Original Article

Survey of *Ehrlichia canis*, *Babesia* spp. and *Hepatozoon* spp. in dogs from a semiarid region of Brazil

Pesquisa de *Ehrlichia canis*, *Babesia* spp. e *Hepatozoon* spp. em cães de uma região semiárida do Brasil

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Abstract

This study assessed the occurrence of *Ehrlichia* spp., *Babesia* spp. and *Hepatozoon* spp. infections in 100 tick-harboring dogs from a semiarid region of the State of Paraíba, Northeastern Brazil. Blood samples and ticks were collected from the animals, and a questionnaire was submitted to dog owners to obtain general data. Blood samples were used to perform hemogram, direct blood smear and immunological and molecular hemoparasite detection. The 1,151 ticks collected were identified as *Rhipicephalus sanguineus*; direct smears revealed *E. canis*-like morulae in the monocytes of 4% (4/100) of the non-vaccinated female dogs, and 34% and 25% of the dogs tested positive for *Ehrlichia canis* by indirect immunofluorescence assay (IFA) and polymerase chain reaction (PCR), respectively. Blood smear examination revealed *Babesia*-suggestive merozoites in the erythrocytes of 2% (2/100) of the animals. *Babesia vogeli* was detected by PCR in ten animals (10%) and was correlated with young age (p = 0.007) and thrombocytopenia (p = 0.01). None of the animals showed *Hepatozoon* spp. positivity. These results indicate that *E. canis* is the main tick-borne canine pathogen in the study area and provide the first report of *B. vogeli* infection in dogs from Paraiba State.

Keywords: *Ehrlichia*, *Babesia*, *Hepatozoon,* dogs, Northeastern Brazil.

Resumo

O presente estudo avaliou a ocorrência de infecção por *Ehrlichia* spp., *Babesia* spp. e *Hepatozoon* spp. em 100 cães, infestados por carrapatos, oriundos de uma região semiárida do Estado da Paraíba, Nordeste do Brasil. Amostras de sangue e de carrapatos foram coletadas dos animais, e um questionário foi submetido aos proprietários dos cães para obter dados gerais. As amostras de sangue foram utilizadas para realização de hemograma, esfregaço sanguíneo e detecção molecular dos hemoparasitos. Os 1.151 carrapatos coletados foram identificados como *Rhipicephalus sanguineus*; os esfregaços sanguíneos revelaram mórulas sugestivas de *E. canis* em 4% (4/100) de cães fêmeas não vacinadas, e 34% e 25% dos cães foram positivos para *Ehrlichia canis* pela imunofluorescência indireta (IFI) e reação em cadeia pela polimerase (PCR), respectivamente. Os esfregaços sanguíneos revelaram merozoítas sugestivas de *Babesia* em eritrócitos de 2% (2/100) dos animais. *Babesia vogeli* foi detectada por PCR em dez animais (10%) e foi correlacionada com a idade jovem (p=0,007) e trombocitopenia (p=0,01). Nenhum dos animais apresentou positividade para *Hepatozoon* spp. Esses resultados indicam que *E. canis* é o principal patógeno canino transmitido por carrapato, na área estudada, e fornece o primeiro relato de infecção por *B. vogeli* em cães do Estado da Paraíba.

Palavras-chave: *Ehrlichia*, *Babesia*, *Hepatozoon*, cães, Nordeste do Brasil.

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Introduction

Tick-borne diseases are important cause of morbidity and mortality in dogs worldwide, and the brown dog tick *Rhipicephalus sanguineus* has been implicated as vector of several pathogens (DANTAS-TORRES, 2008). In Brazil, the main tick-borne pathogens that have been described for dogs are *Babesia vogeli*, *Ehrlichia canis*, *Anaplasma platys*, *Hepatozoon canis*, and *Mycoplasma haemocanis* (RAMOS et al., 2010; SANTOS et al., 2009; SPOLIDORIO et al., 2009, 2011).

E. canis is the primary etiologic agent of canine monocytic ehrlichiosis (CME), which is a multisystemic disease manifesting in acute, subclinical or chronic forms, according to the virulence level of the *E. canis* strain and the presence of co-infection with other arthropod-borne pathogens, such as *Babesia* spp. and *H. canis* (GAL et al., 2008).

Babesiosis is an emerging tick-borne disease affecting animals and humans, caused by intraerythrocytic protozoa of the genus *Babesia* (IRWIN, 2009). In Brazil, two *Babesia* species infecting dogs have been reported, *B. vogeli* (PASSOS et al., 2005) and *B. gibsoni* (TRAPP et al., 2006). *B. vogeli* is the most widespread canine piroplasm due to the cosmopolitan nature of its invertebrate host, the brown dog tick *R. sanguineus*. All *Babesia* species may cause pyrexia, anorexia, splenomegaly, anemia and severe thrombocytopenia (IRWIN, 2009).

The *Hepatozoon* species known to infect dogs are protozoans transmitted through the ingestion of infected ticks (SPOLIDORIO et al., 2009, 2011). Veterinarians usually misdiagnose the infection because the general symptoms are similar to those of other tick-borne diseases, such as ehrlichiosis and babesiosis (MURATA et al., 1991). Two *Hepatozoon* species have been described in dogs, including *H. canis* and *H. americanum* (BANETH et al., 2000, 2007); the former is responsible for all Brazilian cases reported in domestic dogs (SPOLIDORIO et al., 2011).

This study aimed to assess the occurrences of *E. canis*, *Babesia* spp. and *Hepatozoon* spp. infections in dogs from the semiarid region of the State of Paraíba, Northeastern Brazil, using direct examination, serology and molecular detection methods and to identify factors associated with these infections.

Materials and Methods

Study area and dog sampling

This study was carried out in the municipality of Patos (7°1'S, 37°19'W) located in the State of Paraíba in the semiarid region of Northeastern Brazil. This locality has an average temperature of 32°C, relative humidity of 55% and annual rainfall of 700 mm. One hundred tick-harboring dogs attended at the Veterinary Hospital of the Federal University of Campina Grande from April to September of 2012 were selected for this study. A questionnaire was submitted to dog owners to obtain general information, such as the education and income of the owner (minimum wage), the sex and age of the dog, breed, management, food intake, vaccination and deworming statuses, contact with other animals (other dogs, cats, horses, pigs, and wild animals), the condition of the floor of the dog's premises, the cleaning of the dog's premises, and the frequency of cleaning.

Tick collection and identification

Ticks were randomly collected from the animals during a five-minute examination of each animal, and they were then submerged into 70% ethyl alcohol in polypropylene tubes and stored at room temperature until identification. Tick taxonomic identifications were performed according to Barros-Battesti et al. (2006).

Blood sample collection

Blood samples were collected by cephalic or jugular venipuncture into Vacutainer tubes containing sodium citrate. Direct examinations of the hemogram and smear were carried out immediately after blood collection. Reference values (RV) according to Jain (1993) were as follows: leukocytes (RV: $6\text{-}17\times10^3/\mu l$), erythrocytes (RV: $5.5\text{-}8.5\times10^6/\mu l$), and platelets (RV: $2\text{-}5\times10^5/\mu l$). Aliquots of plasma and whole blood were stored at $-20°C$ for subsequent use.

Indirect immunofluorescence assay (IFA)

The presence of anti-*E. canis* antibodies in the plasma was assessed by an Indirect immunofluorescence assay IFA using glass slides coated with crude antigens of *E. canis* (São Paulo strain) as previously described (AGUIAR et al., 2007). Plasma samples were initially tested at a screening dilution (1:80) (KRAWCZAK et al., 2012) and then re-tested with serial two-fold dilutions of up to 1:1280. A commercial fluorescein-labeled rabbit anti-canine IgG (Sigma-Aldrich, 3050 Spruce St. Louis, MO 63103, USA) was used as the secondary antibody.

Molecular analyses

Total DNA was extracted from canine whole blood using a commercial DNA extraction kit (Wizard Kit for DNA Extraction®) according to the manufacturer's instructions (Promega) and eluted in 50 μL of the elution buffer accompanying the extraction kit.

Assays to detect *E. canis* DNA were performed using a TaqMan real-time PCR (qPCR) system targeting a portion of the *Ehrlichia* disulfide bond formation protein-encoding gene (*dsb*) with the primers dsb-321 (5'-TTGCAAAATGATGTCTGAAGATATGAAACA-3') and dsb-671 (5'- GCTGCTCAACCAAGAAATGTATCCCCTA–3') and the *E. canis*-specific probe (5'-FAM AGCTAGTGCTGCTTGGGCAACTTTGAGTGAA-BHQ-1–3') at a concentration of 25 μmol/L as previously described (DOYLE et al., 2005). Positive (DNA from *E. canis*-cultured DH82 cells) and negative (DH82 cells only) controls were included for all PCR assays. Samples with negative results, as shown by the *E. canis*-specific qPCR, were evaluated using conventional PCR with the primers dsb-330 (5'-GATGATGTCTGAAGATATGAAACAAAT-3') and dsb-728 (5'-CTGCTCGTCTATTTTACTTCTTAAAGT-3'), which were designed to amplify a 409-base pair (bp) fragment of the *dsb* gene of *Ehrlichia* spp. (LABRUNA et al., 2007).

For the detection of *Hepatozoon* spp. and *Babesia* spp. DNA, five µL of extracted DNA were used for conventional PCR with the primers HEP144169 (5'-GGTAATTCTAGAGCTAATACATGAGC-3') and HEP743718 (5'-ACAATAAAGTAAAAAACAYTTCAAAG-3'), which amplify a 574bp fragment of the 18S rRNA gene of *Hepatozoon* spp., and the primers BAB143-167 (5'-CCGTGCTAATTGTAGGGCTAATACA-3') and BAB694-667(5'-GCTTGAAACACTCTARTTTTCTCAAAG-3'), which amplify a 551-bp fragment of the 18S rRNA gene of *Babesia* spp., as previously described (ALMEIDA et al., 2012).

PCR products were electrophoresed on 1.5% agarose gel (Invitrogen, Carlsbad, CA), stained with ethidium bromide (AMRESCO, Solon, OH), and examined under UV illumination. Amplicons of the expected sizes were purified with ExoSap (GE Healthcare, Pittsburgh, PA) and sequenced with an automatic sequencer (Applied Biosystems, model ABI 3500 Genetic Analyzer) according to the manufacturer's protocol. The partial sequences obtained were subjected to BLAST analysis (ALTSCHUL et al., 1990) to determine the presence of any similarities to corresponding sequences.

Statistical analysis

Risk factor analyses were performed considering the serological or molecular detection of *E. canis*, *Babesia* spp. and *Hepatozoon* spp. as the dependent variables and the data obtained from the questionnaire and hemogram as independent variables. The association between the dependent and independent variables was assessed in 2 steps with univariate and multivariable analyses. The variables presenting $P \leq 0.20$ as determined by the chi-square test in the univariate analysis were selected and subjected to multivariate analysis using the logistic regression stepwise forward method with a 5% significance level (HOSMER & LEMESHOW, 1989). The serological results were analyzed using the following dichotomic variable: (i) dogs serologically negative or with a titer <80 (0), and (ii) dogs with a titer ≥80 (1) for *E. canis*. The kappa index (THURSFIELD, 2007) was used to compare the IFA with the PCR and direct blood smear results for *E. canis*. Mean tick infestations among dogs positive and negative for *E. canis* or *Babesia* spp. were compared by the *t*-Student test. All analyses were performed with SPSS 13.0 for Windows.

Ethical considerations

The present study was submitted to and approved by the Research Ethics Committee of the Universidade Federal de Campina Grande, protocol number 07/2012, prior to initiation.

Results

Hemogram analysis revealed that 52% (52/100) of the dogs were anemic, 12% (12/100) were leukopenic, and 37% (37/100) exhibited decreased platelets. Direct blood smear examination revealed *E. canis*-like morulae within the monocytes of 4% (4/100) of the dogs and *Babesia*-suggestive merozoites in the erythrocytes of 2% (2/100) of the animals.

E. canis antibodies were detected in 34% (34/100) of the tested dogs, with titers ranging from 80 to ≥ 1280. Among the 100 tested dogs, 9, 6, 6, 3, and 10 had endpoint titers of 80, 160, 320, 640, and ≥ 1280, respectively. *E. canis* DNA was detected by qPCR in 25% (25/100) of the dogs. None of the qPCR-negative samples were positive according to the *Ehrlichia* genus-specific PCR, indicating the exclusive occurrence of *E. canis* in the studied population.

From the 100 canine samples examined, 10 yielded PCR amplicons for *Babesia* spp., which were confirmed by DNA sequencing. By BLAST analysis, the sequences obtained from the *Babesia*-specific PCR were shown to be 100% identical to available sequences from *B. vogeli* (JF825145.1, AY371196.1, EF052627.1, AY371195.1, and DQ297390.1). A partial sequence (18S rRNA) generated from one of the dogs was deposited into GenBank under the accession number KJ494656. None of the canine blood samples yielded amplicons in the *Hepatozoon*-specific PCR.

Age was revealed to be a risk factor for babesiosis (p = 0.007; odds ratio [OR] = 0.197; confidence interval [CI] 95% = 0.061-0.640; standard error [SE] = 0.601), with a higher frequency observed in the younger dogs (6-12 months, Table 1). A significant correlation was found between decreased platelets and *B. vogeli* occurrence (p = 0.01). Based on the serological results, the act of not vaccinating dogs was associated with an increased risk of ehrlichiosis (p = 0.007; OR = 3.921; 95% CI = 1.462–10.512; SE = 0.503). Based on the qPCR results, the females were 2,705 more likely to develop ehrlichiosis than the males (p = 0.041; 95% CI = 1.040-7.036; SE = 0.488).

Table 1. Selected variables in univariate analysis associated with the *Babesia* spp.-specific PCR results for the dogs attended at the Veterinary Hospital of the Federal University of Campina Grande, Patos, Paraíba, Brazil.

Variable	Category	Number	Positive (%)	*P*-value
Age (months)	6-12	36	8 (22.2)	0.009
	12-48	23	1 (4.3)	
	>48	41	1 (2.4)	
Food	Commercial	19	3 (15.8)	0.170
	Homemade	23	0 (0)	
	Commercial / Homemade	58	7 (12.1)	
Floor of dog's premises	Ground	11	0 (0)	0.105
	Cement	49	8 (16.3)	
	Ground / Cement	40	2 (5)	

The kappa index revealed fair agreement between the qPCR and IFA results in terms of *E. canis* diagnosis (p = 0.0037), i.e., 55% (55/100) of the dogs were negative according to qPCR and IFA, and 14% were positive according to both tests. The kappa value for both tests was 0.2619. Poor agreement was observed between the IFA and PCR (k = 0.03) and between the IFA and blood smears (k = 0.0741). The hematological disorders of anemia, leukopenia and thrombocytopenia showed statistically significant associations (p ≤ 0.05) with the qPCR results for *E. canis*.

From the 100 tick-harboring dogs, 1,151 ticks were collected, giving an overall mean infestation of 11.5 ticks/dog (range 1 – 34). Mean tick infestations were statistically similar (*P* >0.05) among positive and negative dogs for *E. canis* or *B. vogeli*, with mean values of 10.2 and 12.2 ticks/dog for *E. canis* serologically positive and negative dogs, respectively; 10.0 and 12.0 ticks/dog for *E. canis*-PCR positive and negative dogs, respectively; and 14.6 and 11.2 ticks/dog for *B. canis*-PCR positive and negative dogs, respectively.

Discussion

R. sanguineus was the only tick species found on the dogs evaluated in this study. According to Tanikawa et al. (2013), the warm weather that occurs in the city of Patos, Paraiba State, which is located in the semiarid region of Northeastern Brazil, is an important factor contributing to the dissemination of this tick species, while at the same time precluding the establishment of many other tick species, which usually require higher moisture during their off-host developmental stages. *R. sanguineus* ticks are considered less dependent for survival upon moisture-rich habitats, allowing for their establishment in regions with unfavorable water balances (YODER et al., 2006). This characteristic contributes to the establishment of these ticks in semi-arid regions, such as that of the present study.

IFA antibody titers for *E. canis* ranging from 80 to ≥ 1280 were found in 34% of the animals. A similar seroprevalence has been previously observed by Carlos et al. (2007) and Souza et al. (2010) in Northeastern Brazil. These high titers are consistent with prolonged infection and chronic antigenic stimulation (BARTSCH & GREENE, 1996).

E. canis DNA was detected by qPCR in 25% of the dogs. This prevalence is higher than those previously observed by Carvalho et al. (2008) and Tanikawa et al. (2013). The high sensitivity of qPCR (DOYLE et al., 2005; HARRUS & WANER, 2011) may explain this difference in comparing the present results with previous results based on conventional PCR. Furthermore, the selection of tick-harboring dogs may have contributed to the higher *E. canis* detection in our study.

According to Solano-Gallego et al. (2006), epidemiological factors related to climatic conditions, vector distribution, population, animal behavior and habitat as well as the investigation methods used can affect the calculation of the prevalence of canine ehrlichiosis in Brazil. None of samples that were negative according to qPCR were positive according to the *Ehrlichia* genus-specific PCR, indicating the sole existence of *E. canis* in the studied population. At least four *Ehrlichia* species have been reported in Brazil:

E. canis, infecting mainly dogs (VIEIRA et al., 2011); *E. ewingii* infecting dogs (OLIVEIRA et al., 2009), *E. chaffeensis* infecting deer (MACHADO et al., 2006), and *E. mineirensis* infecting cattle (CRUZ et al., 2012; AGUIAR et al., 2014); however, only *E. canis* has been reported in the Northeast region (SOUZA et al., 2010, VIEIRA et al., 2011), which is likely related to the higher occurrence of the tick *R. sanguineus* in this region.

In 20% (20/100) of the samples, *E. canis* antibodies were detected by the IFA test in the absence of DNA amplification by qPCR. The IFA test as an additional serological method can generate false-positive results; furthermore, it does not differentiate active infection from previous exposure to an organism (VIEIRA et al., 2011). In addition, anti-*E. canis* antibodies can persist and be detected after healing. On the other hand, the ability of conventional PCR to detect *Ehrlichia* DNA in seropositive dogs depends on the sample source and target gene (HARRUS et al., 2004). The ability of qPCR to detect ehrlichial DNA, indicating active infection, rather than detecting an anti-*E. canis* antibody, which is suggestive of exposure, constitutes an advantage that allows for clinicians to better monitor treatment progress (HARRUS & WANER, 2011).

These results demonstrate that CME is endemic in the city of Patos, suggesting either previous exposure to the agent or the presence of active infection. Hence, this disease must be included as a part of the differential diagnosis in routine veterinary care in this locality and considered as a public health issue. Some *E. canis* strains are capable of infecting humans (PEREZ et al., 2006), and although it is unusual, the parasitism of humans by *R. sanguineus* has been described (DANTAS-TORRES, et al., 2006).

Out of the samples analyzed for *E. canis*, 55% (55/100) were qPCR and IFA negative, and 14% were positive by both tests. The kappa value (k = 0.2619) showed fair agreement between these two tests, with a significant p-value (p = 0.0037), indicating the lack of a significant difference between the two techniques. Poor agreement was observed between the IFA and PCR and blood smear tests (k = 0.03 and k = 0.0741, respectively). This can be attributed to the poor sensitivity of the cytology test, which was successful in only 4% of the cases and has been shown to be more sensitive during the acute phase of infection (WOODY & HOSKINS, 1991). Furthermore, false positives can occur if morulae are misidentified as platelets, lymphocytic azurophilic granules or nuclear phagocytosed material (BREITSCHWERDT et al., 1998).

Based on the serological results, the non-vaccination of dogs was considered a risk factor for ehrlichiosis (Table 2). Although commercial canine vaccines for different bacterial and viral diseases do not specifically protect dogs against *E. canis*, in general, immunization against the main dog diseases promotes good health and prevents the immune decline that increases vulnerability to ehrlichiosis. Furthermore, the simple act of vaccinating is reflective of a careful owner who is attentive to their dog's well-being, likely resulting in the less frequent exposure of the animal to ticks, and consequently, to ehrlichial infection.

The qPCR results revealed that the females were 2.705 more likely to develop the disease than the males (p = 0.041). No correlation has been found between the gender of the dog and disease occurrence by Carvalho et al. (2008) and Santos et al. (2013). The processes of estrus and parturition that occur in females lead to immune system depression. This could explain

Table 2. Selected variables in univariate analysis associated with the *Ehrlichia canis*-specific IFA and PCR results for the dogs attended at the Veterinary Hospital of the Federal University of Campina Grande, Patos, Paraíba, Brazil.

Variable	Category	Number (N)	IFAT (Titer ≥80)		PCR	
			N positive dogs (%)	*P*-value	N positive dogs (%)	*P*-value
Dog´s owner income (minimum wage)	< 2	38	9 (23.7)	0.215	7 (18.4)	0.618
	2-4	53	23 (43.4)		15 (28.3)	
	5-6	4	1 (25)		1 (25)	
	>6	5	1 (20)		2 (40)	
Dog's gender	Male	50	18 (36)	0.833	8 (16)	0.065
	Female	50	16 (32)		17 (34)	
Age (months)	6-12	36	19 (52.8)	0.011	9 (25)	0.4
	12-48	23	6 (26.1)		8 (34.8)	
	>48	41	9 (22)		8 (19.5)	
Breed	Mixed breed	59	21 (35.6)	0.850	12 (20.3)	0.197
	Pure breed	41	13 (31.7)		13 (31.7)	
Floor of dog's premises	Ground	11	1 (9.1)	0.158	2 (18.2)	0.838
	Cement	49	17 (34.7)		13 (26.5)	
	Ground/Cement	40	16 (40)		10 (25)	
Vaccination	Yes	78	21 (26.9)	0.011	20 (25.6)	1.000
	No	22	13 (59.1)		5 (22.7)	

the vulnerability of this gender to ehrlichiosis. The hematological disorders of anemia, leukopenia and thrombocytopenia showed statistically significant associations (p ≤ 0.05) to *E. canis*-positive qPCR results. Bulla et al. (2004) and Dagnone et al. (2003) have recommended that ehrlichiosis should be included in the differential diagnosis of anemic and thrombocytopenic dogs.

Out of the 100 canine samples examined, 10 yielded PCR amplicons for *Babesia* spp., which were confirmed as *B. vogeli* by DNA sequencing. This is the first report of *B. vogeli* in dogs from Paraiba State, and the frequency of positive animals obtained (10%, 10/100) is higher than that reported by Ramos et al. (2010) in another area of Northeastern Brazil. This higher frequency could be related to the fact that all sampled dogs were from areas where they had probably been exposed to *R. sanguineus*, since all of them were infested by this tick species when they were sampled for this study.

Age was considered a risk factor for babesiosis (p = 0.007), with a higher frequency observed in the younger dogs. According to Brown et al. (2006), young dogs that have been weaned are particularly likely to be exposed to concomitant diseases because malnourishment due to a scavenging lifestyle greatly predisposes these animals to infection. A significant correlation was observed between a decrease in platelets and *B. vogeli* (p = 0.01) occurrence, which has been previously reported by Santos et al. (2009) and Brown et al. (2006). The mechanism of platelet damage by *B. vogeli* is poorly understood. In human chronic babesial infections, it has been suggested that spleen enlargement results in an increase in the pooling of platelets, diminishing circulating thrombocytes (PANTANOWITZ, 2002).

None of tested samples was positive for *Hepatozoon* spp., as determined by PCR. This could be attributed to the fact that *R. sanguineus* was the sole tick found on the studied animals. *R. sanguineus* is considered to be the main *H. canis* vector in the Old World (BANETH et al., 2007), and its presence on dogs has been associated with pathogen infection (SPOLIDORIO et al., 2009). However, until now, there is no report of *R. sanguineus* naturally or experimentally infected with *H. canis* in Brazil (FORLANO et al., 2005; SPOLIDORIO et al., 2009; DEMONER et al., 2013). These negative results could be related to the different susceptibilities of *R. sanguineus* populations to *H. canis* because it has been shown that the taxon *R. sanguineus* is represented by at least two different species in Latin America (MORAES-FILHO et al., 2011).

PCR revealed that three animals possessed co-infection with *E. canis* and *B. vogeli*. This co-infection, which has been reported elsewhere (SANTOS et. al., 2009; RAMOS et al., 2010), occurs because these pathogens share the same vector, the brown dog tick *R. sanguineus*, which was predominant in the animals evaluated in this study.

Conclusions

The results from the present study revealed that *E. canis* was the main tick-borne pathogen of dogs in Patos and showed that *B. vogeli* is an important hemoparasite that should be included in the differential diagnosis performed by veterinary medical professionals. These findings are very important for the understanding of the epidemiology of tick-borne pathogens of domestic dogs in this semiarid region of Brazil.

Acknowledgments

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