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A survey of Ixodid ticks in stray dogs and molecular detection of *Ehrlichia canis* from ticks in central Iran

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Abstract: Ticks are well known as hematophagous ectoparasites, which cause different diseases in humans and animals. This investigation was designed in central Iran to assess the prevalence, intensity, and abundance of tick infestation in stray dogs and detection of *Ehrlichia canis*. Overall, 962 ticks were collected from 260 stray dogs between April and August 2018. DNA samples with the goal of amplification of pathogen were screened by real-time PCR assay. The prevalence of tick infestation on stray dogs was 60.3%. *Rhipicephalus sanguineus* sensu lato (s.l.) was only species found in stray dogs. Out of 42 tick pools, 4 (9.5%) were positive for *E. canis* in real-time PCR assay. This study showed the presence of *E. canis* for the first time in ticks infesting stray dogs in central Iran and proved that these ticks can emerge as a zoonotic disease.

Key words: Tick, *Rhipicephalus sanguineus* (s.l.), ehrlichiosis, real-time PCR assay, Iran

1. Introduction

Ticks are well known as hematophagous ectoparasites of significant health public and medical throughout the world [1]. After mosquitoes, ticks are the most common arthropods that may carry pathogens to humans and animals [2]. Canines are successively bitten by ticks, so they are a potential cause for vector-transmitted pathogens [3]. In general, stray dogs are more susceptible to tick infestation compared with owned and pet dogs [4]. Most species of tick are pathogenic in dogs because they can play a role as potential vectors in the incidence of infectious diseases to dogs due to their feeding behavior [4, 5]. One of the primary vectors that can feed on dogs is *Rhipicephalus sanguineus* (s.l.) [6]. However, other ticks species can also be parasites of dogs in several different parts of the world [7]. Infested dogs usually have no clinical symptom; however, in some cases, skin irritation, toxicosis, paralysis, dermatophytosis, allergy reaction, and myiasis due to skin damage may occur in these animals [8]. This tick naturally prefers dogs as the primary host, but accidentally it can adapt to other animals like birds, cats, rodents as well as humans by attaching to the body and feeding on blood [6]. *R. sanguineus* (s.l.) is involved in spread of many infectious diseases in dogs and humans that can affect both hosts. Most of these diseases also are considered zoonotic. The Mediterranean spotted fever, Rocky Mountain spotted fever, Babesiosis, Anaplasmosis, and Ehrlichiosis are diseases transmitted through this vector [9].

Canine monocytic ehrlichiosis (CME) is an emerging tick-borne disease caused by *Ehrlichia canis*, which is an intracellular obligatory parasite belonging to the order Rickettsiale with tropism of canine mononuclear cells [10]. According to investigation performed, E. *canis* has been detected in Iran's neighbor countries, such as Turkey [11], Pakistan [12], Saudi Arabia [13], Palestine [14], Israel [15], and even in southeastern Asia [16, 17, 18, 19, 20]. In Iran, this agent has been reported in domestic dogs for the first time in Kerman province [21]. Epithelial cells of salivary glands, midgut, and hemocyts of the tick are places where E. *canis* multiplies. Transmission in tick happens transstadially. So far, the transovarial transmission of the bacteria from adult stage to eggs has not been observed [22]. The diagnosis of CME is performed using specific techniques including blood smears, serology, and Polymerase change reaction (PCR) [23]. PCR assay is a technique for identifying and determining ehrlichiosis compared with other methods. To date, many varieties of PCR techniques have been applied for the diagnosis of this organism with degrees of sensitivity and specificity. Currently, quantitative real-time PCR (qPCR) as a sensitive method can be utilized to recognize *E. canis* in sick canines that are naturally and experimentally infected [10].

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To the best of our knowledge, the brown dog tick, *R. sanguineus* (s.l.) is the potential vector of CME in the world. But this agent has been identified in other ixodid ticks, including *R. bursa* in Italy [24] and France [25], *Dermacentor marginatus* and *Ixodes canisuga* in Hungary [26], *I. icinus*, *I. hexagonus*, *D. marginatus*, *R.bursa* and *R. pusillus* in some part of the Mediterranean basin [27], and *Haemaphysalis longicornis* in Korea [28]. In the northwest of Iran, it has been demonstrated that hard ticks such as *R. sanguineus* (s.l.) can contain *E. canis* [29]. However, there is a scarce information about this disease in the population of stray dogs and their tick in some parts of Iran particularly in central regions; therefore, this investigation was designed in Isfahan province, central Iran, to evaluation the prevalence, intensity, and abundance of tick infestation in stray dogs and detection of *E. canis* in the ticks isolated by using molecular assay.

2. Material and methods

2.1. Study area

This investigation was carried out in Isfahan province, which are located in central of Iran (Figure 1).

It includes an area of 107044 km^2 with coordinates 42′3832° N, 03′5140° E. This province is surrounded by South Khorasan and Yazd provinces to the east, Lorestan and Chaharmahal and Bakhtiari provinces to the west, Fars and Kohgiluyeh and Boyer-Ahmad provinces to the south and Semnan, Gom and Markazi provinces to the north. The province has four various seasons: winter (January to March), spring (April to June), summer (July to September), and fall (October to December). Despite being located in the highlands, Isfahan has hot summers with the maximum temperature of about 35 °C and dry climate. However, with low humidity and moderate temperatures at night, the climate is entirely pleasant. During the winter, days are cool while nights can be very cold. Snow falls an average of 7.8 days each winter.

2.2. Sampling method and tick collection

According to probable prevalence and statistical calculations with 10% accuracy and 95% confidence coefficient, the number of two hundred sixty stray dogs (125 male, 135 female) with an age range 2 months to 13 years were randomly chosen and inspected individually for the presence of ticks at the animal shelter for a

Figure 1. Location of Isfahan city in Iran, which this study is conducted, is shown on the map of Iran.

period of 4 months from April to August 2018. Most of the examined dogs were of mixed or indigenous breeds, which were brought to the animal shelter for numerous reasons including castration, vaccination and dewormed. Initially the gender and age of each dog were determined. Age index was obtained by dental formulary. Dogs were classified into three age groups (<1 year as puppies, 1-5 year as adolescent and 5< year as adult). Before removing of ticks from the whole body, each dog was restrained with a muzzle or injection of anesthetic drugs like ketamine. Eight body area (ear, head, neck, nose, belly, tail, backside, and inter-digital spaces) were completely checked for the existence of ticks. In case of an infestation in animals, ticks were carefully harvested with forceps, to ensure avoiding hurt to the mouthparts. The collected ticks were preserved into holding containers containing 70% ethanol alcohol solution, and characteristics of each sample including date, sex, age, sampling month, and number of ticks were recorded. The specimens were transmitted to the veterinary parasitology laboratory of Shahid Bahonar University of Kerman. In the laboratory, all specimens were investigated, and their gender, species and instar (larva, nymph, or adult) were identified on the basis of valid identification keys [30].

2.3. DNA extraction

After identification, specimens were rinsed with 0.9% physiological serum for 3 times to remove the remaining alcohol. Later, ticks were categorized into 42 pools with average number of 5–7 ticks each. Then, they were placed on a piece of sterile paper for 10 min to dry. Ticks were crushed by a sterile scalpel and transferred to a 1.5 cc sterile microtube, and 100 µL of pre-lysis buffer and 30 µL of protease K were added to them. DNA was extracted with a commercial DNA extraction tissue kit (Sinapure, Iran) according with the manufacturer's instructions. Then, the quantities of DNA extracted by Nano drap BioTek ELISA (model Epoch) were measured at the wavelength of 260 nm. After quantification, DNAs were stored at –20 °C until PCR amplification.

2.4. Touchdown-PCR assay (TD-PCR)

After DNA extraction from ticks**,** firstly touchdown-PCR was performed using specific primer pair, including Forward EHR16SD (5/-GGTACCYACAGAAGAAGTCC-3/) and Reverse EHR16SR (5/-TAGCACTCATCGTTTACAGC-3/) [15], designed with the purpose of amplifying 345 bp fragment of 16SrRNA gene of *Ehrlichia* spp. using Thermocycler (MJ-MIN model) manufactured by BIORAD company. The PCR was prepared in a total reaction volume of 20 µL, containing 2.5 µL of DNA pattern, 0.5 µL of each primer, 10 µL of prepared master mix (Amplicon, Denmark) along with 6.5 µL of distilled water. In this reaction, distilled water was used as a negative control in this reaction. PCR amplification was conducted under the following conditions: 94 °C for 3 min, 94 °C (2 cycles) for 30 s, 62 °C for 30 s, 72 °C for 30 s, 94 °C (2 cycles) for 30 s, 60 °C for 30 s, 72 °C for 30 s, 94 °C (2 cycles) for 30 s, annealing: 58 °C for 30 s, extention: 72 °C for 30 s, 94 °C (2 cycles) for 30 s, 56 °C for 30 s, 72 °C for 30 s, 94 °C (2 cycles) for 30 s, 54 °C for 30 s, 72 °C for 30 s, 94 °C (39 cycles) for 30 s, 52 °C for 30 s, 72 °C for 30 s and then final extention, 72 °C for 30 s. PCR products, such as the desired DNA fragments, were electrophoresed through 2% dyed agarose gel with DNA Green Viewer containing fluorescence dye and were visualized and photographed under UV in TBE buffer.

2.5. Real-time PCR assay (qPCR)

After TD-PCR assay and identification of *Ehrlichia* genus, all positive samples with the goal of amplification 350 bp fragment of *E. canis* dsb gene using a pair of specific primer designed including dsb Forward (5/-TTGCAAAATGATGTCTGAAGATATGAAACA-3/)with dsb Reverse (5/- GCTGCTCAACCAAGAAATGTATCCC CTA-3/) [31] were screened by real-time PCR. The reaction was run using the light cycler (Roche Diagnostics GmbH, Mannheim, Germany) in a total volume of 20 µL including 2.5 µL DNA, 0.5 µL of each primer, 10 µL ready master mix contains Eva Green fluorescent dye with 6.5 µL of distilled water. In this reaction, distilled water was used as a negative control. Then, the reaction was conducted in 96 wells plates (light cycler-Tube Strips White). qPCR reaction was conducted under the following conditions: primary denaturation: temperature 95 °C for 15 min in 1 cycle, denaturation: temperature 95 °C for 15 s in 40 cycles, annealing: temperature 65 °C for 20 s in 40 cycles, extention stage: temperature 72 °C for 20 s in 40 cycles, melting: first stage: temperature 95 °C for 10 s in 1cycle, second stage: temperature 65 °C for 60 s in 1 cycle and the third stage: temperature 97°C for 1 s. After amplification, the products were analyzed by IQ software v 3.1.

2.6. Statistical analysis

Data were entered and evaluated by Excel, and the significant relationship between variables such as age, sex, and months of the year with the prevalence of tick infestation was compared using chi-square tests or Fishers exact tests and performed by SPSS 20.0 statistical software. The level of statistical significance p-value <0.05 was considered.

3. Results

3.1. Collection and identification of tick species

Overall, 962 ticks including 797 (82.8%) mature, 129 (13.4%), nymph and 36 (3.7%) larvae were collected from 260 stray dogs. All ticks were identified based on certain characteristics such as reddish-brown, stretched shape, and hexagonal base of capitulum of *R sanguineus* (s.l.). One

hundred and fifty-seven stray dogs (60.3%) were positive for ticks. The mean severity and frequency of infection were 6.1 and 3.7, respectively.

3.2. Infestation of ticks in relation to sex, age, season and different organs

The prevalence of *R. sanguineus* (s.l.) tick infestation was 63.7% in female dogs and 56.8% in male dogs, so female dogs had the highest infestation compared to male dogs. Also, the highest prevalence of infestation found in <1 years old followed by 1–5 years old are shown in Table 1. The highest prevalence of infection was related to June and July, and the lowest prevalence was related to April ($p < 0.05$). In addition, the highest severity and frequency of infestation were related to April (7.2) and July (5.8), respectively, and the lowest severity and frequency of infestation were related to May (4.8) and April (2.06), respectively (Table2).

In the survey of tick distribution in different organs, ear (36.06%) followed by head and neck (23%) and backside (19.6%) had the highest and inter-digital space with 2.04% had lowest tick density (Figure 2).

3.3. Detection of *E. canis* in ticks

According to the TD-PCR results out of 42 tick pools, 4 (9.5%) were approved to be positive with *Ehrlichia* genus (Figure 3). So, the tick pools that were confirmed to be positive with those of *Ehrlichia* genus were detected as *E. canis* by real-time PCR assay (Figure 4).

4. Discussion

Ticks are obligatory ectoparasites whose survival depend on feeding their hosts blood. It is estimated that about 10% of them are vector for many diseases that can affect humans and animals, both domestic and wild. One of the general concerns about dog infestation with ticks is the increase in zoonotic tick born disease. The present survey aimed to investigate the faunal of tick in stray dogs and to confirm *E. canis* infection in ticks in Isfahan. In total 962, ticks including adult, nymph and larvae stages were isolated, all of which were detected based on morphological characteristics of *R. sanguineus* (s.l.).

This tick was only species found in our study among stray dogs in Isfahan. The results corresponded with studies conducted in some parts of Iran such as Ilam [32], Ahvaz [33], Qazvin, and Guilan [2] as well as countries such as Pakistan [34], Nigeria [35], Taiwan [36], and Algeria [37]. *R. sanguineus* (s.l.) has global distribution and is considered as a common species in dogs although it can feed on other animals randomly. From an ethological

Table 1. Infestation of ticks in relation to risk factors of sex and age of dogs.

Risk factors	Number of examined dogs	Number of infested dogs	Tick prevalence %	p-value
Sex				
Male	125	71	56.8	
Female	135	86	63.70	$p > 0.05$
Total	260	157	60.3	
Age				
<1	71	43	60.5	
1-5	137	85	62.04	$p > 0.05$
>5	52	29	55.7	
Total	260	157	60.3	

Table 2. Month prevalence of ticks' infestation of dogs in the Isfahan city.

Month	Number of examined dogs	Number of infested dogs	Tick prevalence %	Level of infestation	Number of ticks	Tick abundance	Infestation intensity	P-value	Temperature °C		Rainfall (mm)	Humidity (%)
									Mean	Min-Max		
April	87	25	28.7	Low	180	2.06	7.2	$p < 0.05$	16.4	10.4-22.4	23.4	46
May	66	42	63.6	Medium	204	3.09	4.85	-	20.7	14.2-27.2	17.7	44
June	57	46	80.7	High	285	5	6.19	-	28.5	21.2-35.8	2	21
July	50	44	88	High	293	5.86	6.65	-	29.7	21.7-37.8	0.0	13
Total	260	157	60.3	-	962	-	-	-	-	-	-	-

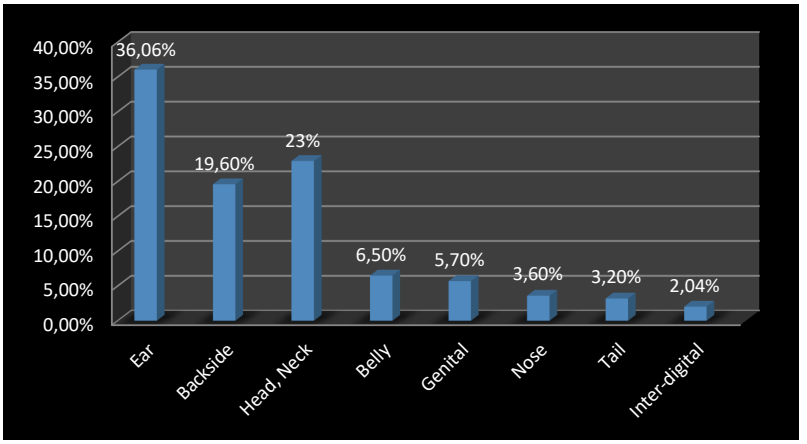

Figure 2. Distribution of the ticks in different parts of the dogs' body.

viewpoint, this tick is an endophilic, monotropic, and three-host tick species. These species are seen frequently in tropical and subtropical areas [38].

In the present study, 60.3% of dogs were infested with ticks, which were consistent with the findings of Sahu et al. [4] and Abuzeid et al. [39], which have been recorded as the prevalence of 58.3% and 60% in dogs, respectively. One of the reasons for the high incidence of tick infestation in stray dogs can be attributed to their roaming in natural environments and different places. In addition, this group of dogs is less treated against diseases than owner dogs, so the possibility of them of being infected is not unexpected with ectoparasites, especially ticks [34].

In terms of relationship between infestation and sex, female dogs (63.7%) had the highest levels of infestation compared to male dogs (56.8%) ($p > 0.05$), that was similar with studies conducted by Memon et al. [34] and Shitta et al. [40], which observed higher tick infestation in female dogs.

One of the reasons for the susceptibility of female dogs to infestation can be attributed to their feeding habit during pregnancy because they usually feed more than male dogs during this period and are searching for food in the environment, which facilitates their contact with ticks. Also, female dogs are usually less active when taking care of their puppies, which can lead to infestation with ticks [41]. In the present study, adolescent dogs less than 1 year old were more contaminated with ticks compared to adult dogs (Table 1). These results were similar to the results of Hadi et al. [42], Hassissen et al. [37], and Opeyemi et al. [35], which stated in their research that young dogs were less resistance to tick infestation than middle-aged and adult dogs. This situation could be due to lack of resistance in younger dogs and can be attributed to the immune system because they don't have efficient immune response against infestation compared to older dogs [37]. According to the

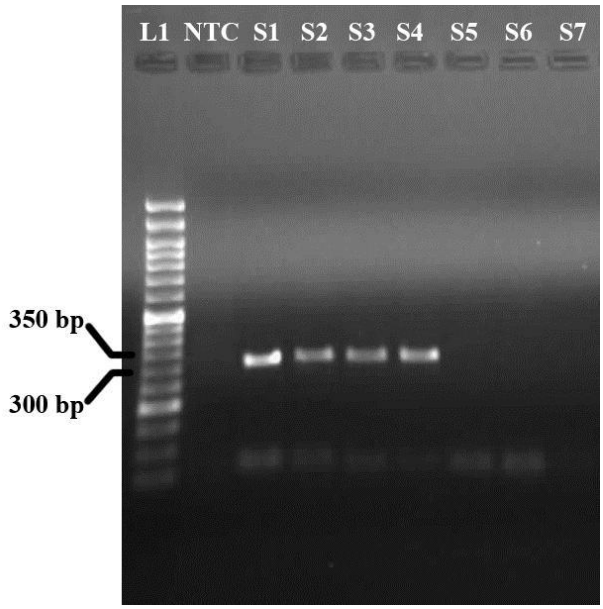

Figure 3. Agarose gel containing a number of samples infected with *Ehrlichia* spp. L1:50bp, NTC: negative, S1, S2, S3, S4, positive samples containing 345 bp fragment, S5, S6, S7, negative samples.

results of table 2, ticks were found on dogs in 4 months of the hot seasons of the year. An increase in number was seen in June and July with a maximum temperature of 35.8 and 37.8 °C, respectively and there was a significant relationship between infestation and sampling month ($p < 0.05$).

These results were consistent with the report obtained from the United States, France [38], and Nigeria [41], showing the peak activity of ticks in temperate areas and warm months of the year. However, it was not similar to the results obtained from Algeria [37], which reported the peak of *R. sanguineus* (s.l.) activity in April and May.

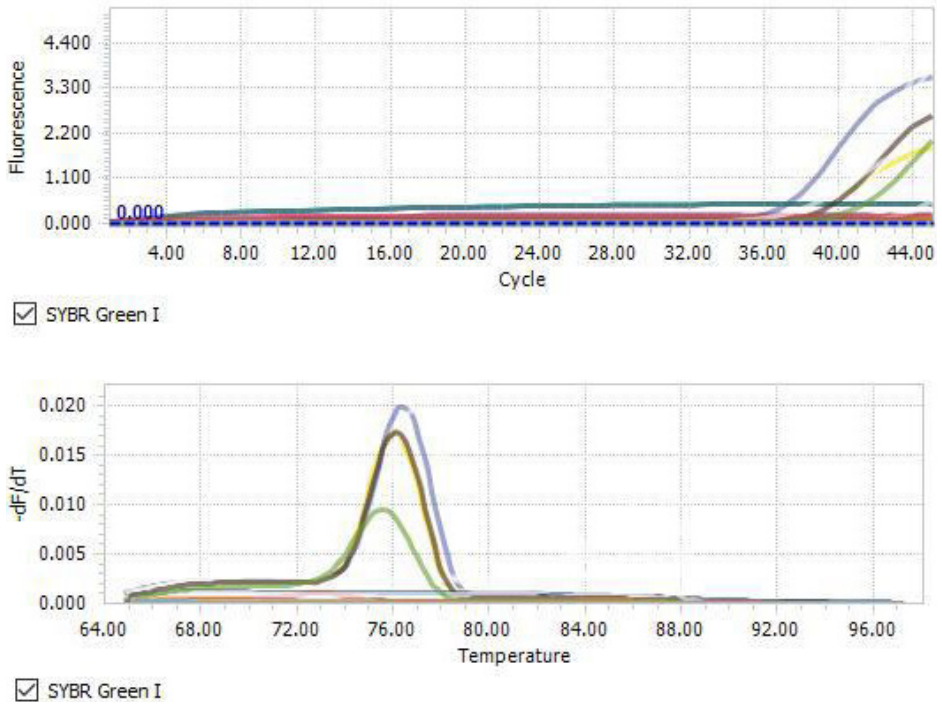

Figure 4. Curves related to CQ (High) and TM (bottom) of positive samples in terms of *Ehrlichia canis*. In the image above, the curves of 4 positive samples in terms of *Ehrlichia canis* in blue, brown, yellow, and green colors are observed with CQ of 36.9, 39.1, 39.1 and 40.7, respectively. In the bottom image, the curves related to Tm are the same positive examples in terms of the existence of *Ehreliccia canis* in blue, brown, yellow, and green colors, all ranging from 75.9 to 76.1 °C.

Isfahan city has a temperate and dry climate and most of the climate change, especially the increase in temperature and the downward trend of rainfall pattern usually occur in the warm months of the year, which can have favorable effects on growth and development as well as the population of ticks in these months of the year. In the study of tick distribution in different parts of the body, ear, backside, head, and neck had the highest infestation compared to other parts, which was in accordance with previous reports [42, 43, 37]. In another study, hind leg, head, and tail were showed to be preferred sites [34]. One of the possible causes of infestation of such sites can be attributed to the lack of access of dogs to remove ticks from these areas using their claws [44].

In this survey, we confirmed the existence of *E. canis* for the first time in central Iran with using real-time PCR assay in 9.5% *R. sanguineus*(s.l.) ticks and revealed that this tick can be a competent vector for Canine monocytic ehrlichiosis in stray dogs in this region.

These findings were in accordance with studies conducted in Cameroon [45] and Israel [10] that recorded the evidence of *E. canis* in *R. sanguineus*(s.l.) ticks 6% and 10%, respectively but lower than that reported in northwestern Iran [29] and Malaysia [19] with prevalence rates of 16.6% and 52.2%, respectively.

The presence of this pathogen in ticks in our study represents that this agent could be endemic in this region. In addition, previous studies have shown that this disease is endemic in Iran [21] such as Middle East countries [46]. Clinical signs of the disease in dogs in terms of steps acute, subacute, and chronic phases might be characterized by splenomegaly, lymphadenopathy, anorexia, lethargy, fever, anemia, and leukopenia. Most dogs usually carriers for a long period of time without overt evidence of disease [47, 20].

Canines serve as the main reservoir host for this microorganism in nature because this agent does not survive in the primary vectors tick for more than a generation [48]. Other than dogs, this pathogen can involve human; therefore, they are important in zoonotic and public health aspects. Recently, human cases of the disease with clinical symptoms have been detected in some countries of the American continent like Venezuela [49], Brazil [50], and Costa Rica [51]. In Iran, the disease was observed in the northern regions and in Mazandaran province between 2000 and 2002 in the patients who had clinical symptoms [52].

This study showed the high prevalence of *R. sanguineus*(s.l.) ticks in stray dogs and existence of *E. canis* for the first time in central Iran and proved that this tick

as a principal vector can play a major role in the incidence of epidemiology of this pathogen in stray dogs in this area and other parts of Iran as an emerging zoonotic disease. Due to the infestation, other animals with brown dog tick, further investigation by using other molecular method is required to screen this species and its role in transmitting ehrlichiosis in other animals and humans in Iran. So, the necessary measures could be taken to control and prevent this disease in dogs to be focused on the importance of ehrlichia infection in animals and its effects on human health.

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Conflict of interest
The authors declare that they have no conflict of interest.

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