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Molecular Epidemiology, Characterisation of *Hepatozoon canis* in Dogs as well as in Ticks and Haemato-biochemical Profile of the Infected Dogs in Chennai

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ABSTRACT

Background: Canine hepatozoonosis is caused by *Hepatozoon canis* and is transmitted through ingestion of an ixodid tick, *Rhipicephalus sanguineus sensu lato* containing mature oocysts. Periodical screening of vectors and their host for the presence of pathogen acts as reliable method for understanding the pattern of the disease which thereby reduces the chance of disease and the cost of treatment.

Methods: A total of 482 blood samples and 47 pooled tick samples collected from 482 dogs during the study period were screened through conventional and molecular techniques for the presence of *H. canis*. The haematology and biochemical profile of infected animals were evaluated. Positive PCR products were sequenced, compared with the existing sequences and were analysed phylogenetically.

Result: Out of 482 blood samples collected from dogs, only 5 were found to be positive microscopically for *H. canis* while 32 blood samples and 8 tick samples were found positive by polymerase chain reaction (PCR). Amongst epidemiological attributes, age and gender were found to have no significant relationship for the occurrence of the disease while, breed of the dog had a significant association. Haematology and biochemistry revealed profound anaemia, monocytosis and elevated blood urea nitrogen in dogs with *H. canis*.

Key words: Blood profile, Dogs, *Hepatozoon canis*, Molecular epidemiology, Phylogenetic analysis, *Rhipicephalus sanguineus sensu lato,* Ticks.

INTRODUCTION

Globalization and increased international trade, urbanization, climate change, as well as increased travel and mobility of pets, causes rapid extension of the zoogeographical range for many tick species (Shaw *et al.* 2001). In view of transmitting infections, ticks have historically been placed second to mosquitoes (Hillyard, 1996). Canine vector-borne diseases are a group of infectious diseases caused by a variety of pathogens that are transmitted by arthropod vectors such as ticks, mosquitoes, fleas and lice causing serious human health hazard worldwide (Otranto *et al.* 2017). Many microbes with zoonotic significance, such as protozoa, rickettsia spp. can be transmitted by ticks, which are ubiquitous ectoparasitic arthropod vectors (Sparagano *et al.* 1999).

Otranto *et al.* (2011) reported that because of the frequent infestation by the tick *R. sanguineus*, Hepatozoonosis is one of the most common canine vector-borne diseases. *R. sanguineus* is found in temperate and tropical regions worldwide and cases of *H. canis* have been reported from different places like southern Europe, Asia, Africa, the Middle East and South America (Baneth and Vincent, 2005). *H. canis* infection (HCI) was first described in India in 1905 (Craig, 2006).

The infection with *H. canis* is caused primarily by the ingestion of infected ticks or tick parts containing mature oocysts with infective sporozoites through oral route (Aktas *et al.* 2013). It is an intracellular, malaria like parasite affecting leucocytes of dogs. Leucocytes containing gamonts of *H. canis* are usually seen in peripheral blood smear (Hendrix *et al.* 2006).

In most of the cases, *H. canis* causes a chronic infection in its host with relatively mild or no clinical effects. However, symptoms like fever, paralysis, inappetence, anaemia, ocular discharge, hind limb weakness and emaciation were

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recorded in severe and fatal cases of the disease (Gondim *et al.* 1998). Roux and Raoult (1999) recommended screening of the animals and vectors periodically for pathogen carrier status because these blood sucking vectors carry infected host blood and pathogens. They suggested that vectors act as reliable indicators for the existence of pathogens in a specific area.

Hence, the present study was conducted to know the prevalence and molecular epidemiology of the canine hepatozoonosis and to characterize the organism in both dogs as well as in ticks and also to find out the changes in the haematology, biochemical parameters in infected animals if any. PCR products obtained from pooled tick samples collected from the corresponding *Hepatozoon* positive dogs were sequenced and further phylogenetic analysis of the parasite was also done.

MATERIALS AND METHODS

Sample collection

Sampling of dogs

Dogs presented with tick infestation and clinical signs of haemoparasitic disease such as anaemia, pyrexia, lymphadenopathy and anorexia were included in the study. A total of 482 dogs were selected after examination at Madras Veterinary College Teaching Hospital, Madras Veterinary College and a few private veterinary clinics in Chennai (12°55′N to 13°10′N and 80°0′E to 80°25′E) between December 2019 and August 2021 (Fig 1). Age, sex, breed and tick infestation were all documented for each dog in a structured questionnaire. About 3 ml of blood was collected from the saphenous vein of the dogs, transferred to EDTA coated tubes for haematology and DNA extraction and in clot activator tubes for serum biochemistry. Peripheral blood smears were collected from the ear tip for staining technique. Blood samples for DNA extraction were stored at -80°C (Voltas- TATA, India) until further processing.

Sampling of ticks

A total of 548 ticks from 47 infested dogs out of 482 were collected and placed in test tubes containing absolute ethanol before being transported to the laboratory. They were identified for their morphology (Keirans, 2009) and stored at -80°C until further processing. Ticks collected from each dog were considered as one pooled sample. 47 tick pools were processed for DNA extraction.

Staining method

The blood smears were prepared on glass slides and stained with Leishman-Giemsa cocktail stain (Senthil *et al.*, 2015). Stained smears were examined under light microscopy for the presence of ellipsoidal shaped *H. canis* gamonts in neutrophils. Each smear was examined for 100 microscopic fields under an oil immersion objective.

Haematology and biochemistry

The whole blood was analysed for Haemoglobin, RBC, WBC, Platelet count and differential leucocyte count by auto haematology analyser (Mindray BC-2800 Vet, China). Serum biochemical parameters like BUN, Creatinine, total protein, blood glucose, SGPT and bilirubin were analysed by A15 auto analyzer (Biosystems, Spain).

DNA extraction

Blood samples

Genomic DNA was extracted from EDTA coated blood samples using DNeasy blood and tissue kit (Qiagen, Netherland) as per the protocol recommended by the manufacturer. The quality of DNA in the final elutes were estimated using Nanospectrophotometer (Nano drop™ one, Thermoscientific, USA).

Ticks

Each pooled tick sample was frozen and ground to fine powder using liquid nitrogen in an autoclaved mortar and pestle (Tajedin *et al.,* 2016). Powdered tick tissue samples

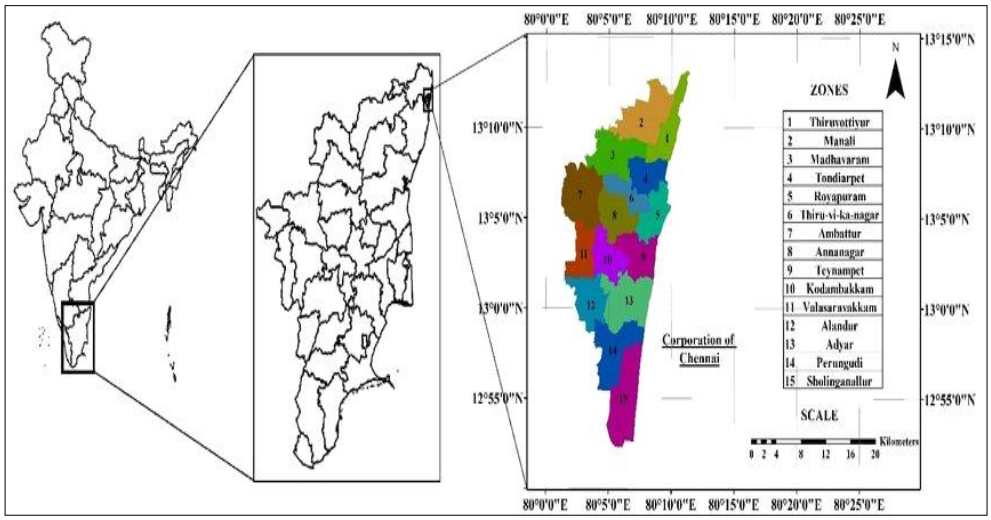

Fig 1: Geographical Map showing the sampling area.

were used for DNA extraction using DNeasy blood and tissue kit (Qiagen, Netherland) according to the manufacturer's instructions. The final volume of DNA was adjusted in 50 µl of TE buffer and stored at -80°C until further use.

Conventional PCR for the detection of *H. canis*

The extracted DNA samples from blood and tick samples were screened for the presence of *H. canis* with species specific PCR. The organism was targeted for 18S rRNA gene using primers as previously described by Inokuma *et al.* (2002). Primers were custom synthesized from Sigma, India. Master mix of 2x (Ampliqon, Denmark), Nuclease free water (Himedia, India), PCR tubes of 200 µl capacity (Bio-Rad, USA), DNA molecular weight marker (100 bp, Gene Direx, USA) were used. A 666 bp product size was amplified using the Forward primer 5′- ATACATGAGCAAAATCTCAAC- 3′ and Reverse primer 5′- CTTATTATTCCATGCTGCAG- 3′ by standardization of PCR cycles using thermal cycler (Bio-Rad, USA). Conventional PCR was performed using 25 µl reaction volume containing 12.5 µl of master mix, 1.5 µl of each forward and reverse primer (10 pico moles), 3 µl of template DNA and 6.5 µl of Nuclease free water. Cycling conditions of Initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, Annealing at 60°C for 30 sec, Extension at 70°C for 1.30 min and final extension at 72°C for 5 min with slight modifications. Amplified PCR product was subjected to electrophoresis in 1.5% agarose gel (Seakem LE agarose, USA) and the gel was visualized under gel doc mega UV transilluminator (Bio-Rad, USA) with 100 bp reference molecular weight marker.

Sequencing and phylogenetic analysis

Three positive PCR products were selected randomly, one from dog blood sample and two from tick samples were purified and sequenced in both directions using the forward and reverse primers (Eurofins, India). The obtained *18S* rRNA gene sequences of *H. canis* were submitted to GenBank under accession numbers OK181857, OK181864 and OK181882. DNA sequence data were compared to sequences of the *18S* rRNA gene deposited in GenBank using the basic local alignment search tool nBLAST. These sequences were aligned using ClustalW multiple gene sequence alignment software (Thompson *et al.* 1994). The Mega X program (Kumar *et al.* 2018) was used for construction of the neighbour-joining (NJ) tree (Saitou and Nei, 1987) with Kimura 2-parameter model (Kimura, 1980) with uniform rates and bootstrap of 1000 replicates (Felsenstein, 1985). The 18S rRNA partial sequences of this study were compared with the available sequences from GenBank.

Statistics

The statistical association between the presence of infection by PCR from both positive and negative blood samples with other epidemiological attributes like age, sex and breed of the dog were analysed using Pearson's chi-square test. Haematology and serum biochemical parameters for the positive and negative animals were tested for their equality of variances using Fischer's exact test and then analysed by independent two sample t-test. All the data was computed using the SPSS software. P values <0.05 were considered as significant.

RESULTS AND DISCUSSION

Parasitic DNA in dog blood

Out of 482 blood smears examined, five were found positive for *H. canis* gamonts which were seen within neutrophils by Leishman- Giemsa cocktail stain in peripheral blood smear examination (Fig 2) revealing the prevalence of 1.03%. A similar low prevalence of 1.08% and 1.41% was reported by Singh *et al.*, 2012 and Bhattacharjee and Sarmah (2013) respectively. A higher prevalence of 4.8% (out of 4190 blood smears tested) was found in the earlier studies conducted by Senthil Kumar *et al.*, 2009 in Tamil Nadu. This difference in the prevalence of the infection could be due to the difference in number of samples screened. In contrast, 32 dog blood samples were found positive for *H. canis* by conventional PCR products amplified at 666 bp targeting 18S rRNA gene (Fig 3) with a prevalence of 6.63%. All five samples which were found positive with blood smear were also found positive by PCR. Abd Rani *et al.* (2011) found that PCR is more sensitive in detecting haemoparasites than microscopic examination of the blood film. The low

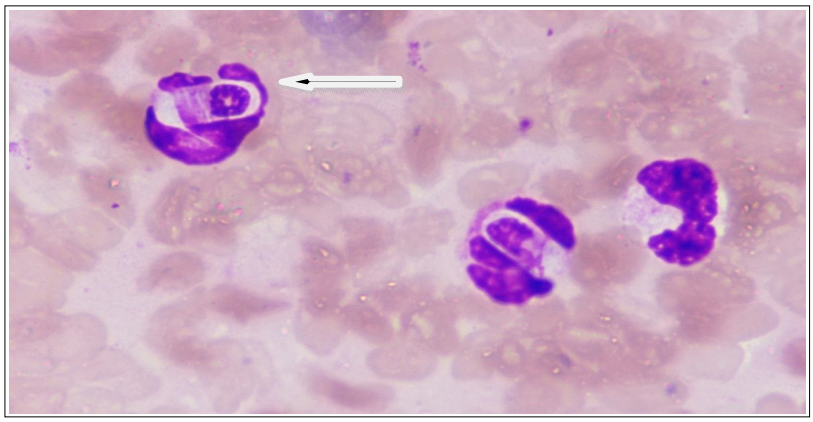

Fig 2: Arrow mark showing *H. canis* gamonts in neutrophils stained with Leishman-Giemsa cocktail stain in 100X.

prevalence recorded in this study via blood smear examination could be attributed to light microscopy's low detection limit which is approximately 0.001 per cent parasitaemia (Matsuu *et al.*, 2005). The overall prevalence of 6.63% was in accordance with the earlier molecular studies of Thomas *et al.* 2020. However, higher prevalence 41.4% was reported in Nigeria by Kamani *et al.* 2013. The low prevalence of *H. canis* in our study could be explained by sampling, study population characteristics, social factors (raising and caring for pets), the immune status of the dogs, climatic conditions and geographic location, all of which influence the abundance and distribution of vector ticks. (De Miranda *et al.* 2011).

Among 482 dogs screened, 19 (87.5%) were females and 13 (49%) were males (Table 1). Although female dogs had a slightly increased number, there was no statistically significant risk of contracting *H. canis* infection with gender in the present study which was in accordance to previous surveys of Licari *et al.* (2017). The percentage of age groups that are infected were below 2 years, 4.11%; 25-48 months, 3.77%; 49-72 months, 9.68%; 73-96 months, 6.25%; 97-120 months, 13% and above 10 years, 14.7%. These outcomes could suggest a greater susceptibility of adult dogs (> 4 years) to *H. canis*. The above could be explained by the host's immunologic status (which changes with age) or increased exposure to tick vectors in adult and older dogs, giving them more opportunities and time to become infected (Tsegay *et al.* 2016). However, our study found no statistically significant correlations between age and positive *H. canis* cases. In the present study, there was a significant association with the dog breed (P= 0.03). This was in contrast to the earlier findings of Manoj *et al.* (2020) who found that there was no statistical association between different breeds of dogs. However, Pacifico *et al.* (2020) found significant association with dog's breed category and hair coat length for acquiring *H. canis* infection. This could be due to the dogs fighting or biting habits, which put them at a higher risk of ingesting an infected tick on the prey or being exposed to ticks, which then infest the dogs (Baneth, 2011).

In haematology, there was no significant difference except for haemoglobin and monocyte count which revealed anaemia and monocytosis (Table- 2). These findings were in accordance with the studies of Sarma *et al.* (2012) and Lilliehook *et al.* (2019) reported that the *H. canis* infection was associated with anaemia and profound monocytosis. Elevated Blood urea nitrogen was found to have significant difference when compared to other parameters. This increase might be due to dehydration or due to renal amyloidosis or secondary glomerulonephritis in the chronic stage of the disease (Pawar and Gatne, 2005).

Parasitic DNA in ticks

A total of 548 ticks were collected (213 males, 335 females) from 47 dogs out of 482 dogs included in the study. Out of 47 pooled samples, eight were found positive for the DNA of *H. canis* in ticks amongst which, only one sample was positive in the host blood. The DNA of *H. canis* was found in the remaining seven tick samples, although these infected ticks were present on uninfected dogs. The possibility of these uninfected animals picking up the *H. canis* infection through the infected ticks is high. The tick being a three host tick, could have picked up *H. canis* from some other dogs during its larval or nymphal stages. Similar findings were observed by Azmi *et al.* (2017) who reported dogs carrying infected ticks were not positive themselves. Baneth (2011) stated that there was no evidence of *Hepatozoon* spp. during blood meal, salivary transfer from the final hematophagous vector host to the vertebrate intermediate host and ingestion of infected tick was the only mode of transmission.

Sequencing and phylogenetic analysis

The sequences obtained for the three *H. canis* isolates from Chennai, Tamil Nadu were highly similar to one another (99.4-100%) and > 99% identical to those of *H. canis* isolates

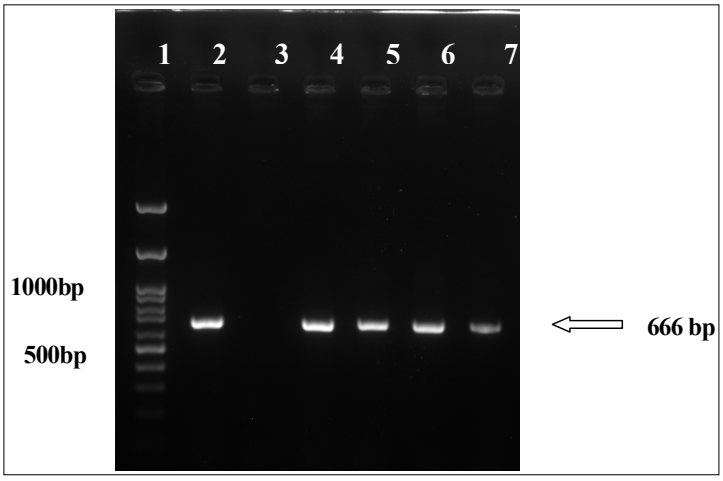

Fig 3: 666 bp PCR product of 18S rRNA gene of *H. canis* in 1.5 percent agarose gel.
Lane-1 Molecular weight marker, Lane-2 Positive Control, Lane-3 No template Control, Lane-4-7 Field samples tested.

Table 1: Statistical analysis on prevalence of *H. canis*.

Epidemiological determinants		Total number screened N= 482	No. of positives (n= 32)	Per cent positives	P-value
Age of the animal	Below 2 years	170	7	4.11	0.0731^{NS}
	25-48 months	106	4	3.77	
	49-72 months	93	9	9.68	
	73-96 months	48	3	6.25	
	97-120 months	31	4	13	
	Above 10 years	34	5	14.7	
Sex of the animal	Female	217	19	87.5	0.132^{NS}
	Male	265	13	49	
Breed of the animal	Beagle	10	1	10	0.0357*
	Cocker Spaniel	7	1	14.3	
	Dalmatian	7	1	14.3	
	Dobermann	27	2	7.4	
	Golden Retriever	12	2	16.7	
	Labrador	170	13	7.64	
	ND	72	3	4.17	
	Rottweiler	14	2	14.3	
	Spitz	50	6	12	
	Terrier	3	1	33.3	
	Others	110	0	0	

P value > 0.05^{NS} Non-significant, P value < 0.05 * significant difference.

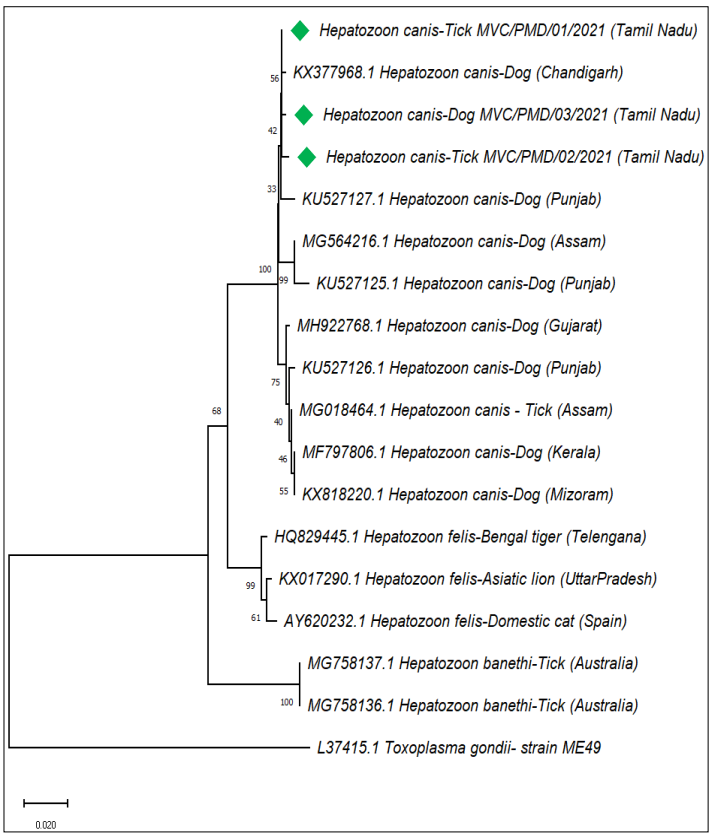

Fig 4: Phylogenetic analysis of *Hepatozoon canis* isolates identified in Chennai, Tamil Nadu. Phylogenetic analysis of *H. canis* partial (609bp) *18S rRNA* gene sequences identified in dogs and ticks from Chennai. GenBank accession numbers, *Hepatozoon* spp., host species, place of origin from where these sequences were derived are included for each sequence. The scale bar indicates the number of substitutions per nucleotide site. *Toxoplasma gondii* was used as an out group.

Table 2: Statistical analysis of blood parameters in *H. canis* infection.

Blood parameters		Reference values	*H. canis* positive mean± SE	P value
Haematology	Haemoglobin	12- 18 mg/L	9.5±0.66*	0.01
	RBC	5.5- 8.5 m/ cmm	4.3±0.27	0.7
	WBC	6000-17000/ cmm	13400±1512	1.96
	Platelets	200000-500000/ cmm	84787.5±16515.7	0.71
	Neutrophils	60-70%	76.6± 1.15	0.53
	Lymphocytes	20-30%	19±1.7	0.39
	Monocytes	0-5%	5.25±0.27*	0.01
Biochemical parameters	BUN	8-28 mg/dL	52.9±9.9*	0.03
	Creatinine	0.5-1.5 mg/dL	3.37±0.87	0.09
	ALT	10-109 IU/L	69.7±9.4	0.16
	Total Bilirubin	0.15-0.5 mg/dL	0.92±0.21	0.74
	Total protein	5.4-7.5 g/dL	7.12±0.2	0.68
	Blood glucose	76-119 mg/dL	100.7±8.68	0.77

Data was expressed as Mean ± SE, P values were calculated for individual parameter, P value >0.05; ^{NS}Non-significant, P value < 0.05; * significant difference.

from Brazil (KP233215), Croatia (FJ497018), Pakistan (KU535868), Nigeria (JX027010) and Italy (GU376453). The phylogenetic analysis produced a tree (Fig 4) with the Indian isolates of *H. canis* positioned within a clade with strong bootstrap support (100%) that contained all other isolates of *H. canis* from India to the exclusion of all other species in the genus (*e.g.*, *H. felis*, *H. banethi* and *H. americanum*). The results of the phylogenetic analyses revealed that *H. canis* from dogs and ticks in Chennai, Tamil Nadu belonged to the same clade with all other isolates of *H. canis* from India and there was very strong statistical support for separation of this clade from all other species of *Hepatozoon* that included *H. americanum*, *H. banethi* and *H. felis*. Sequences of *H. canis* obtained from two ticks and one dog blood sample were assigned the accession numbers OK181857, OK181864 and OK181882 respectively. To the best of our knowledge, this is for the first time 18S rRNA gene of *H. canis* was sequenced in tick pooled sample which was collected from the corresponding infected dog.

CONCLUSION

This report provides the molecular evidence regarding the existence of *H. canis* in *R. sanguineus* ticks collected from dogs in Chennai. *H. canis* detected in the present study revealed a high sequence identity with *H. canis* isolates from Brazil, Croatia, Pakistan, Nigeria and Italy. Because dogs can serve as hosts for infected ticks, determining the prevalence of *Hepatozoon* infection in *R. sanguineus* ticks is required for canine hepatozoonosis prevention. Further research focusing on the detection of *Hepatozoon* species in various tick vectors parasitizing stray and domestic dogs would help to clarify the significance of *Hepatozoon* genetic diversity in Chennai.

Ethical statement

All the blood samples and tick samples were collected from the infected dogs for the diagnostic purpose. Animals were treated in a humane non-invasive method and all the procedures carried out in this study were in accordance with the ethical standards of the institution where the study was conducted.

Conflict of interest: None.

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