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Original Article

Conventional and ITS1 Gene-PCR Studies on Suspected Cutaneous Leishmaniasis Patients Referred to Different Public Health Hospitals in Quetta, Pakistan

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Abstract

Background: Cutaneous leishmaniasis (CL) is a neglected tropical disease that remains endemic in several areas of Balochistan Province, including Quetta city. We aimed to evaluate the effectiveness of conventional and molecular diagnostic methods for the detection and identification of CL.

Methods: Overall, 100 suspected patients were examined using conventional techniques, including microscopy, culture, and histopathology during 2024-2025, from various public hospitals of Quetta city, Pakistan. In molecular methods *ITS1* gene was used to detect *Leishmania* on species level. Specific primers of *ITS1* gene were used to amplify the targeted regions.

Results: Microscopy and PCR amplification of the *ITS1* gene confirmed CL infection in 86% of cases, Culture techniques yielded promastigote growth in 16 samples, while 14 slides showed histopathological effects, indicating comparatively lower detection rates. Molecular characterization through *ITS1* sequencing revealed that the isolates shared a 99% and 100% similarity with *Leishmania tropica*. Phylogenetic analysis further supported this relationship, confirming the predominance of this species in the study area.

Conclusion: The findings highlight the limitations of conventional diagnostic methods and focus on the implication of molecular methods, particularly PCR, for exact and early detection of CL on species-level, which is critical for effective disease management and control strategies in endemic regions like Quetta. Other demographic characteristics such as gender, age, education status, and site of lesions were also recorded.

Introduction

Cutaneous Leishmaniasis is a vector-borne parasitic disease, which is caused by a protozoan of the genus *Leishmania*, and transferred by female sandflies of the genus *Phlebotomus* (1). Leishmaniasis is prevalent in 99 countries (2). More than 350 million people are at risk and 30,000 deaths each year globally because of this disease (3). Almost 12 million people are affected worldwide by leishmaniasis (4). The WHO has considered CL amongst the six main infectious diseases in tropical regions worldwide (5).

Cutaneous leishmaniasis is typically heals itself; however, it can lead to disfiguring skin lesions that may result in permanent scares (6). About 20 species of *Leishmania* have been identified as causing humans infection (7). *L. tropica* predominates in Pakistan Afghanistan, northern Syria, and Turkey (8). Dogs are reported as the main reservoirs (9). The lesions usually start as a tiny, single, non-supportive papule where the sand fly bit, usually on areas of the face and limbs that are well exposed (10). These lesions gradually enlarges and changes into ulcers. These ulcers may heal spontaneously over a period of several weeks to months, and in some cases, may persist for a year or longer (11).

CL identification can be performed using various methods, including clinical assessment, microscopic analysis, culture, and molecular techniques (12). Among these PCR-based methods offer high sensitivity and specificity; enabling the detection of *Leishmania* species even at low parasite load (13). Various *Leishmania* genes have been used to study phylogenetic relationships at genus, species and strain levels (14). A universal ITS1-region PCR is highly effective for direct diagnosis and species identification (15).

We aimed to highlighting the significance of conventional and molecular methods in Quetta city where CL is endemic in different rural and urban areas. Additionally, it aimed to determine associations between the disease's incidence and various epidemiological and demographic factors, including age, gender, and living circumstances.

Material and Methods

Quetta is the capital and largest city of Balochistan, Pakistan. It covers an area of about 3,447 square kilometers and is located at an altitude of 1,680 meters (5,510 feet) above sea level. The main inhabitants are Pashtuns, Baloch, Hazaras, Brahuis, Sindhi and Punjabi. The major languages spoken are Pashto, Balochi, Brahui, Hazaragi (persion), Urdu, Sindhi and Punjabi. The average temperature in the summer season (June–August) can reach up to 40 °C, while the winter season (November–March) with temperatures dropping as low as -12 °C (Fig. 1). According to 2023 census, the total population of Quetta District was 2, 595, 492 (16).

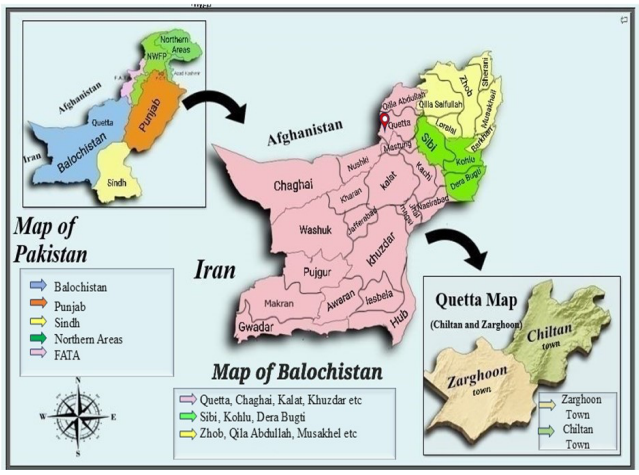

Fig. 1: Study area of Quetta, Balochistan Province, Pakistan showing bordering countries like Iran and Afghanistan

One hundred wound scrapings of the inner edge of the lesions were collected during 2024-2025, from various public hospitals (i.e.,

Sandeman provincial Hospital, Mohtarma Shaheed Benazir Bhutto General Hospital, & BMC hospital and Civil Hospital) of Quetta City. Patient's information and complete history such as their gender, age, socio-demographic features, clinical and sub-clinical signs were collected through questionnaire.

Ethical statement

The Institutional Ethical Committee of the University of Balochistan Pakistan sanctioned the study under reference number 366/Zool/UOB.

Microscopy

The skin lesions were disinfected with 70% alcohol and then tissue smears were collected from the edge of each nodule or papule by using a disposable scalpel or surgical blade (17). The prepared smears were fixed by methanol for one minute, and then stained with Giemsa for 10 min. The prepared smears were examined under microscope by using 100X magnification.

Biopsy Samples

The skin lesions were sterilized with 70% ethanol then a sterile blood lancet was used to collect lesion scrapings from CL suspected patients, then these were distributed into micro tubes containing 50 µl of PBS solution (Phosphate buffered saline solution) and stored at -20 °C (18).

Culture

Leishmania can be cultured from biopsy and aspirate samples, with promastigotes grown in the Novy, MacNeal, Nicolle (NNN) medium. To make (NNN) media, blood base agar, NaCl, 10% defibrinated rabbit blood, and 1% penicillin (50µg/ml) and streptomycin (50µg/ml) solution was used. The mixture was distributed into sterile containers, and placed in a slope position till solidification, then kept in incubator at 25 °C. After 14 d cultures were examined for parasite growth (19).

Histopathology

All tissue samples from biopsies were dehydrated and cleaned and then from these samples formalin-fixed paraffin-embedded (FFPE) tissue blocks were formed. The cutting sections of these blocks were applied to Hematoxylin and eosin staining method. Then the degree of inflammation, the development of granulomas, and additional dermal and epidermal characteristics was examined (20).

DNA extraction

Genomic DNA was extracted from lesion biopsies and also from filter paper samples by using DNeasy Blood & Tissue Kit (QIAGEN Cat. No.69506).The procedure was carried out according to the manufacturer's instructions. The extracted DNA was then stored at -20 °C until used for PCR.

ITS1gene primers

Primers were provided by Macrogen Company, Korea (Table 1). Conventional PCR (Thermal cycler 2720) was used for amplification of *ITS1* region.

Table 1: Primers Used in ITS1-PCR

Primers	Target	Primers Sequences 5'-3'	Length(bp)	Product size
LITSR(Forward)	ITS 1	CTT-GGATCATTTT CCGATG	19	350 bp
L5.8S(Reverse)		TGATACCAC-TTATCGCATT	19	

PCR conditions

The PCR process started with an initial step of denaturation at 95 °C for 5 min. Then it was succeeded by 35 cycles, each comprising of denaturation at 95 °C for 30 sec, then annealing at 53 °C for 30 sec, and extension on 72 °C for 30 sec. Upon completion of amplification cycles, an ending extension step was carried out at 72 °C for 6 min, then hold at 4 °C.

Gel electrophoresis

The 5 µl PCR products from each sample were analyzed by gel electrophoresis comprising of 1.5 gm agarose (1.5%) mixed with 100 ml of 1X TAE buffer then heated for 1-2 min until the gel was fully dissolved. After cooling to 35-40 ºC, 3 µl of ethidium bromide solution was added. In the gel, the DNA ladder was loaded in the first, and the PCR products were loaded in other wells. The amplified DNA fragments were subsequently observed on gel by using an ultraviolet transilluminator (21).

Phylogenetic analysis

It was performed by using the Neighbor-Joining method (22) with 1000 bootstrap replicates. Evolutionary distances were estimated using the Kimura 2-parameter model in MEGA X (23).

Statistical Analysis

The data was evaluated in SPSS (ver. 22.0) (IBM Corp., Armonk, NY, USA) by using Chi-square ($\chi 2$) test. The *P*-value was 0.001 which is of great importance and showed a considerable link among various parameters.

Results

Quetta is the main capital city of Balochistan. The rural, as well as urban areas of Quetta District are endemic for cutaneous leishmaniasis. Out of 100 lesions samples, 86 (86%) samples were found positive, for microscopy, as well as ITS1-PCR and 14 (14%) samples were found negative. Sixteen samples were positive for culture, and 14 slides showed histopathological effects. Highly Significant ($P<0.001$) correlation was observed between diagnosis methods, as showed in (Table 2).

Table 2: Comparison of conventional and molecular methods for the diagnosis of CL

S/No	Method	Positive	Percentage	Negative	Percentage	Total	*P-value*
1	Microscopic	86	86	14	14	100	0.001
2	ITS1-PCR	86	86	14	14	100	
3	Culture	16	16	84	84	100	
4	Histopathology	14	14	86	86	100	

Microscopic analysis of Leishmaniasis

Microscopic examination showed that out of 100 lesion samples, 86(86%) were positive and 14(14%) of the samples showed negative results for CL, as shown in the (Fig. 2a).

Culture

Out of 100 samples only 16 (16%) samples showed growth of promastigote stage of *Leishmania*. On 14th day of the culture, the numbers of motile promastigotes were seven which increased to sixteen at the end of the month, as shown in (Fig. 2b).

Histopathological Findings

Only 14 slides showed parasite load. Higher parasite loads were significantly associated with more intense and deeper dermal inflammation, often involving the entire dermis, while low loads showed only superficial inflammation. Increased parasite burden also correlated with a rise in macrophage infiltration. In contrast, granuloma formation showed a strong negative relationship with parasite load: most patients with well-formed granulomas had no detectable parasites (58%), whereas those with higher parasite levels commonly had poorly formed or absent granulomas (42%) (Fig. 2c).

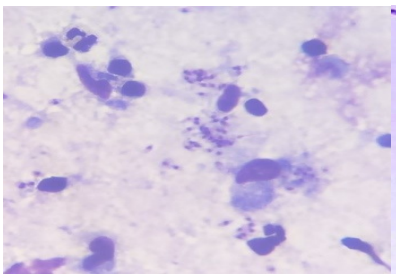

Fig. 2a: Smear stained with giemsa showing macrophage infected with amastigote stage of *Leishmania*

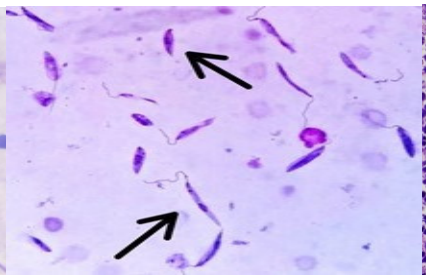

Fig .2b: Culture smear showing Promastigote stage of *Leishmania* under 100X as shown by arrows

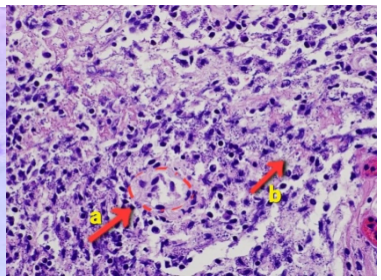

Fig. 2c: Dermal and epidermal features of Cutaneous leishmaniasis Granuloma formation (a) and presence of amastigotes (b)

Molecular detection of ITS1 gene

Out of 100 positive CL patients 86 (86%) were positive for *ITS1-PCR* However, 14 lesion samples were negative for *ITS1* gene. Agarose gel electrophoresis results showed DNA band size of 350bp for *ITS1* gene *L. tropica* was the predominant species. The positive samples were examined in lane 1, 2, 3, 4, 6, 8, 9, and 10.L represented ladder, as shown in (Fig. 3).

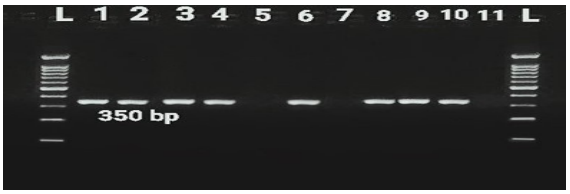

Fig. 3: ITS1-PCR of *L.tropica* showing bands at 350 bp. The positive samples were observed in lane 1,2,3,4,6,8,9 and 10.L represent ladder

Sequencing of ITS1-gene

The amplified ITS1 region of seven *L. tropica* samples was visualized on an agarose gel, purified, and submitted for Sanger sequencing to Apical Scientific Sdn. Bhd., Malaysia. The resulting chromatograms were examined for sequence quality and the sequences were edited and assembled using BioEdit. Low-quality terminal bases were trimmed. The edited sequences were compared with sequences available in the NCBI GenBank database using BLASTn to determine sequence similarity and confirm the identity of the isolates (Table 3). Reference *L. tropica* sequences representing different geographic regions were retrieved from GenBank and aligned with the sequences generated in the present study using ClustalW.

Phylogenetic analysis

Phylogenetic analysis of the *L. tropica* sequences was carried out using the Neighbor-Joining (NJ) method in MEGA X based on the ITS1 region. Evolutionary distances were estimated using the Kimura 2-parameter (K2P) model and the reliability of the resulting tree was assessed using 1,000 bootstrap replicates. Pairwise deletion was applied to exclude ambiguous positions, resulting in a final dataset of 18 nucleotide sequences comprising 282 positions. The sequences generated in the present study were distributed into two main clusters rather than forming a single cluster according to their Pakistani origin. Three sequences from the present study (PZ806687, PZ806485, and PZ806694) clustered together, with isolates 22 and 25 forming a well-supported subclade (97%). This group was also strongly supported (98%) and was closely related to the Pakistani sequence PQ303789.

Table 3: GenBank accession numbers and percentage identity of sequences generated in the present study compared with NCBI GenBank (BLASTn) reference sequences

Species Studied	GenBank Accession No (present study	Sequence length (bp)	Query coverage (%)	E-Value	Sequence identity (%) on NCBI	Closest NCBI Match Accession No.
***Leishmania tropica* isolate 1**	PZ806687	278 bp	96	2e-132	99.62	PQ303789
***Leishmania tropica* isolate 31**	PZ827147	277 bp	96	2e-122	97.36	MH347945
***Leishmania tropica* isolate 32**	PZ823868	229 bp	100	9e-111	99.13	PQ470334
***Leishmania tropica* isolate 21**	PZ830671	283 bp	98	1e-140	100	JX289848
***Leishmania tropica* isolate 22**	PZ806485	276 bp	97	2e-133	99.63	PQ303789
***Leishmania tropica* isolate 24**	PZ830672	252 bp	94	6e-118	100	MG755821
***Leishmania tropica* isolate 25**	PZ806694	277 bp	97	2e-133	99.63	PQ303789

The remaining four sequences formed a separate group. Isolates 31 and 32 clustered together with 100% bootstrap support and were further grouped with isolate 21 (96%). Isolate 24 was closely related to the Iranian sequence MG755821 (99%). Overall, the sequences from the present study were divided into two main groups and the clustering did not consistently reflect geographic origin. Several relationships showed strong bootstrap support, although some deeper branches had lower support (Fig. 4).

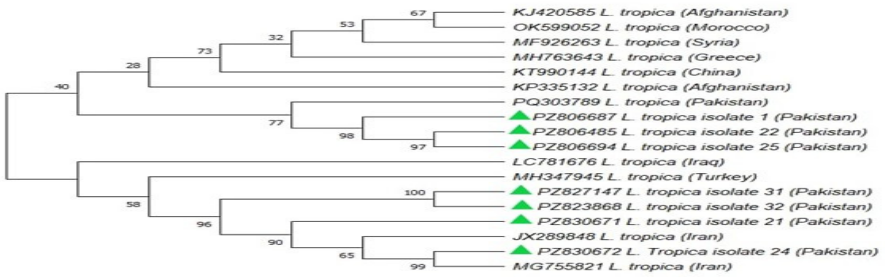

Fig. 4: Neighbor-Joining phylogenetic tree of *L.tropica* based on ITS1 sequences constructed in MEGA X. The tree was constructed using the Kimura 2-parameter method with 1000 bootstrap replicates. Bootstrap values are shown at the nodes. Green triangles indicate sequences generated in the present study; other sequences were retrieved from GenBank accession numbers

Evaluation of Risk Factors of Leishmaniasis

Demographic and some other data concerning the subjects are included in Table 4. Lesion characteristics of cutaneous leishmaniasis are depicted in Fig. 5 and Table 5.

Table 4: Demographic characteristics of cutaneous leishmaniasis

Factors		Number of CL patients (%)	*P*-value
Gender	Male	46 (53.48)	0.001
	Female	40 (46.51)	
Age, years	1-10	16 (18.60)	
	11-20	22 (25.58)	
	21-30	18 (20.93)	
	31-40	14 (16.27)	
	41-50	10 (11.62)	
	51-60	06 (6.97)	
House type	Mud Made	52(60.46)	
	Cement made	34(39.53)	
Travel history	Outside country	14 (16.27)	
	Inside country	06 (6.97)	
Education Level	Illiterates	47 (54.65)	
	Literates	39 (45.34)	
Use of repellents	Yes	20(23.25)	
	No	66(76.74)	

Table 5: Lesion characteristics of cutaneous leishmaniasis

Number of lesions		Number (%)	*P*-value
	Single	60 (69.76)	
	Double	21 (24.41)	0.001
	Multiple	5 (5.81)	
Site of Lesions	Face	39 (45.34)	
	Upper limb	23 (26.74)	
	Lower limb	20 (23.25)	
	Other sites	4 (4.65)	
Lesion type	Dry	54 (62.79)	
	Wet ulcerative	32 (37.20)	

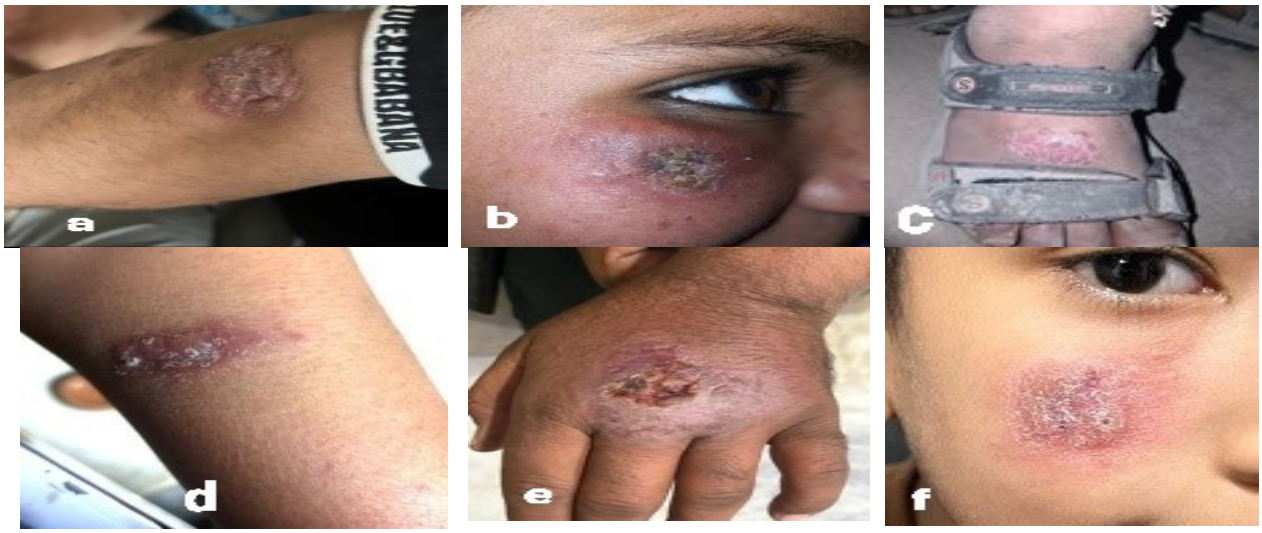

Fig. 5: (a, b, c, d, e, f) Lesions on different sites of body

Treatment of cutaneous leishmaniasis

In Quetta, Balochistan, these two drugs are used in hospitals: Glucantime (meglumine antimoniate intramuscular) and Impavido (miltefosine oral). (Fig. 6a & 6b). The dose of IM/IV antimonial is 20 mg/kg once daily for 21 days. Treatments with Glucantime caused drug resistance and severe secondary infection in 50% CL patients. Contributing factors to resistance are incomplete treatment courses due to painful injections, weak immunity of patient, high costs or unavailability of drugs lead to persistent infections and drug tolerance. Miltefosine offers successful second line oral therapy for patients showing relapse. Its treatment duration is 28 d and weight-based dosing. In view of several outbreaks of CL in Quetta city it is strongly recommended that new effective drugs and vaccines should be introduced to minimize the CL burden.

Fig. 6a: Glucantime (meglumine antimoniate) intermuscular drug for CL

Fig. 6b: Impavido (miltefosine) oral drug for CL

Discussion

CL is endemic in the deprived areas of Quetta Balochistan, Pakistan. The current study is based on combination of both molecular techniques and traditional methods to investigate the frequency of CL, as well as the risk factors related to CL in Quetta city of

Balochistan Province. Pakistan shares long borders with Iran and Afghanistan, where CL is highly prevalent (24).

Routine CL diagnosis relies on microscopy and parasite culture, which typically identify the organism only to genus level (25). ITS1-PCR was found to be a more sensitive and specific method as compared to other traditional methods for the detection of parasites. The study showed 86(86%) positive cases for *ITS1* gene. Similar findings 81% were presented by (26), where ITS1-PCR was also found more specific method as compared to microscopy for *leishmania* species identification.

The present results showed increased frequency of CL in males 46 (53.48%) as compare to females 40 (46.51%). These findings were in agreement with prior studies by (27), where males had higher infection rates as compare to females. However, our study's results are in contrast with (28). The increase number of males is because they are actively involved in outdoor activities (29), and the females remained mostly covered because of cultural and religious values in the proposed study area (30).

The current study revealed the highest infection rate 22 (25.58%) in age group (11–20) and lowest percentage was found in the age group 51-60 with infection rate 6(6.97%). Similar results were presented by (31). The increased infection rate in age group (11–20) is because this age group spent more time outside home, when sandflies are most active, particularly from dusk to early night (32). Lower infection rates in the elderly may result from lasting immunity built during childhood exposure to CL (33)).

The most affected region of the body was face with highest proportion of lesions 48(55.81%). A previous study has similar findings with most lesions on face (34), it was in contrast to our research study that revealed hands and arms were the most common sites 56 (62%) (35). The highest number of facial lesions is significant because the face represents a highly visible and sensitive anatomical site.

In our study 16 CL patients had travel history; this suggests that travel may expose individuals to diverse environments with different types of disease vectors (36). The structure of home has great influence on disease prevalence, present study shows 60% CL patients lived in mud made houses while 40% lived in brick made houses. Earlier studies confirmed that inhabitants of mud-walled houses have higher infection risks, as cracks and crevices provide ideal shelter for sand flies (37).

In present study the CL patient showed 50% resistances with pentavalent antimonials. Pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) are first-line therapy recommended by WHO, while, miltefosine is an oral alternative; however, these drugs can cause various adverse and toxic effects (38).

Limitations of study

The present study in Quetta faced geographic restrictions, security challenges, and patient unwillingness toward invasive sampling. Getting enough tissue samples for histopathology may be challenging. High costs and limited availability of specialized laboratory chemicals, DNA kits, culture media, PCR reagents, advanced sequencing facilities, poor DNA quality, and contamination, all these factors restricted molecular analysis on large-scale.

Conclusion

CL is a major growing health issue in Quetta City, Balochistan, where the number of reported cases continues to increase. *L. tropica* is the primary causative and predominant species in Quetta, it affects children, and low-socioeconomic populations. Combining conventional diagnostics with sensitive molecular techniques, such as ITS1-PCR, is vital for early, species-level detection, while government-led awareness campaigns, vector control, and

new treatments strategies are required to reduce the disease burden.

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Conflict of interest

The authors have no conflict of interest.

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