Prevalence Risk Factor Analysis and Molecular Characterization of Canine Monocytic Ehrlichiosis in Maharashtra

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ABSTRACT

Background: The research work was undertaken with the aim to investigate the prevalence and influence of associated risk factors on canine monocytic ehrlichiosis (CME), by employing conventional and molecular techniques in naturally occurring ehrlichiosis in dogs from the geographical area of Pune and Satara, Maharashtra, India.

Methods: Evaluation of 60 blood samples from dogs exhibiting illness clinically suggestive of CME was performed for the detection of *Ehrlichia canis* by conventional microscopy and PCR-based assays targeting 16S rRNA gene. The collected blood samples were also utilized immediately for estimation of haemato-biochemical parameters. Descriptive statistics along with chi-square test of association were performed by using SPSS software.

Result: Microscopic examination of Geimsa stained thin blood smears revealed *Ehrlichia canis* morulae in 5.00% (03/60) samples. Processing of purified DNA from blood samples by Genus-specific 16SrRNA PCR, with known primers resulted in amplification of 477 bp product of 6.66% (04/60) samples evident of CME. Further two step nested PCR assay using published species-specific primers yielded 387 bp product in 16.66% (10/60) samples indicative of *E. canis*. Younger, exotic cross breed females dogs were found more prone compared to adults. Indian non-descript male counterparts and winter season being more favorable for CME. Sequence analysis of randomly selected representative samples from Pune and Satara each when aligned with nine isolates from India and western countries showed complete homology.

Key words: Canine monocytic ehrlichiosis, *Ehrlichia canis*, Nested PCR, Risk factors, Sequencing.

INTRODUCTION

India has a wide range of climatic zones ranging from arid, semi-arid to wet tropics, environment is greatly suitable for wide range of vector breeding and thus transmitting life threatening diseases in animal population. Canines are known to suffer from emergence and re-emergence of diseases caused by bacteria, rickettsia and protozoa transmitted by vector ticks leading to high morbidity and mortality throughout the world. Published evidences show that, canine monocytic ehrlichiosis (CME), a tick borne rickettsial infection of canines has gained significant importance during recent years in India (Bhattacharjee and Sarmah, 2013). The etiological agent *Ehrlichia canis,* an obligate, intra-cytoplasmic, pleomorphic, gram-negative rickettsial member of the family Anaplasmataceae, is a highly pathogenic agent transmitted by *Rhipicephalu ssanguineus*, commonly known as brown dog tick. Disease is manifested by a wide variety of clinical signs including depression, lethargy, weight loss, anorexia, pyrexia, lymphadenomegaly, splenomegaly and bleeding tendencies being common. In severe chronic phase, there is decrease in platelet production due to bone marrow depletion leading to thrombocytopenia. Adequate knowledge about the prevalence of disease along with associated risk factor in a particular geographical region is need of hour in order to combat the disease promptly and effectively. In Indian sub-continent most cases of CME are diagnosed with traditional microscopic observation of stained smear for demonstration of morulae formation in infected blood leukocytes (monocytes), lung aspirates, spleen and lymph nodes. However, the detection of morulae of *E. canis* in Romanosky stained blood smears apart from being time consuming; lacks sensitivity especially for sub-clinical and chronic disease (Singla *et al.*, 2016). Serology is the most commonly used diagnostic method in CME (Singla *et al.*, 2011; Mann *et al.*, 2017); however, cross reactivity and an inability to differentiate between current and past infections are disadvantages of these methods (Waner *et al.,* 2001). Therefore, molecular technique such as polymerase chain reaction (PCR) with the help of species-specific primers provides a better diagnostic tool in terms of both sensitivity as well as specificity (Singla *et al.,* 2016). Further, though

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correlation of various risk factors with the prevalence of the disease has been documented, however, similar reports from this part of the country using molecular assays are lacking (Abd Rani *et al.,* 2011). Therefore, the current study was undertaken to investigate the prevalence and molecular characterization of CME and its correlation with various risk factors.

MATERIALS AND METHODS

Blood samples (n=60), were collected aseptically from the cephalic vein of selected dogs suspected for CME, presented to the Teaching Veterinary Clinical Complex, KNP College of Veterinary Sciences, Shirwal and from the private veterinary clinics considering the criteria of tick infestation and clinical signs *viz.* anorexia, depression, lethargy, mild weight loss, fever and splenomegaly were consistent with CME. Giemsa stained thin blood smears prepared from all sample were observed under oil immersion for presence of *E. canis* morulae. The collected blood samples were also utilized immediately for estimation of haematological parameters (haemoglobin, TLC and total platelet count) and were then kept at -20°C until DNA extraction. Purified DNA from the blood samples was subjected for molecular analysis with PCR and nested PCR using known primers. Samples were also processed for genomic finger printing and phylogenetic alignment with Gen Ome bio Technologies Pvt. Ltd., Pune using EMBL, Clustal Omega software.

Genomic DNA isolation

For conducting the PCR assays, genomic DNA was extracted from whole blood using HiPurA Blood Genomic DNA Miniprep Purification kit following the manufacturer's recommendations. Twenty microlitres of proteinase K were pipetted into a 2 ml microcentrifuge tube and 200 µl microliters each of anticoagulant-treated blood and PBS were added to it. Then two hundred microliters of buffer (C1) was added to the tube and the contents were mixed thoroughly by vortexing, before incubation at 55°C for 10 min. Two hundred microliters of ethanol (Diluent for DNA extraction, Himedia) was added and the contents were mixed thoroughly by vortexing. The mixture was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 10,000 rpm for one min (KHSC Hi-speed centrifuge, KEMI). The flow-through and collection tube then discarded. The spin column was then placed in a new 2 ml collection tube and 500 microliters of Buffer (PW) were added. Subsequently, centrifugation was performed at 10,000 rpm for one min. The flow-through and collection tube was discarded. The spin column was placed in a new 2 ml collection tube and 500 microlitersof Buffer WS were added. Centrifugation was performed at 14,000 rpm for three minutes. The flow-through and collection tube was discarded. The spin column was transferred to a 2 ml microcentrifuge tube. The DNA was eluted by adding 200 microliters of Buffer AE to the center of the spin column membrane. After incubation at room temperature for one minute, centrifugation was carried out at 10,000 rpm for one minute. Last step was repeated for increasing DNA yield and the samples thus obtained were stored at -20°C until further use.Purity of DNA was measured by Nanodrop (Thermo Scientific) at OD of A260/A280 nm and the value obtained was close to 1.8. Purity of GenomicDNA was checked by the absorbance method in a spectrophotometer equipped with a UV lamp, UV-transparent cuvettes. Purified DNA of known PCR positive isolate of *E. canis* was prepared and used as a positive control. Genomic DNA was also isolated from the whole blood of an infection-free day old pup and was used as a negative control along with nuclease-free water.

PCR protocols

Purified DNA (4 µL) was used as a template to amplify a portion of 16SrRNA gene *E.canis* (Murphy *et al.*, 1998). The primer sequences selected for primary PCR assay were ECP1F: 5′AGAACGAACGCTGGCGGCAAGC3′ ECP1R: 5′CGTATTACCGCGGCTGCTGGCA3′ and for Nested PCR assay: ECP2F:5′CAATTATTTATAGCCTCTGGCTCTGGCT ATAGGA3′ and ECP2R: 5′TATAGGTACCGTCATTATCTTCC CTAT 3′. Reaction was carried out in 25 µL volumes. Each PCR reaction mixture comprised of 12.5 µL Himedia PCR mix, 1 µL each of forward and reverse primers, 4 µL of DNA and volume was made upto 25 µL using nuclease free water. Nuclease free water in 4 µL volumes was used in negative control reactions. PCR amplification conditions consisted of: Initial denaturation at 94°C for 01 min; followed by 29 amplification cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min and extension at 72°C for 2 min, final extension step at 72°C for 5 min, before cooling to 4°C. In order to carry out the nested PCR, the master mix was the same as described above, but instead of template DNA, 1 µl of the primary PCR product was used and amplified with 20 pmol each of primers. The Nested PCR conditions were as follows: initial denaturation at 94°C for 3 min, first step consisted of three cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 1.5 min while the second step consisted of 37 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 1.5 min and the final extension was performed at 72°C for 8 min.

The primary as well as nested PCR products were checked for amplification by electrophoresis on a 1.5% agarose gel and visualized using a gel documentation system. In order to check the specificity of the assays isolated genomic DNA of *Babesia canis* and *Hepatozoon canis* isolated from the microscopically positive cases was also tested in the PCR to determine amplification, if any.

Hematological evaluations

A complete blood count, including the following parameters: Hb, VPRC (Volume of packed red cells), TLC, RBC and platelets in anticoagulant treated blood was performed using an Automatic Hematology Analyzer (Orphee, Mythic Vet 18).

Statistical analysis

The IBM- SPSS-20 software was used to analyze numerical data gathered in the present study. Descriptive statistics was employed to find out the prevalence of the disease and chi-square of association was applied by using IBM-SPSS-20 software to study the associated risk factors influencing prevalence of Canine monocytic ehrlichiosis (CME). All results were considered statistically significant when P≤0.05.

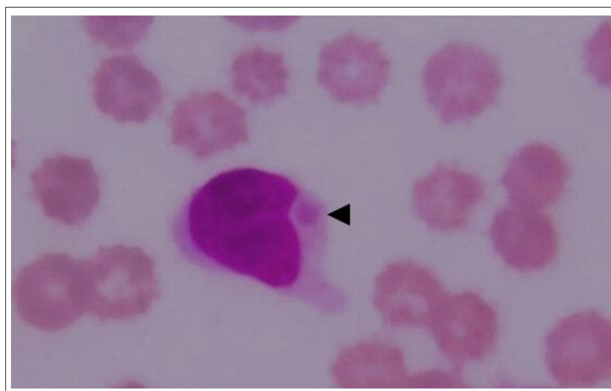

Fig 1: Giemsa stained blood smear of dog from a clinical case showing cytoplasmic inclusions within monocytes or lymphocytes. *i.e* morula of *Ehrlichia canis*.

RESULTS AND DISCUSSION

Blood smear examination

Examination of Romanosky-stained peripheral thin blood smears revealed 5.00% (03/60) samples to be positive for presence of darkly stained compact morulae of *E. canis* in the leukocytes (Fig 1).

Polymerase chain reaction

Out of all blood samples processed with conventional PCR targeting 16S rRNA gene, 6.66% (04/60) were found positive for generating 477 bp amplicons of *E. canis*. Further processing of the samples for Nested PCR resulted in16.66% (10/60) samples to be positive for *E. canis* producing amplicons of 389 bp (Fig 2.1 and 2.2). This result generated by nested PCR in detection of *E.canis* showed significantly higher (P<0.05; P value 0.017*) prevalence (Table 1) as compared to blood smear examination and conventional PCR.

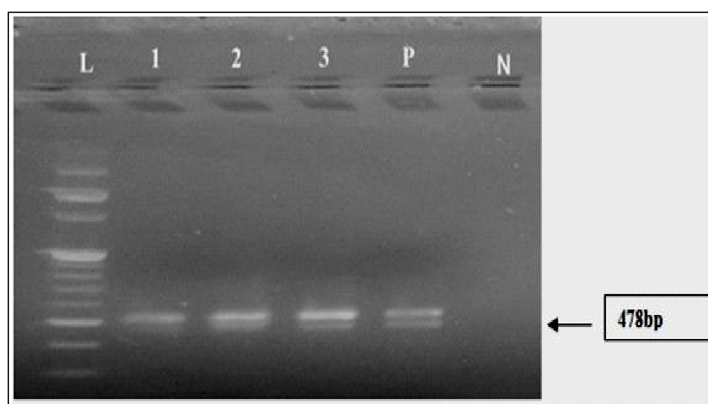

Fig 2.1: Primary PCR amplification of *E. canis* (genus specific): Positive detectable amplification at 478 bp. Lanes 1 to 3 - test samples; in lanes. Lane 4- positive control; Lane L: DNA ladder 100 bp.

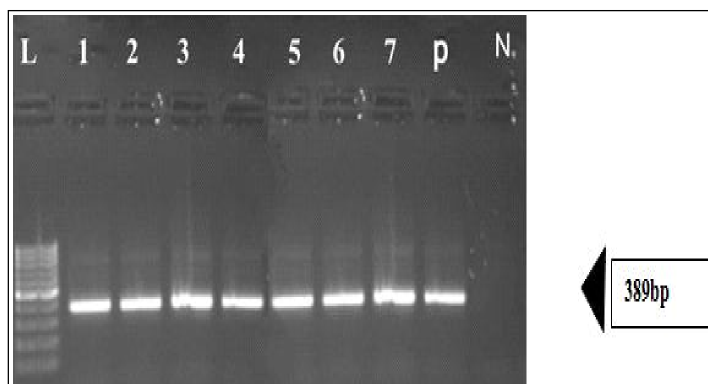

Fig 2.2: Nested PCR amplification of primary PCR with inner pair of PCR primers for species specific amplification of *E. canis* at -389 bp: Lanes 1 to 8- Samples where 1 pi product from primary PCR has been used as template for nested PCR, all samples, are positive by nested PCR (389bp); Lane L- DNA ladder 100 bp.

Influence of external risk associated factors on prevalence of CME

In dogs, below one year of age *E. canis* infection was observed in 5.55% blood smears and by conventional PCR as well, while 11.11% of these dogs were found positive by nested PCR (Table 1). While in adult dogs of above one year age, none was positive by blood smear, 6.66% were positive by PCR and 66.66% by nested PCR, respectively indicating significantly lower ($p<0.01$) in the young dogs than that of adults by nested PCR and higher by blood smear examination.

Out of 29 samples tested from females, 09 (31.03%) were positive, while 01 male out of 31 (3.22%) tested positive for CME by nested PCR (Table 3). Out of total 10 positive samples confirmed for *E. canis* infection with nested PCR, 90% belonged to females while 10% were of male dogs, indicating more occurrence ($p<0.01$) in females than that of males. Significantly higher rate of infection of *E. canis* ($p<0.01$) was observed exotic breeds (25.71%) than that of non-descript Indian dog breeds (Table 3). Higher ($p<0.01$) number of *E. canis* cases was reported in winter season (60%) than in summer (40%).

Table 1: Prevalence of *E. canis* employed during the study period.

Samples examined	Technique	Positive (%)	Chi square χ^2	P value
60	Blood smear	03 (5.00%)		
60	Primary PCR	04 (6.66%)	12.000	0.017*
60	Nested PCR	10 (16.66%)		

*Significant at P<0.05, **Significant at P<0.01, ns: non-significance.

Hematological profile

The values of total leukocytes (3.11±0.25) were found decreased in CME positive dogs indicative of slight leukopaenia (Table 2). Higher PCV values (40.76±2.79) in *E. canis* infected dogs than the normal dogs were indicative of dehydration. Mean platelet count (1.88±0.15) indicated slight thrombocytopaenia in CME positive dogs.

Serum biochemical profile

Slight increase in alkaline phosphatase (U/L), bilirubin (mg/dl) and creatininne (mg/dl) in CME positive animals was seen. Mean ALT (189.66±43.084) and bilirubin (16.266±1.681) values of CME positive group was found to be higher than that of normal range. While other parameters like total serum protein, albumin, globulin and creatinine were within the normal range.

Sequencing and phylogenetic analysis

Phylogenetic analysis was performed at GenOmbio Technologies Pvt. Ltd. Baner, Pune, India and the phylogenetic tree was prepared using EMBL Clustal Omega software. On the basic of phylogenic analysis, the amplification product for *E.canis* obtained in the present study using primers ECP2R and ECP2F presented 100% homogeneity with *E. canis* isolates in database from Bengaluru (MN994343.1), Assam (MG050140.1) and Mannuthy (MN484597.1), India. It also resembled the same genomes sequences with that of *E. canis* isolated from other countries *viz.* Texas (MH620199.1), Mexico (MH374119.1), US (MG029067.1), Mexico (MG029075.1), Brazil (MG793444.1) and Brazil (MF153959.1) (Fig 3).

Table 2: Distribution of canine monocyticehrlichiosis in accordance with various risk factors. Hematological profile of dogs diagnosed with CME (Mean±S.E).

Risk factor	Parameter	Number	Blood smear (%)	Primary PCR %	Nested PCR %
Age	Y	54	3(5.55%)	3(5.55%)	6(11.11%)
	A	6	0	1(16.66%)	4(66.66%)
	χ^2-value		0.351	1.071	12.000
	P value		0.75^{ns}	0.351^{ns}	0.005**
Sex	Male	31	0	0	1(3.22%)
	Female	29	3(10.34%)	4(13.79%)	9(31.03%)
	χ^2-value		3.376	4.581	8.343
	P value		0.107^{ns}	0.049*	0.004**
Breed	Cross breed	35	3(8.57%)	3(8.57%)	9(25.71%)
	ND	25	0	1(4%)	1(4%)
	χ^2-value		2.256	0.490	4.951
	P value		0.191^{ns}	$.443^{ns}$	0.026*
Season	Summer	10	2(2%)	4(4%)	4(4%)
	Winter	50	1(2%)	0	6(12%)
	χ^2-value		5.684	21.429	4.704
	P		0.069^{ns}	0.000**	0.052*
Total		60	3	4	10

*Significant at P<0.05, ** Significant at P<0.01, ns: non-significance, Y= Young dog, A= Adult dog and ND= Non descriptive dog.

In the present study, examination of blood smears revealed 5.00% samples to be positive for presence of morulae of *E. canis* in the leukocytes. A lower prevalence of morula of *E. canis i.e.* 2.34%, 1.3% and 2.12% in blood smears was observed by Milanjeet *et al.* (2014), Mittal *et al.* (2017) and Bhattacharjee and Sarmah (2013), respectively. Procajło *et al.* (2011) indicated this conventional method a reliable technique for the diagnosis of CME, however, Abd Rani *et al.* (2011) reported inefficiency of stained blood smear due to lack of sensitivity. Similarly, Singla *et al.* (2016) had also discussed failure of microscopic blood smear examination in detection of *E. canis* owing to the low Rickettsemia. The variation in the prevalence reported by various workers may be attributed to sample size, geographical area and climatic conditions influencing the tick. High prevalence by nested PCR as compared to blood smear examination and conventional PCR is due to the fact that it is 100 times more sensitive than one-step PCR for the diagnosis of CME (Bulla *et al.,* 2004).

Significantly lower ($p<0.01$) prevalence in the young dogs than that of adults by nested PCR and higher prevalence by blood smear examination in the present case may be due to significant variation in the number of dogs examined in each group. Many earlier scientists had reported higher prevalence of CME in young dogs (Lakshmanan *et al.,* 2007; Milanjeet *et al.,* 2014; Bhadesiya and Raval, 2015). In contrast Bai *et al.* (2016) reported higher prevalence of ehrlichiosis in age group of more than 1 year while, some reports have suggested that there is no age predilection for Ehrlichiosis in dogs (Jafari *et al.,* 1997; Harrus *et al.,* 1996; Smitha, 2003). The higher prevalence ($p<0.01$) of *E. canis* in female dogs might be due to higher stress levels in females because of pregnancy, hormonal changes, nutritional deficiency *etc.* than that of male dogs which is in agreement with Milanjeet *et al.* (2014) and Bhadesiya and Raval (2015). Whereas, in contradiction to our findings, Harrus *et al.* (1996) observed no sex predilection for Ehrlichiosis while, Bai *et al.* (2016) reported higher incidence in males than in females. Higher cases of Ehrlichiosis were recorded in exotic cross bred (25.71%) dogs than that of non-descript Indian (4%) dogs. As in present case, other workers (Bhadesiya and Raval, 2015; Kottadamane *et al.,* 2017) had reported higher prevalence of Ehrlichiosis in exotic breeds especially German shepherd and Labrador retriever dogs. Significantly more ($p<0.01$) cases were reported during winter season (60%) than in summer (40%) suggested that season may contribute an important role in CME. On similar note Kalaivanan *et al.* (2020) had earlier reported higher prevalence of Ehrlichiosis during winter season.

Though slight leucopenia and thrombocytopaenia were observed among *E. canis* infected dogs when compared with healthy controls (Table 2), however it showed statistically insignificant variation. Similar findings indicating

Table 3: Hematological profile of dogs diagnosed with CME (Mean±S.E).

Parameter (units)	Normal range*	Blood smear (n=03)	PCR (n=04)	Nested PCR (n=10)
Hb (g/dl)	10-16	12.8±1.00	12.97±0.50	12.72±0.93
PCV (%)	30-35	41.33±1.98	43.0±5.92	40.76±2.79
TLC (/µl)	6-16	7.00±1.35	15.12±5.48	3.11±0.25
TEC (/µl)	5-8	7.43±027	5.72±0.26	7.04±0.45
Neutrophil (%)	60-70	80.33±5.78	57.5±11.97	63.8±5.87
Eosinophil (%)	2-10	1.33±0.33	1.25±0.40	1.20±0.59
Lymphocyte (%)	15-30	35.66±14.37	47.75±2.71	34.60±4.90
Monocyte (%)	3-8	1.00±0.577	1.00±0.40	1.30±0.47
Platelet (%)	2-8.5	1.09±0.106	1.4±0.20	1.88±0.15

*Radostits *et al.* (2006).

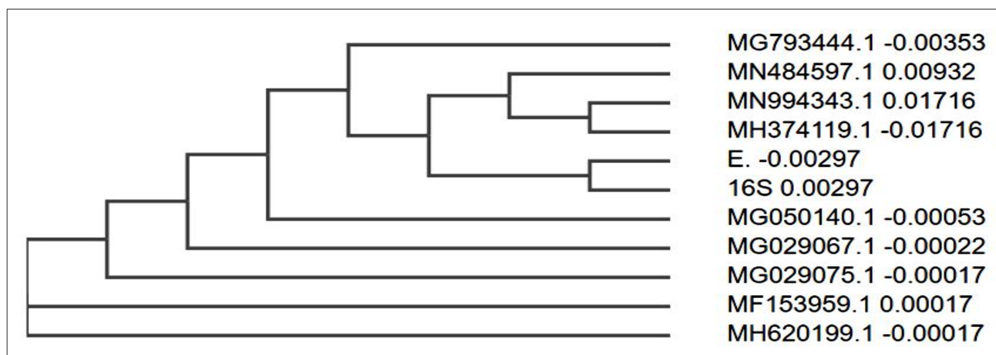

Fig 3: Phylogenetic tree of *E. canis*. This phylogenetic analysis showed high genetic diversity among different strains of *E. canis*. Consensus sequences obtained in this study is indicated as “Present study *E. canis* 16S rRNA 0.00297 Pune and Satara region Maharashtra, India”.

leucopaenia and light thrombocytopenia in dogs positive for *E. canis* by PCR have been previously reported (Alexandre *et al*., 2009; Milanjeet *et al*., 2014). Serum biochemical profile revealed slightly elevated alkaline phosphatase, bilirubin and creatininin CME positive dogs. These findings were in agreement with other workers (Srivastava and Srivastava 2011; Agnihotri *et al*., 2012; Bhardwaj, 2013; Kottadamane *et al*., 2017).

Phylogenic analysis of the amplification product for *E. canis* obtained in the present study indicated complete homogeneity with the database of isolates from Bengaluru (MN994343.1), Assam (MG050140.1) and Mannuthy MN484597.1), India. It also resembled the homologous genomes sequence with that of *E. canis* isolated from abroad *i.e.,* Texas (MH620199.1), Mexico (MH374119.1), US (MG029067.1), Mexico (MG029075.1), Brazil (MG793444.1) and Brazil (MF153959.1).

CONCLUSION

Two step nested PCR showing 16.66% prevalence of *E. canis* in dogs from Pune and Satara districts of western Maharashtra proved to be superior technique compared to blood smear examination and PCR in diagnosis of *E. canis*. Non-significant alterations in biochemical and hematological parameters indicate that most of the present cases were of subclinical nature exerting lesser effect on liver and kidney dysfunction and haematology. This was the first report of genome sequencing of *E.canis* from western Maharashtra, India indicating no genetic alterations and stability in the strains isolated in the region. High positivity for *E. canis* detected by nested PCR in dogs warrants the screening for the disease in suspected dogs by this technique as compared to routine blood smear examination in order to elucidate the true spectrum of the disease.

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Conflict of interest

Authors declare there is no conflict of interest's regarding the publication of this research paper.

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