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Clinical implications of early detection of *Ehrlichia canis* using nested polymerase chain reaction

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Abstract
Aim: The primary objective of this study was to thoroughly evaluate and refine molecular methodologies specifically designed for the accurate detection of *Ehrlichia canis* in dogs.
Methods: This study involved the collection of blood samples from a cohort comprising 73 dogs, which were subsequently screened for the presence of *Ehrlichia canis* utilizing an innovative nested PCR assay. Positive samples identified through this screening process underwent genetic characterization, involving amplification and sequencing of a segment approximately 389 base pairs in length within the Ehrlichia Spp. 16S rRNA gene. The application of nested PCR was specifically employed to ascertain the species identification of Ehrlichia, strategically utilized to heighten the sensitivity and specificity of detecting *E. canis* within blood specimens.
Results: The utilization of the Nested PCR method proves highly sensitive in identifying Ehrlichia infections, even in subclinically affected dogs. Detection through this method prompts timely treatment recommendations for PCR-positive dogs, potentially serving as a life-saving intervention in numerous cases.
Conclusion: Drawing from the initial molecular evidence highlighting the presence of *Ehrlichia canis* in dogs, the suggested protocol stands poised to enhance the landscape of epidemiological investigations concerning canine ehrlichiosis. This innovative approach has the potential to significantly contribute to a more comprehensive understanding of the prevalence and spread of this disease among canine populations.

Keywords: Nested PCR, ehrlichiosis, thrombocytopenia, petechiae

Introduction
Over the last two decades, *Ehrlichia* diseases have emerged as pressing concerns affecting both humans and animals. The initial discovery of *Ehrlichia canis* in a dog in Algeria dates back to 1935 [1]. Prior to a notable outbreak among military working dogs in Southeast Asia in 1967, canine ehrlichiosis was commonly perceived as a mild illness typified by symptoms such as fever, vomiting, and naso-ocular discharge [2]. However, following this pivotal event, the disease rapidly disseminated among dogs globally [3], leading to significant and severe health consequences.
Canine ehrlichiosis, also known as tropical pancytopenia, manifests as an acute, subacute, or chronic tick-borne ailment attributed to *E. canis*, a gram-negative intracellular bacterium [4]. The prevalence of this disease is notably concentrated in tropical and subtropical regions owing to the geographical spread of its primary vector, the tick species Rhipicephalus sanguineus [5].
Diagnosing ehrlichiosis relies on multiple methods, including assessment based on clinical signs, identification of morulae within monocytes, serological testing to detect antibodies against *E. canis*, and Polymerase Chain Reaction (PCR). However, the visualization of morulae in blood smears from dogs in the subclinical and chronic stages proves challenging and often yields a low sensitivity rate. This limitation stems from the typically low concentrations of the organism, rendering it unsuitable as a standalone diagnostic tool for the disease [6].
PCR is a technique used in the early diagnosis of ehrlichiosis and also for identification of the infecting species from blood of dogs as well as from various tissues and can be used for confirmatory testing [7].
PCR testing plays a crucial role in diagnosing canine ehrlichiosis. The sensitivity of a single-step PCR assay targeting the *E. canis* 16S rRNA gene in blood and tissues was frequently inadequate to reliably detect a minimal number of organisms.

Therefore, nested PCR test was performed to increase sensitivity and specificity in the detection of *E. canis* in blood specimens. This method is highly sensitive for detection of ehrlichial infections in dogs and treatment is recommended and initiated for PCR positive dogs, even for subclinically infected, as they can deteriorate, and develop clinical disease. It helps to initiate prompt therapy which can be life saving in many instances and also to know the recovery state of an animal after treatment [8].

Tereza *et al*. (2012) [9] demonstrated that nested PCR was highly sensitive in detecting *Ehrlichia* sp than the direct stained blood smear examination of dogs with clinical signs suggestive of ehrlichiosis. Results showed that 50 percent false negative rate would occur when only direct examination is used for diagnosis. In contrast, all animals with morulae in the blood smears were positive by nPCR with whole blood or fraction samples.

Materials and Methods

Samples and DNA extraction: A total of 73 blood samples were collected which are suggestive of ehrlichiosis. Blood samples were collected in anti-coagulant tubes (EDTA). Blood collected in EDTA was utilized to extract DNA following the method of rapid isolation of mammalian DNA [10]. Quality of the DNA was tested by measuring the ratio of A_{260} and A_{280} as well as by carrying out the agarose gel electrophoresis. The good quality DNA was stored at – 80 °C until further use. DNA quality was checked by electrophoresis on an agarose gel, and the DNA was then quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific).

PCR amplification and DNA sequencing

Molecular diagnosis of *Ehrlichia* spp. Done using a new nested PCR assay based on the partial amplification of the 16S rRNA gene of Ehrlichia spp. The primers specific for 16S rRNA gene [11] was used in this assay for the detection of *E. canis* using nested PCR. The details of the primers used were given in the table 1.

Table 1: Details of the primers specific for *E. canis*

PCR Round	Primer	Oligonucleotide sequence (5'-3')	Target Gene	Amplicon size (bp)	Specificity
I	ECC(F)	AGA ACG AAC GCT GGC GGC AAG C	16SrRNA	478	*Ehrlichia* species
	ECB(R)	CGT ATT ACC GCG GCT GCT GGC A			
II	ECAN5(f)	CAA TTA TTT ATA GCC TCT GGC TAT AGG	16SrRNA	389	*Ehrlichia canis*
	HE3(r)	TAT AGG TAC CGT CAT TAT CTT CCC TAT			

The first round of amplification was carried out in 50 µL reaction mixture with 10–20 ng of the DNA template, 1 µL of each primers (ECC and ECB), master mix 20 µL and ultra pure water 18 µL. The amplification reaction consisted of 30 cycles of 1 min at 94 °C, 2 min at 68 °C, and 2 min at 72 °C, preceded by 3 min at 94 °C and followed by 3 min at 72 °C. The second round of amplification was also carried out in 50 µL reaction mixture with 2 µL of the first-round PCR product, 1 µL of each primer (ECAN5 and HE3), master mix 18 µL and ultra pure water 28 µL. The amplification reaction consisted of 37 cycles of 1 min at 94 °C, 2 min at 58 °C, and 1.5 min at 72 °C, preceded by 3 min at 94 °C and followed by 3 min at 72 °C. All the PCR products were visualized in 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer, using GelRedTM Nucleic Acid stain (Biotium) and an ultraviolet transilluminator after electrophoresis. A 100 bp molecular marker (Invitrogen DNA ladder) was used to assess the size of amplified fragment. The samples that produced fragment size of 389 bp for the second round PCR were considered to be positive and the PCR product was sent for custom sequencing. The specificity of the sequence obtained were determined using 'BLAST' (Basic Local Alignment Search Tool) by the method of [12] (www.ncbi.nlm.nih.gov).

For molecular diagnosis, DNA of *E. canis* was used as the positive control, while sterile bi-distilled water was used as the negative control. BioEdit software was used to align forward and reverse sequences.

Results

Agarose gel electrophoresis of nested PCR product revealed the amplification at 478 bp (Fig 1 & 2) with first round of PCR and amplified at 389 bp (Fig. 3 & 4) with second round of PCR and the product which supported the infection due to *Ehrlichia canis* (Fig 3, 5, 6, 4). Further, the PCR product was gel purified and sequenced. The BLAST analysis of sequences is shown in Fig 7 and Table 2, 3 & 4).

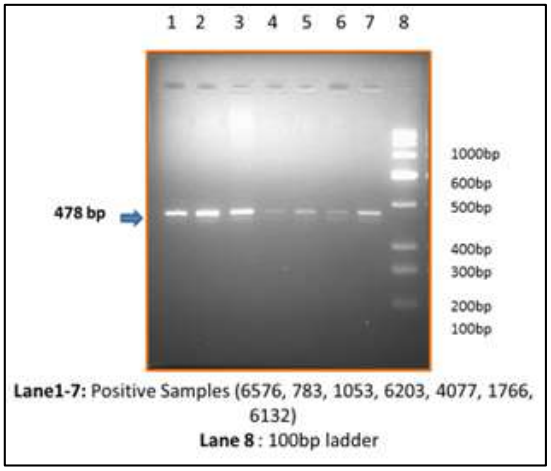

Fig 1: PCR Detection of *E. canis* – Agarose gel electrophoresis of PCR product (I Round)

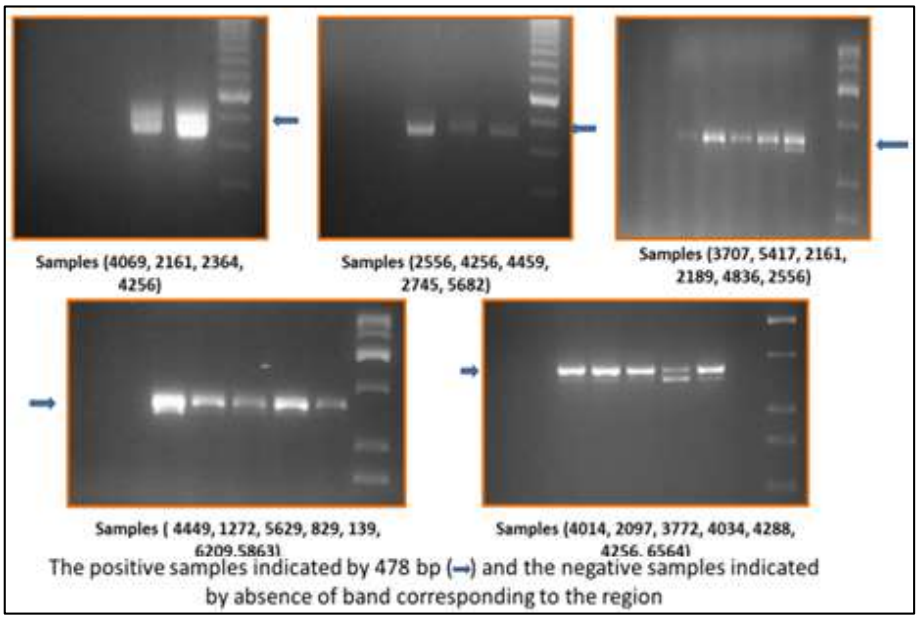

Fig 2: PCR (I Round) screening of field samples for *E. canis*

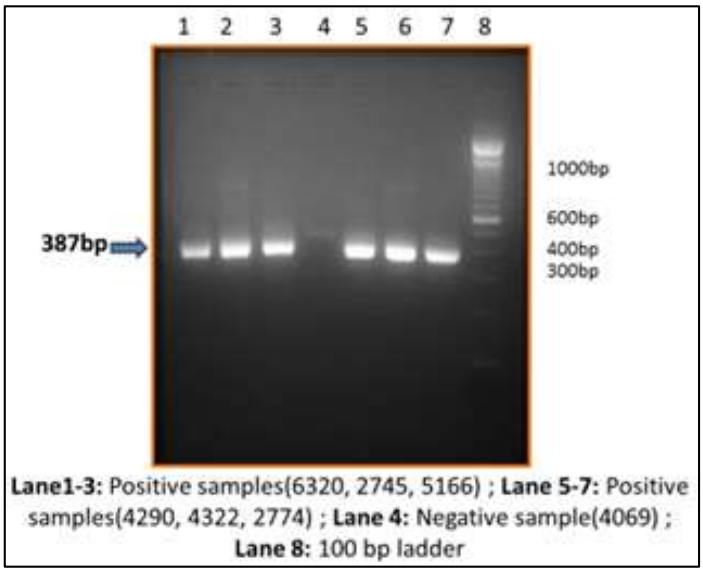

Fig 3: PCR Detection of *E. canis* – Agarose gel electrophoresis of PCR product (II Round)

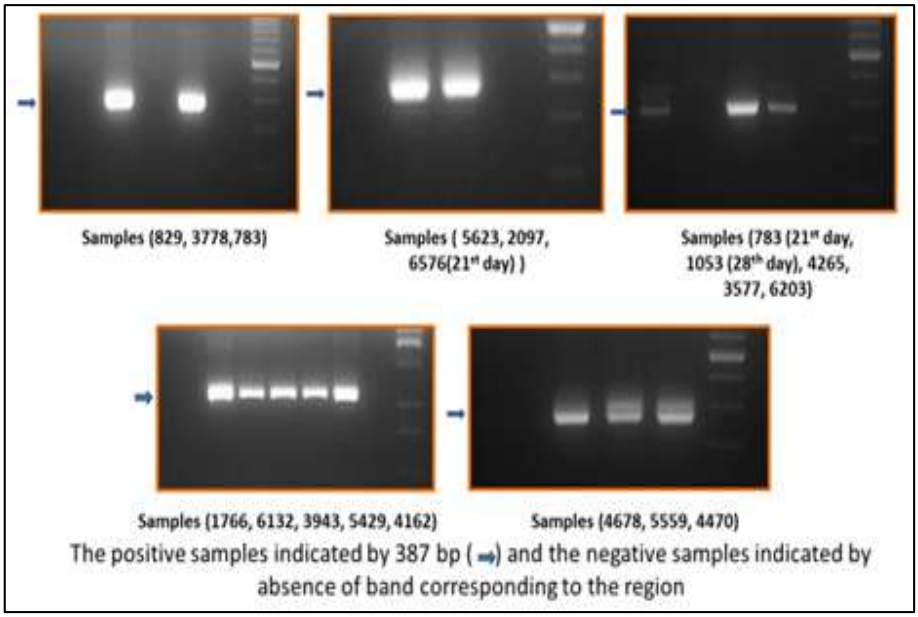

Fig 4: PCR (II Round) screening of field samples for *E. canis*

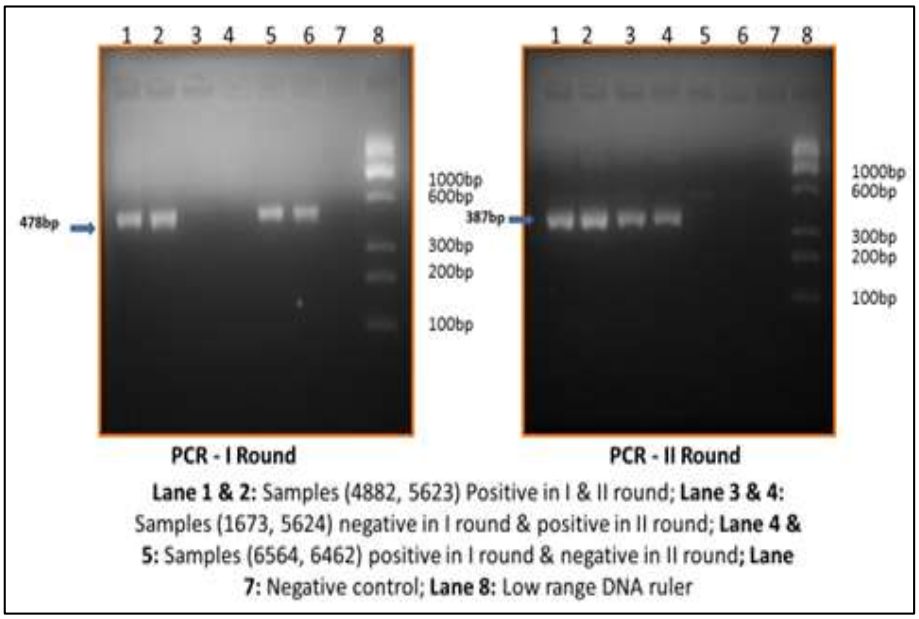

Fig 5: PCR Detection of *E. canis* – Agarose gel electrophoresis of PCR product (I & II Round)

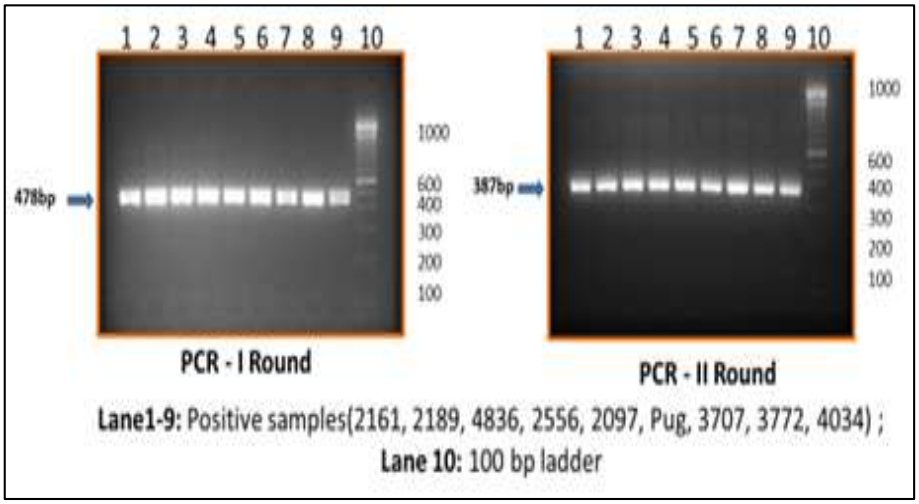

Fig 6: PCR Detection of *E. canis* – Agarose gel electrophoresis of PCR product (I & II Round)

The accession number obtained after submission of sequence to the Gene Bank and is given in the Table.5.

Table 1: Gen Bank Accession numbers allotted for the partial sequences of 16S rRNA gene of *E. canis*

Sl. No	Sample No	Organism*	Gene Bank Accession No.
1	2745	*E. canis*	KF536734
2	4459	*E. canis*	KF536737
3	5682	*E. canis*	KF536738

*Based on PCR positive / sequence similarity to *E. canis*

Suspected samples were subjected to first and second round PCR. Thirty seven samples were positive for both first and second PCR. Nine samples were negative by first round and positive by second round PCR (Fig. 5). Nine samples (12.33 percent) were positive by first round but found negative by second round PCR (Table 6)

Table 6: Screening of first and second round of PCR

Technique	II Round (+)	II Round (-)	Total
I Round (+)	37	9	46
I Round (-)	9	18	27
Total	46	27	73

The representative sequences obtained from this study has been deposited in the GenBank database under accession numbers KF536737, KF536734 and KF536738 (Table 5). The sequence had 100% identity with the corresponding sequences from *E. canis* isolates in Czech Republic (GenBank accession number KC479024), USA (GenBank accession number NR074283), Romania (GenBank accession number KC305491) and Japan (GenBank accession number AB723712).

Table 2: The sequence similarity of 16S rRNA sequence (partial) of the representative samples

Case No.	Extent of identity (%)				
	***Sphingomonas* sp.**	***Neorickettsia* sp.**	***Anaplasma* sp.**	***Ehrlichia* sp.**	***Wolbachia* sp.**
4240	97-99	90-91	87-89	82-88	81
6364	97-99	90-91	86-89	81-86	80-81
4394	97-99	89-90	87-88	82-87	80-82

The representative sequences obtained from this study has been deposited in the GenBank database under accession numbers KF536735, KF536736 and KF536739 (Table 11).

Table 3: Gen Bank Accession Numbers allotted for the partial sequences of 16S rRNA gene of *Sphingomonas* spp.

Sl. No	Sample No	Organism*	Gene Bank Accession No
1	4240	*Sphingomonas* sp.	KF536735
2	4394	*Sphingomonas* sp.	KF536736
3	6364	*Sphingomonas* sp.	KF536739

*Based on PCR positive / sequence similarity to *Sphingomonas* sp.

The sequence had 99% identity with the corresponding sequences from *Sphingomonas* spp. isolates in USA (GenBank accession number GU429324), India (GenBank accession number KF536739), Canada (GenBank accession number JN796467) and China (GenBank accession number JF912452).

Discussion

Ehrlichiosis is one of the tick borne disease of dogs which is caused by *E. canis.* Morulae inclusions in blood monocytes are transient and hence cannot be used for confirmatory diagnosis of the disease. Early diagnosis of canine monocytic ehrlichiosis by nested polymerase chain reaction will help in initiating prompt therapy which can be lifesaving in many instances. Very few literature are available in relation to nPCR of canine monocytic ehrlichiosis in veterinary field. Therefore, the current research endeavor was initiated to investigate molecular diagnostic methods employing nested polymerase chain reaction (nPCR) for the detection of canine ehrlichiosis.

Out of 73 samples screened by PCR, 46 samples (63.01 percent) were found positive by nested PCR. Out of the forty six samples positive, thirty seven (80.43 percent) were positive on first and second round PCR and nine samples (19.57 percent) were negative by first round PCR but positive on second round PCR. The amplification of genomic DNA using species-specific primers in a single-step PCR did not produce positive results. Consequently, the adoption of nested PCR proved to be a dependable and sensitive technique for diagnosing low-level parasitemia, as documented by [13].

The sequences obtained from this study has been deposited in the GenBank database under accession numbers KF536737, KF536734 and KF536738. The sequence had 100% identity with the corresponding sequences from *E. canis* isolates in Czech Republic (GenBank accession number KC479024), USA (GenBank accession number NR074283), Romania (GenBank accession number KC305491) and Japan (GenBank accession number AB723712).

Nested PCR has been widely used in the laboratory diagnosis of CME, especially during the acute and subclinical phase of disease and treatment is recommended even to those that are sub clinically affected as they can decompensate and develop into clinical disease [14]. While IFAT has been employed in the diagnosis of CME, there exists a potential for false positive interpretations attributable to the production of cross-reactive antibodies in animals. Moreover, its reliability and applicability are constrained by the requirement for a high level of expertise, low sample output, lack of standardization, and the expense associated with microscopic equipment, as highlighted in [15]. IFA may cross-react with different species of Ehrlichia or microorganisms from other closely related genera. The inability to distinguish between current infection and prior exposure is widely recognized as a weakness of IFA tests as described by Murphy *et al.* (1998) [11]

Occasionally, during the acute phase of the disease, further microscopic examination may reveal the presence of typical intracytoplasmic *E. canis* morulae in monocytes. However, only about 4% of blood smears from dogs with ehrlichiosis exhibit these characteristic *E. canis* morulae, as reported in [17]. While thrombocytopenia is a common observation in dogs infected with CME, it can also manifest in other diseases and infections involving different Ehrlichia species and parasites, as highlighted in [18]. Hence, relying solely on thrombocytopenia and other clinical signs as indicators of *E. canis* infection is no longer deemed adequate for establishing the diagnosis of CME, as similarly underscored by Santos *et al*. (2009) in their findings [19].

The diagnosis of ehrlichiosis necessitates a blend of clinical, hematological, and molecular biology examinations. Nested PCR emerges as a superior choice due to its sensitivity, specificity, ease of use, rapidity, and capacity to analyze numerous samples simultaneously, enabling the detection of early and persistent canine ehrlichiosis, as emphasized in [20]. Considering all these aspects, it is deduced that the diagnosis of ehrlichiosis through nPCR stands out as the most rational and expeditious method, underscoring the significance of molecular techniques in the prompt detection of CME.

Out of 73 samples, 9 samples (12.33 percent) which turned positive by first round PCR was found negative on second round PCR. Representative samples were selected and were custom sequenced. BLAST analysis revealed 97-99% identity to *Sphingomonas* spp. The sequences of the PCR amplified product of 16S rRNA gene obtained only in the first round PCR using primer specific for genus *Ehrlichia* had shown sequence identity with *Sphingomonas* spp. The natural habitat of Sphingomonas remains incompletely defined, yet it is extensively found in the natural environment, particularly in water and soil, as noted in [13]. Additionally, it has been isolated from various hospital settings, encompassing tap water, distilled water, nebulizers, respirators, dialysis fluid, and various equipment, as reported in [21].The sequences obtained from this study has been deposited in the GenBank database under accession numbers KF536735, KF536736 and KF536739. The sequence had 99% identity with the corresponding sequences from *Sphingomonas* spp. isolates in USA (GenBank accession number GU429324), India (GenBank accession number KF536739), Canada (GenBank accession number JN796467) and China (GenBank accession number JF912452).

Sphingomonas paucimobilis (previously known as Pseudomonas paucimobilis) [22] is a Gram-negative bacterium characterized by its yellow pigmentation, aerobic nature, motility facilitated by a polar flagellum, and nonfermentative properties [23]. This bacterial species has been isolated in a pure culture from a leg ulcer, from the blood of an individual with septicemia, and from the cerebrospinal fluid of a person suffering from acute meningitis. Nevertheless, instances of endemic and epidemic animal infections caused by this organism have been infrequently reported. To our knowledge, this is the first time that *Sphingomonas* spp. bacteremia has been epidemiologically linked to canine blood stream using molecular typing methods. *S. paucimobilis* had already been identified in feline blood by Pinyoowong (2009)[24] using molecular typing methods. Hence, further studies are suggested in this aspect.

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