 2007). After entry organisms invade monocytes, macrophages, and epithelial cells. They cause monocytes to multiply excessively, filling the cytoplasm and leading to the destruction of leukocytes and thrombocytes. Thrombocytopenia occurs due to increased consumption of platelets because of inflammation in blood vessel linings, increased sequestration in the spleen, and immune-mediated destruction (Harrus *et al*., 1997; Ramakant *et al*., 2020). *E. canis* causes a range of clinical outcomes, from subclinical infections to severe illness characterized by fever, anemia, thrombocytopenia, and immunosuppression, which can lead to fatal complications in untreated cases (Harrus and Waner, 2011).

Molecular tools such as PCR have proven instrumental in the accurate detection and characterization of *E. canis*, surpassing the limitations of traditional diagnostic methods like Giemsa-stained blood smear examinations, which have low sensitivity and specificity (Jose *et al*., 2018). Genetic studies of *E. canis* using 16S rRNA gene sequencing have also revealed regional variations, underscoring the need for localized studies to unravel strain diversity and evolutionary patterns (Sainz *et al*., 2015).

In India, despite the growing recognition of vector-borne diseases, limited studies exist focusing on the molecular and genetic characterization of *E. canis* (Jose *et al*., 2018). Odisha, a state with favourable conditions for tick proliferation, lacks comprehensive epidemiological data on *E. canis*. This study aimed to address this gap by conducting molecular detection and characterization of isolates from Gajapati district. Specifically, the study evaluated the prevalence of *E. canis* in canine blood samples using Giemsa smear examination and PCR targeting the 16S rRNA gene, followed by genetic sequencing to assess strain diversity.

Materials and methods

Study area and sample collection

The study was conducted during Feb – June (2024) in Gajapati district, Odisha, India, which has a warm and humid climate conducive to tick proliferation (ODSMA, 2017). Blood samples were collected from 64 dogs (Male: 42 and Female: 22) exhibiting clinical signs consistent with ehrlichiosis, such as fever, anorexia, lethargy, lymphadenopathy, and epistaxis. Blood samples (approximately 2–3 mL) were collected aseptically in EDTA vials. All samples were transported to the laboratory under cold chain conditions for further processing.

Blood smear examination

Thin blood smears were prepared from each sample, air-dried, and stained with Giemsa stain accordingly to the method of Coles (1986). The slides were examined under oil immersion (100x magnification) using a light microscope for the presence of intracytoplasmic morulae within mononuclear cells.

Molecular detection

DNA extraction from blood samples

The Genomic DNA was extracted from each blood sample using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The DNA concentration and purity were assessed using

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a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and subsequently stored at - 80°C until further use.

Amplification of 16S rRNA gene of E. canis

The DNA samples were tested for detection of *E. canis* using species specific oligonucleotide primers of 16S rRNA (ECA-F: 5` AACACATGCAAGTCGAACGGA 3`; HE3R-R 5`TATAGGTACCGTCATTATCTTCCCTAT 3`) as suggested by Jain *et al.* (2018) and Prameela *et al.* (2020). The PCR reaction mixture, comprising 12.5 µl of GoTaq® Green Master Mix (2X), 1.25 µl each of forward and reverse primers (20pmol/µl) and 150 ng of DNA was prepared with a final volume of 25 µl using nuclease-free water. The PCR assay was conducted in Proflex PCR systems (Applied Biosystems) following the standardized cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30sec, annealing at 59°C for 30sec, and extension at 72°C for 1min, with a final extension step at 72°C for 5 min. The PCR amplified products were analyzed by gel electrophoresis in a 1.5% agarose gel and observed under UV illumination using the BIO-RAD gel documentation system.

Nucleotide sequencing and Phylogenetic analysis

Positive PCR products from two samples (GEC-1 and GEC-2) were sequenced at Barcode Biosciences Private Limited, Bangalore, Karnataka, India. The obtained sequences were edited for errors using Codon Code aligner, Version 10.0, (Sequence Assembly and Alignment software). Homology searches were conducted using the NCBI program BLAST. The phylogenetic analysis was performed in MEGA 11 utilizing ClustalW and a maximum likelihood tree was constructed. The significance of deduced phylogenetic tree was verified by bootstrap analysis of 1000 replicates (Tamura *et al.*, 2021). The nucleotide sequences of the two isolates have been deposited in GenBank.

Results

Microscopic examination of blood smears stained with Giemsa stain

Giemsa-stained blood smears upon microscopic examination revealed the presence of morulae in monocytes indicating *E. canis* in 15 out of 64 samples (23.4%) tested (Fig. 1).

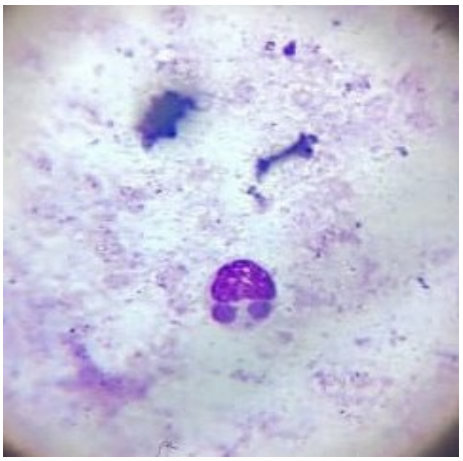

Fig. 1. Blood smear of a dog infected with *E. canis* under oil immersion lens showing morulae within monocytes.

Detection of E. canis by PCR

All the DNA samples were tested for detection of *E. canis* using species specific primers of 16S rRNA gene. Out of 64 samples 35 samples (54.68%) were positive of which 23 were male (35.9%) and 12 were Female (18.75%) and produced the amplification product of 412bp (Fig. 2).

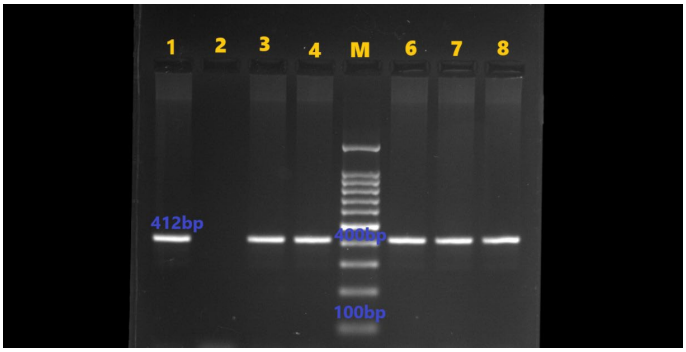

Fig. 2. Amplification of *E. canis*16s rRNA gene. Lane M: DNA Ladder, Lane 1: Positive control (412bp), Lane 2: Negative control, Lane 3-8: Positive samples of *E. canis*.

Nucleotide sequencing and Phylogenetic analysis

The coding region of 16S rRNA gene from two isolates, namely GEC-1 and GEC-2 was sequenced to determine the degree of genetic relatedness among *E. canis* strains found in various nations and those originating in India. The obtained sequences submitted to the GenBank with the accession numbers OR770191 and OR770192 respectively. A Phylogenetic tree (Fig. 3) was constructed, which revealed that current study isolates clustered together at the bottom of the tree and are distinctly separated from other sequences, forming their own clade.

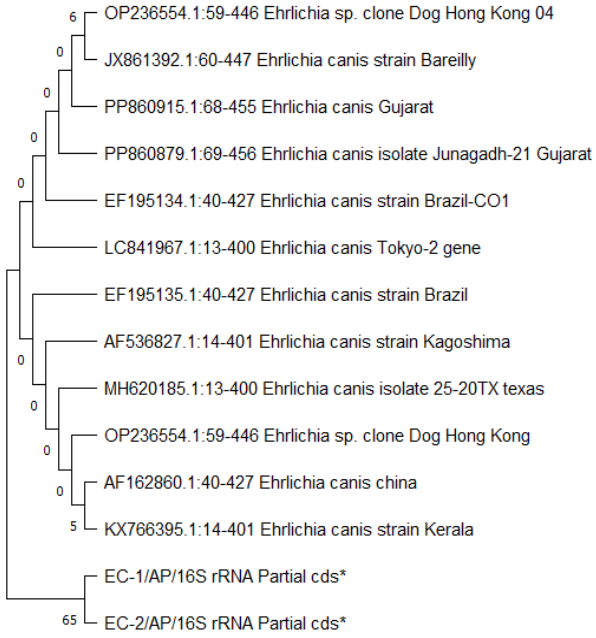

Fig. 3. Phylogenetic tree by maximum likelihood method.
Note: The sequences obtained through this investigation were identified by (*) labels. Other *E. canis* sequences used in this analysis were obtained from GenBank.

Discussion

The findings of this study highlight the presence of *E. canis* in the Gajapati district of Odisha, marking the first molecular characterization of this pathogen in the region. The higher prevalence detected through PCR compared to Giemsa staining underscores the need for molecular diagnostics as a superior tool for early and accurate detection of *E. canis* infections. This is in accordance with Prameela *et al.* (2020). Similar observations were also reported by Lakshmanan *et al.* (2007) who found that nested PCR detected *E. canis* in 50% of samples, whereas blood smear examination identified morulae in only 19.38% of cases, reinforcing the reliability of PCR as a diagnostic method.

The phylogenetic analysis revealed that the *E. canis* isolates from Gajapati district, Odisha (GEC-1 and GEC-2), form a distinct clade indicating a unique evolutionary lineage. These isolates are genetically distant from other global strains, suggesting localized genetic divergence, likely due

to regional ecological and evolutionary pressures. This highlights the potential for unique traits in these isolates. This, finding aligns with studies conducted in other parts of India, such as work by Parthiban *et al.* (2024), highlighting evolutionary changes that may impact virulence and drug resistance.

The implications of these findings are critical for veterinarians and policymakers. The high prevalence rate indicates that ehrlichiosis may be an emerging threat to canine health in the region, necessitating the implementation of tick control programs and routine molecular surveillance. Similar strategies have proven effective in other regions with high tick-borne disease prevalence. For instance, a study by Mobarak *et al.* (2024) in Egypt as well as a study by Bai *et al.* (2017) in Haryana, India, emphasized the importance of molecular prevalence studies and associated hematological profiles in managing canine monocytic ehrlichiosis.

Further research involving whole-genome sequencing is recommended to explore genetic variations, antimicrobial resistance markers, and virulence factors in *E. canis*. Such studies could lead to better therapeutic strategies and vaccine development, as evidenced in prior genomic research on rickettsial pathogens. For example, the identification of *E. canis* by PCR with phylogenetic analysis in dogs from South India has provided insights into the genetic makeup of the pathogen, aiding in the development of targeted treatments (Kalaivanan and Saravanan, 2019).

Conclusion

The findings emphasize the superiority of PCR in detecting *E. canis* due to its enhanced sensitivity and accuracy. In this study sequencing was done based on limited information so, future research should focus on whole-genome sequencing which will help in exploring potential vaccine development to improve disease prevention and treatment strategies. Implementing effective tick control strategies, routine surveillance programs, and public awareness campaigns are essential to manage and prevent ehrlichiosis. These efforts will contribute significantly to safeguarding canine health and controlling the spread of ehrlichiosis in endemic regions.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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