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SPECIAL ARTICLE

Mosquito-borne pathogens . . . Now Rickettsias?

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Abstract In the last few decades, the emergence of several infectious diseases has increased. Mosquitoes are the most important vectors due to their role in transmitting several pathogens, such as *dengue*, *chikungunya* and *Plasmodium* spp. Recently, mosquitoes have been suspected of transmitting Rickettsias, another emerging pathogen. However, their role in the transmission of this agent remains unclear. In this study, we report the detection of a *Rickettsia* spp. in *Coquillettidia venezuelensis* mosquitoes collected from Corrientes Province, Argentina. The phylogenetic analyses show that this *Rickettsia* is related to *Rickettsia felis*, and the *ompB* sequence shows high homology with *Rickettsia tillamookensis*. The evidence of the detection of *Rickettsia* in mosquitoes shows that several infectious diseases could be transmitted by mosquitoes, and the interaction between them is important to investigate. More studies should be done in order to analyze the impact on public health.

PALABRAS CLAVE
Rickettsias;
Mosquitos;
Argentina

Patógenos transmitidos por mosquitos. . . ¿Ahora *rickettsias*?

Resumen En las últimas décadas, la aparición de enfermedades infecciosas ha ido en aumento. Los mosquitos son los vectores más importantes debido a que actúan como transmisores de diversos patógenos, entre ellos, dengue, chikunguña y *Plasmodium* spp. Recientemente, se ha sospechado que los mosquitos también pueden transmitir *rickettsias*, otro patógeno emergente. Sin embargo, el papel de los mosquitos en la transmisión de este agente es aun poco claro. En este estudio informamos la detección de *Rickettsia* spp. en la especie de mosquito *Coquillettidia venezuelensis*, de ejemplares capturados en la provincia de Corrientes, Argentina. Este

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representa el primer reporte de detección de ADN de *rickettsia* en el país. El análisis filogenético indica que esta *rickettsia* está relacionada con *Rickettsia felis* y la secuencia del gen *ompB* muestra una alta homología con *Rickettsia tillamookensis*. La detección de *rickettsia* en los mosquitos sugiere que estos insectos podrían ser los vectores de múltiples agentes causales de enfermedades infecciosas, lo que resalta la necesidad de investigar la interacción entre ellos. Se deben realizar más estudios con el fin de analizar el impacto de este hallazgo en la salud pública.

Introduction

In recent decades, the impact of several infectious diseases has increased. Their emergence may be attributed to factors such as increasing urbanization, changes in land use, global warming, increased human–animal contact, and the expanded global distribution of their vectors[37].

Rickettsiales are a group of bacteria that are obligate intracellular, Gram-negative microorganisms, primarily vectored by arthropods. Their importance in public health lies in the fact that they can cause severe human diseases, such as anaplasmosis, ehrlichiosis, and rickettsioses. Ticks have been considered the most important vectors of *Rickettsia*; however, other ectoparasites, such as fleas, mites, and midges, also act as vectors for different *Rickettsia* species[7].

Rickettsia is responsible for emerging and re-emerging diseases worldwide[30]. In nature, *Rickettsia* bacteria are spread by both transstadial and transovarial (i.e., vertical) transmission in their arthropod hosts, or by horizontal (co-feeding) transmission through an infected vertebrate. In Argentina, human cases of *Rickettsia* have been reported, including *R. rickettsii* and *R. parkeri*[26,27]. Additionally, *R. massiliae* has been detected in ticks[5], and *R. asembonensis* and *R. felis* have been recorded in fleas[1,17,20,21,29].

Mosquitoes are among the most important insects in terms of public health; their blood-feeding behavior is a critical determinant of pathogen transmission. It is well known that mosquitoes are the primary vectors of several arboviruses, such as dengue, chikungunya, and West Nile virus, and are the major vectors of *Plasmodium* spp., the causative agent of malaria[33]. Moreover, in recent years, *Rickettsia* have been detected in mosquito samples[12,18,19,31,32,36]. However, the role of mosquitoes in the transmission of *Rickettsia* and their potential impact on public health remain unclear. Additionally, no studies on *Rickettsia* involving these insects have been conducted in Argentina.

In order to gather more data on *Rickettsia* species in mosquitoes, the objective of this study was to analyze the presence of *Rickettsia* spp. in mosquito samples from northeastern Argentina using molecular biology methods.

Materials and methods

Mosquito collection and identification

In November 2019, mosquitoes were collected in Tabay, Corrientes (28°18′27″S, 58°17′13″W) during an entomological surveillance for yellow fever virus. This town is located 140 km from the city of Corrientes and has a population of 1700 inhabitants (Censo Nacional de Población, Hogares y Viviendas de la República Argentina, 2010). Manual captures were performed over three consecutive days, during the day, with morning and afternoon sessions lasting four hours (from 8 a.m. to 12 p.m. and from 2 p.m. to 6 p.m., respectively). Mosquitoes were stored in cryovials containing liquid nitrogen and shipped to the Instituto Nacional de Enfermedades Virales Humanas (INEVH-ANLIS) laboratory. Mosquito processing was performed as previously described by Goenaga et al.[11]. Briefly, upon arrival at the laboratory, the specimens were sorted by species and day of capture under a stereoscopic microscope on a chilled table. Specimens were morphologically identified using a dichotomous key developed by Darsie[5].

Pools were assembled with mosquitoes of the same species captured on the same day, and each was homogenized in a microtube containing a 3 mm tungsten bead and 1 ml of phosphate-buffered saline solution with 0.75% bovine albumin, penicillin (100 units/ml), and streptomycin (100 μg/ml) for 1 min at 20 cycles per second using a Bead Ruptor 24 Elite (OMNI International, Kennesaw, Georgia, USA). Homogenates were clarified by centrifugation at 5000 × *g* for 10 min at 4 °C.

Samples were processed using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, North Rhine-Westphalia, Germany) and eluted into final volumes of 60 μl.

The presence of *Rickettsia* spp. was screened by a specific PCR of the following genes: citrate synthase (*gltA*: 401 bp fragment and 834 bp fragment), outer membrane protein A (*ompA*), and outer membrane protein B (*ompB*) genes (Table 1). For the amplification, the PCR program started with an initial denaturation for 2 min at 98 °C, followed by 40 cycles (94 °C for 15 s, gene-specific annealing at 48 °C for 30 s, and 74 °C for 30 s), and a final extension step at 72 °C for

Table 1 Genes, primer names and sequences, and PCR amplification conditions used for *Rickettsia* detection.

Target gene	Primer name	Nucleotide sequence (5′–3′)	Annealing T (°C)	Product length (bp)	Reference
gltA	CS-78	GCAAGTATCGGTGAGGATGTAAT	48	401	15
	CS-323	CTTCCTTAAAATTCAATAAATCAGGAT			
	CS-239	GCTCTTCTCATCCTATGGCTATTAT	48	834	16
	CS-1069	CAGGGTCTTCGTGCATTTCTT			
ompA	Rr190.70	ATGGCGAATATTTCTCCAAAA	46	530	25
	190-701	GTTCCGTTAATGGCAGCATCT			
ompB	120-M59	CCGCAGGGTTGGTAACTGC	48	856	28
	120-807	CCTTTTAGATTACCGCCTAA			

5 min (Table 1). The PCR reaction was set to a final volume of 20 μl, containing: 25–100 ng of template DNA, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM of each dNTP, 1X reaction buffer, 0.5 U of *Taq Platinum* DNA polymerase, and ultrapure sterile water to reach the final volume. For each reaction, a negative control (5 μl of molecular-grade water) and a positive control (5 μl of DNA belonging to *R. prowazekii*) were included. PCR products were electrophoresed through a 2% agarose gel, stained with 10 000× SYBR® Safe (Invitrogen, Eugene, Oregon, USA), and examined by LED transillumination.

Conventional PCR products (*gltA*, *ompA*, and *ompB* genes) were purified using the QIAquick Kit (QIAGEN, Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's protocol. Both strands of the purified DNA were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sanger sequencing was performed using an ABI PRISM 3500 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequences were edited using the BioEdit program[13]. A homology analysis was conducted using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank nucleotide database to elucidate the identity of each sequence. Finally, the Clustal-W method was used for alignment (available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Phylogenetic trees were constructed using the maximum likelihood (ML) method for both the *gltA* and *ompB* genes. For the phylogenetic analysis, the IQ-TREE web server was used[35]. The best-fit model for *gltA* was TIMe+G4, and for *ompB*, TVM+F+G4, both chosen according to the Akaike Information Criterion (AIC)[14]. The confidence levels of the nodes were determined using bootstrapping with 1000 replicates. Tree topology was reconstructed using FigTree software[24].

Results

We captured 357 individual mosquitoes (Diptera: Culicidae) belonging to the genera *Aedes* (0.28%), *Anopheles* (0.28%), *Coquillettidia* (59.6%), *Mansonia* (15.4%), *Psorophora* (20.7%), *Sabethes* (0.84%), and *Wyeomyia* (2.8%).

Of all the pools (25 in total), only one tested positive for both the *gltA* and *ompB* genes by PCR. No amplification was detected using primers targeting the *ompA* gene. Since the *gltA* gene is highly conserved, its amplification only confirms the presence of the genus; therefore, to confirm the species identity, the *ompB* gene was sequenced.

A 750 bp fragment corresponding to the *gltA* gene and a 780 bp fragment corresponding to the *ompB* gene were sequenced and deposited in the GenBank nucleotide database under accession numbers PQ488464 (*gltA*) and PQ488463 (*ompB*).

The positive pool was composed of 39 individuals from *Coquillettidia venezuelensis* (Theobald, 1912). For species confirmation, a PCR targeting the cytochrome c oxidase 1 (COI) gene was performed using the primer pairs from Folmer et al.[8]: LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), which were used to amplify the ∼658 bp fragment of the COI gene. The sequences were deposited in GenBank under accession number PQ315765.

For the *ompB* gene, nBLAST analysis indicated identities of 93.94% (query cover: 100%; e-value: 0) with *Rickettsia tillamookensis* and 91.78% (query cover: 100%; e-value: 0) with *R. felis*, respectively.

In Figure 1, we show the results of the phylogenetic analysis (ML) using a 750 bp fragment from the citrate synthase gene (*gltA*), and Figure 2 shows the analysis using a 780 bp fragment from the *ompB* gene sequences. These analyses demonstrate that the sequences obtained in this study belong to *Rickettsia* (Fig. 1). The *ompB* gene sequence analysis shows that it clusters with *R. felis* and *R. assembonensis*, but not with *R. tillamookensis*, despite the high identity.

Discussion

In this study, we report the presence of *Rickettsia* related to *R. tillamokensis* and *R. felis* in one pool of mosquitoes belonging to *Coquillettidia (Rhynchotaenia) venezuelensis* (Theobald, 1912), collected in Corrientes province, Argentina. This is the first detection of *Rickettsia* DNA in mosquitoes in Argentina.

The genus *Rickettsia* comprises Gram-negative bacteria that are obligate intracellular pathogens. It currently contains 32 species, divided into five phylogenetic groups: the Spotted Fever group (SFGI and SFGII), with the SFGI *Rickettsiae* group containing 24 species (e.g., *R. conorii*, *R. massiliae*, *R. rickettsii*), and the SFGII *Rickettsiae* group (also referred to as the transitional group, TRG), which includes five species (e.g., *R. felis* and *R. australis*); the Typhus group (TG), which includes *R. prowazekii* and

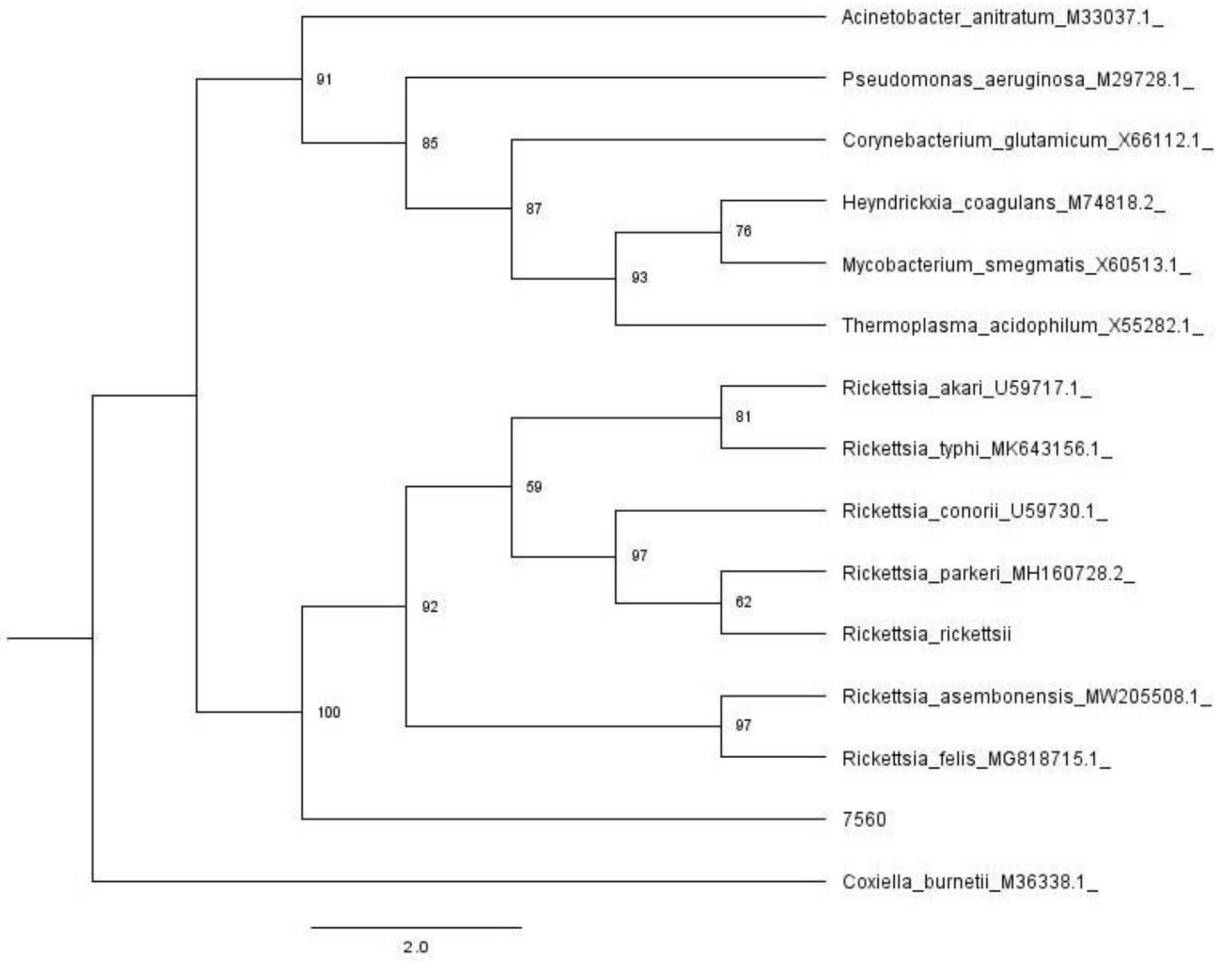

Figure 1 Phylogenetic tree generated using the maximum likelihood method for *Rickettsia* spp., based on the *gltA* gene. The strain of interest is identified as 7560 (GenBank accession number PQ488464), with bootstrap values indicated at the nodes.

R. typhi; the Canadensis group (CG), which includes *R. canadensis*; and the Bellii group (BG), which includes *R. bellii*[7]. In our study, we detected a *Rickettsia* related to the TRG, which includes *R. felis*, *R. australis*, *R. asembonensis*, and *R. tillamookensis* (Fig. 2)[10].

In this study, we detected DNA belonging to *Rickettsia* (94% identity with *R. tillamookensis* and 92% identity with *R. felis*) in the mosquito species *C. venezuelensis*, which showed the highest abundances in the sampling conducted in the study area. This species is an aggressive and zoophilic mosquito. Its distribution in Argentina includes Northeast of Argentina, in the ecoregion of *humedal* (wetland) of Corrientes province[4]. It breeds in natural and permanent water containers, such as swamps, ponds, and lakes. *Mayaro* (Togaviridae) and *Oropouche* (Orthobunyavirus) viruses have been isolated from this species[2,3]. Furthermore, it has been reported as a vector for Eastern Equine Encephalitis (Togaviridae)[9]. This mosquito species is characterized by its daytime activity and strong preference for human hosts[34]. This condition could increase the health significance of the *Rickettsia* detected in terms of human transmission.

Rickettsia tillamookensis was recently characterized by Gauthier et al.[10]. This new *Rickettsia* species was detected in 1967 and several isolations and detections from ticks have since been reported. Recently, in 2021, as a result of whole genome sequencing of the original type strain (Tillamook 23T), it was revealed that this microorganism is a unique transitional group of *Rickettsia* species[10]. Experimental and historical observations suggest that *R. tillamookensis* could be pathogenic to human hosts[22]. Paddock et al.[22] isolated *R. tillamookensis* from the tick *Ixodes pacificus*, in both adults and nymphs, suggesting that it is likely transmitted transstadially in nature. However, it should be noted that the role of mosquitoes as *Rickettsia* vectors remains unclear. Further studies should be conducted to evaluate vectorial competence and capacity.

In the phylogenetic analysis using *ompB* sequences (Fig. 2), our strain formed a distinct clade closely related to *R. felis* and *R. asembonesis*. Only a few sequences of *R. tillamookensis* are available in GenBank: two from complete chromosomes and three from the 16S ribosomal RNA. This limited sequence data may account for the difficulty in aligning with this *Rickettsia* species. However, it is important to note that the tree topology could change if additional *R. tillamookensis* sequences become available.

On the other hand, *R. felis* is another microorganism with zoonotic potential. Several authors have detected *R. felis* worldwide in fleas, and there are also reports of human cases

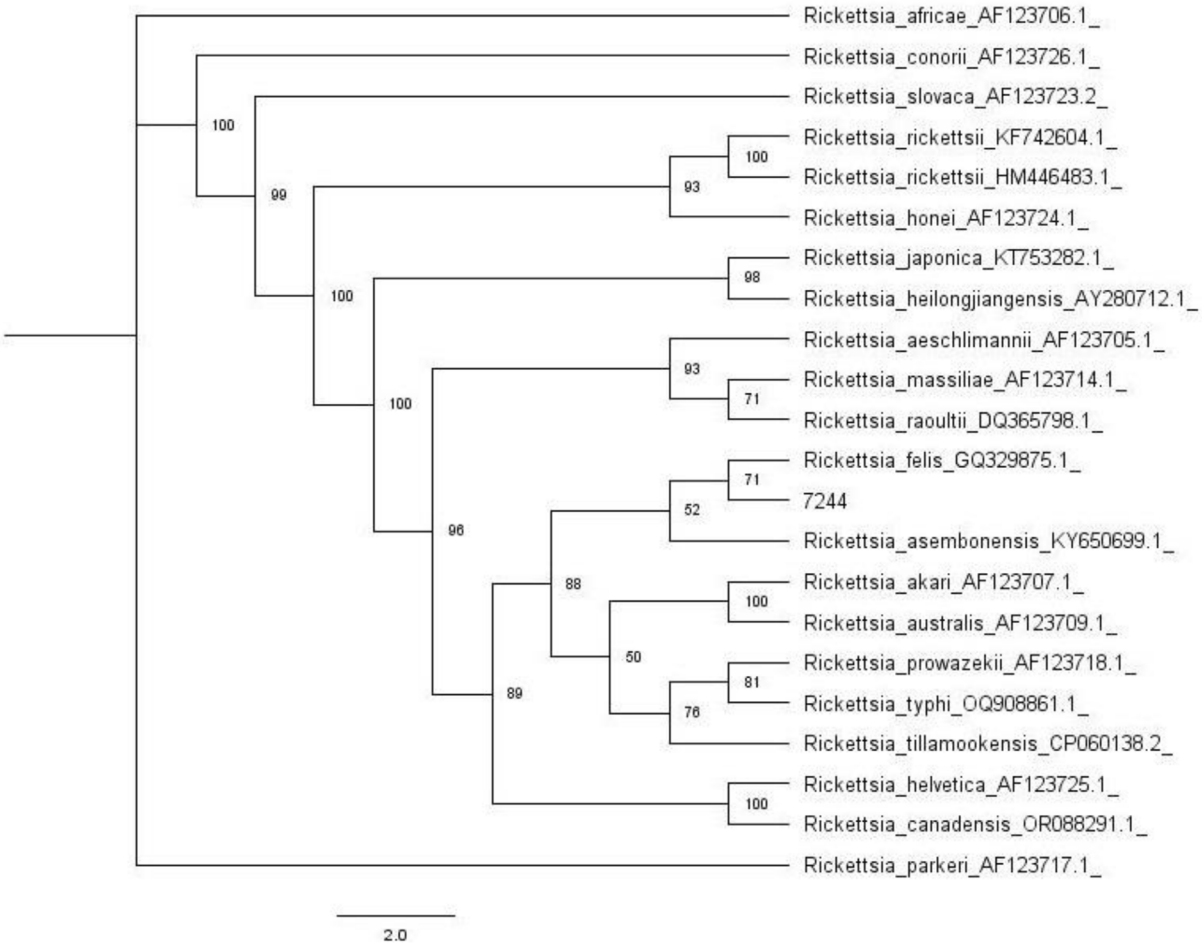

Figure 2 Phylogenetic tree generated using the maximum likelihood method for *Rickettsia* spp., based on the *ompB* gene. The strain of interest is identified as 7244 (GenBank accession number PQ488463), with bootstrap values indicated at the nodes.

associated with this agent[23]. However, in Argentina, despite several reports of *R. felis* detection, there are no reports in patients, and it is possible that the prevalence of the infection is underestimated. Healthcare professionals should be alerted to the possibility of infection with *R. felis* among their patients. The first detection of *R. felis* in mosquitoes was made by Socolovschi et al.[31]. Following that, Guo et al.[12] reported the presence of *Rickettsia* species (*R. japonica*, *R. sibirica*, and *R. monacensis*) in adult mosquitoes using groEL sequences. In addition, *R. japonica*, *R. sibirica*, and *R. monacensis* were detected in all life stages (egg, larva, pupa, and adult). These data show the presence of several *Rickettsia* species; however, there is no evidence of the role mosquitoes play in transmission.

A novel *Rickettsia* species was detected in *Anopheles* mosquitoes in China, showing high similarity to *R. felis*[18]. In the phylogenetic analysis, our strain did not align with this new *Rickettsia*. This result suggests that it could represent a new *Rickettsia* species also related to *R. felis*, but further studies are needed to confirm this hypothesis.

In order to determine the role of mosquitoes as vectors, one approach should be to isolate the bacterium from the saliva or salivary glands of specimens from wild-caught mosquitoes. Additionally, the infection of mosquitoes following experimental feeding on a bacteremic host, as well as the transmission of bacteria to a vertebrate through the bite of a mosquito, has been demonstrated. Dieme et al.[6] infected *Anopheles gambiae* mosquitoes orally with *R. felis* and reported the transmission potential of *R. felis* 15 days post-infection, both via salivation and by detecting *R. felis* in mice after feeding on mosquitoes that had been previously infected. However, there was no report of vertical transmission, which suggests that horizontal transmission occurs in nature.

Overall, the studies indicate that mosquitoes may play a role in the transmission of several species of *Rickettsia*. However, the epidemiological significance of this finding remains uncertain. The detection of *Rickettsia* in mosquitoes suggests that they could transmit various infectious diseases, making it crucial to analyze their interactions. Further research is needed to assess the impact on public health and the potential risk of a new emerging zoonotic disease.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT to improve certain sentences in English. After using this

tool, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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Conflict of interest

The authors declare no conflict of interest.

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