Molecular identification of an old clinical isolate of Indian Kala-azar

Madhumita Manna¹ · Supriya Khanra¹

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Abstract Molecular characterization is an important task for species identification of the isolates belonging to different *Leishmania* species. Clinical symptoms, tissue tropism, vector preference, reservoir and geographical distribution may act as distinguishing parameters but not always decisive. On the other hand, modern taxonomic tools employed to divulge characteristics of the genome or protein molecules of the parasite would be convincing and for *Leishmania* sp., they include nuclear and kDNA buoyant density, multi locus enzyme electrophoresis (MLEE), RAPD, RFLP or use of monoclonal antibodies etc. In the present study, we intend to establish the identification of an old clinical isolate of Indian Kala-azar, familiarly known as ‘UR6’ by MLEE, RAPD, RFLP and species specific monoclonal antibodies. UR6 has been isolated from a confirmed Kala-azar patient admitted in Calcutta School of Tropical Medicine, Kolkata in 1978. From then it is being regularly used for various scientific studies by the *Leishmania* Research Group of India and abroad. The isozyme profile of UR6 showed similar electrophoretic mobility that of WHO reference strain for *Leishmania tropica*, K27. Similar findings were obtained in the RAPD and RFLP assays. Screening with species specific monoclonal antibodies showed its strong reactivity towards *L. tropica*. The Jaccard’s Similarity Indices were calculated.

Keywords UR6 · *L. tropica* · MLEE · RAPD · RFLP · Species specific mAbs

Introduction

Leishmaniasis is a disease complex with variety of clinical forms, ranging from moderately mild cutaneous lesion to serious visceral disease to devastated mucocutaneous form (Desjeux 2004). Leishmaniasis are endemic in 98 countries with an estimated 350 million people at risk (Alvar et al. 2012). 90 % of cutaneous leishmaniasis infections occur in Saudi Arabia, Algeria, Iran, Brazil, and Peru; 90 % of visceral leishmaniasis develops in India, Bangladesh, Nepal, Sudan, and Brazil (Desjeux 2004). Visceral Leishmaniasis or Kala-azar in India is primarily a public health problem and is fatal if left untreated. Some Kala-azar patients (10 %) may develop Post Kala-azar Dermal Leishmaniasis (PKDL) after apparent chemotherapeutic cure. The etiological agent for Indian Kala-azar has long been known to be *L. donovani*. On the other hand, *L. tropica*, the causal agent of Cutaneous Leishmaniasis (CL) does not show co endemicity with *L. donovani* in the eastern part of the country but there are reports that *L. tropica* causes VL in India (Sacks et al. 1995; Khanra et al. 2011, 2012).

With the spectacular development of purification techniques for enzymes, nucleic acids and other complex molecules, new molecular criteria for taxonomic classification of *Leishmania* became available. Excretory factors from various *Leishmania* isolates were also explored for typing and showed some promise (Schnur et al. 1981) but have never been used routinely for taxonomic purposes. In contrast, isoenzyme profile analysis or multilocus enzyme

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✉ Madhumita Manna
madhumita.manna09@gmail.com

¹ Department of Zoology, Barasat Govt. College, 10, K.N.C Road, Kolkata 700124, India

electrophoresis (MLEE) is the most useful taxonomic technique for epidemiological purposes for *Leishmania* spp (Miles et al. 1980; Evans et al. 1984; Rioux et al. 1990; WHO 1990). MLEE is still considered the ‘Gold standard’ by WHO (1994) for *Leishmania* species identification and genetic diversity studies. By scoring the electrophoretic mobility of the encoding enzymes, MLEE detects different alleles of housekeeping genes. It is generally assumed that isolates exhibiting the same electrophoretic mobility for a given enzyme contain the same allele of the underlying gene.

The introduction of Monoclonal antibody (mAb) technique in the field of *Leishmania* research has made it possible to develop a number of species specific and stage specific monoclonal antibodies (McMahon-Pratt and David 1981; McMahon-Pratt et al. 1982). They have been reported to distinguish strains and different life cycle stages of *Leishmania* species (McMahon-Pratt and David 1981; Handman and Hocking 1982). Jaffe et al. developed different mAbs that could distinguish *L. major*, *L. donovani* and *L. tropica* respectively (Jaffe and McMahon Pratt 1983; Jaffe and Sharfstein 1987). These have greatly facilitated the rapid typing of strains for epidemiological, diagnostic and identification purposes.

Molecular biological technologies and other emerging tools brought a spectacular change in quality in the research in protozoan parasites in last three decades. Methods to genotype strains within parasite species using PCR based assays have provided valuable tools to study the genetic diversity of parasites. The PCR-based methods used most often for genotyping is Random Amplified Polymorphic DNA (RAPD) and it provides an effective method for obtaining genetic markers in all sorts of organisms (Williams et al. 1990; Welsh and McClelland 1990). It is rapid and simple method and no prior DNA sequence knowledge is required, only one relatively short oligonucleotide of arbitrary sequence is utilized as a primer. RAPD PCR band profiles can act as DNA ‘fingerprinting’ at species and sub-species levels (Tibayrenc et al. 1993; Bañuls et al. 2000) and is used in differentiating *Leishmania* species and even, it is proved to be informative in differentiating some intra specific lineages (Zemanova et al. 2004).

The PCR-based method to analyze genetic diversity is based on the amplification of some markers such as the internal transcribed spacers (ITS), about 1 kb in *Leishmania* and flanked by highly conserved segments to which PCR primers can be designed followed by digestion with different restriction enzymes (Cupolillo et al. 1995). Other such marker is the gene encoding the heat shock protein of 70 kDa (hsp70) and RFLP of the PCR product of the gene using the restriction enzyme HaeIII (Garcia et al. 2004). This is a simple and universal tool for species

discrimination and has been employed for identification of Old World and New World species (Fraga et al. 2010; Montalvo et al. 2010).

An old clinical isolate of Indian Kala-azar, familiarly known as ‘UR6’, has been isolated from a confirmed Kala-azar patient admitted in Calcutta School of Tropical Medicine, Kolkata in 1978. From then it is being regularly used for various scientific studies by the Leishmania Research Group of India and abroad but its identity faced a crisis. Routine identification of any clinical isolate of KA employing biochemical and/or molecular biological techniques had not been carried forward in order to ascertain its taxonomical status as this kind of work always takes back seat. Same was true for UR6. Conventionally it has been described as *L. donovani* as it is isolated from a confirmed Kala-azar patient but afterwards, doubt arose regarding the species status of the isolate. Then also it has been extensively used by various research groups (Das and Adhya 1990, Bhattacharyya et al. 1993, 1996, Mukhopadhyay et al. 2000) considering as representative isolate of Indian Kala-azar. Serious attempts were taken for the first time to characterize the isolate by MLEE, RAPD and using species specific monoclonal antibodies followed by RFLP study. In the present study, we have characterized the clinical isolate UR6 by using all of the above mentioned techniques in order to ascertain its taxonomical status and clear the doubt.

Materials and methods

Chemicals and reagents

M199 medium, Penicillin, Streptomycin and other reagents used were obtained from Sigma chemical (St. Louis, MO, USA). Fetal Bovine Serum (FBS) was purchased from Invitrogen, USA. Random primers and species specific primers were procured from Eurofins Scientific GmbH, Germany. AluI, EcoRI, MspI, Hae III restriction enzymes were purchased from Bioenzyme, USA.

Clinical history of the *Leishmania* isolate, UR6

UR6 was collected from a confirmed KA male patient aged 27 years admitted in Calcutta School of Tropical Medicine. It has then been stabilized in Indian Institute of Chemical Biology, Kolkata, India and used routinely for many research works.

World Health Organization (WHO) reference strains

Leishmania donovani (MHOM/IN/80/DD8) and *L. tropica* (MHOM/SU/74/K27) were also included in the study. All

strains were grown at 22 °C in M199 (St. Louis, MO, USA) culture medium supplemented with 10 % Fetal Bovine Serum (FBS) (Gibco, USA).

Another old clinical isolate named as AG83 (MHOM/India/1983/AG83), obtained from confirmed Kala-azar patient in the year of 1983 and extensively utilized, has also been included in the study for the purpose of comparison. Part of the isozymes and RAPD profiles of this isolate have already been described elsewhere (Chatterjee et al. 1995; Manna et al. 2005).

MLEE of the isolate UR6

The crude extracts of UR6, AG83 and two reference strains were prepared for Isozyme study according to the method

of Evans DA (Evans 1989). The enzymes studied were Alanine amino transferase (ALAT; EC 2.6.1.2), Aspartate amino transferase (ASAT; EC 2.6.1.1) 6-Phosphogluconic dehydrogenase (6PGD; EC 1.1.1.44), Glucose phosphate isomerase (GPI; EC 5.3.1.9), Malate dehydrogenase (MDH; EC 1.1.1.37), Isocitrate dehydrogenase (ICD; EC 1.1.1.42), Nucleoside hydrolase (NH; EC 3.2.2.2), Phosphogluco mutase (PGM; EC 2.7.5.1) and Mannose phosphate isomerase (MPI; EC 5.3.1.8). Cellulose acetate electrophoresis (CAE) is a WHO recommended method for isozyme characterization of *Leishmania* strains (Evans 1989; Kreutzer and Christensen 1980). Electrophoresis was performed in Helena Gel apparatus (Helena Laboratories, Beaumont Texas, USA). The plates were incubated at 37 °C for 5–10 min except for ALAT and ASAT which

Fig. 1 Photograph of cellulose acetate (CA) electrophoretic mobility of UR6 with respect to other clinical isolate and reference strains, stained for the enzyme, glucose phosphate isomerase (**a**); Nucleoside hydrolase (**b**); 6-Phosphogluconic dehydrogenase (**c**) and Phosphogluco mutase (**d**). Lane 1 *Leishmania tropica* (K27), lane 2 UR6, lane 3 *L. donovani* (DD8), lane 4 AG83

were kept at room temperature. Isozymes of ALAT and ASAT were observed under a UV light source.

Characterization of UR6 by species specific monoclonal antibodies

5–10 ml of promastigote cultures of parasites were grown to stationary phase (about 5×10^7 /ml). The samples were frozen and thawed thrice in liquid nitrogen and protein concentrations were assayed by Lowry method (Sacks et al. 1995). The lysates were prepared for ELISA study and *Leishmania* species specific monoclonal antibodies, LXXVIII 2E5-A8 (*L. donovani* specific mAb, designated as D₂) and XCIV 1H2-A8 (*L. tropica* specific mAb, designated as ϕ) were the generous gifts of Prof. C. L. Jaffe, Israel. Absorbance at 405 was measured in ELISA reader.

Preparation of Nucleic acid from the isolates and reference strains

Total nucleic acids from isolates and reference strains were prepared as per standard protocol (Bhattacharyya et al. 1993). The freshly prepared DNA was dissolved in Tris EDTA buffer and stored at -20°C till use.

Characterization of UR6 by RAPD-PCR study

DNA amplification using random primers were performed according Manna et al. (Manna et al. 2005) with ten decamers and Universal Minicircle Sequence primer. The primer sequences were described elsewhere (Manna et al. 2005; Khanra et al. 2011). PCR amplification was performed in a thermal cycler following the standardized protocol. The products after the PCR run were electrophoresed in 1.2 % Agarose gel in TAE buffer and analyzed.

Characterization by RFLP of genetic markers

ITS, ITS1 PCR amplification and RFLP study of UR6 and reference strains

The ITS & ITS1 regions were individually amplified from DNA (El Tai et al. 2000; Schonian et al. 2003) with the following primers: LITSR (5'-CTGGATCATTTTCCGATG-3')/LITSV (5'-ACACTCAGGTCTGTAAAC-3') and LITSR (5'-CTGGATCATTTTCCGATG-3')/L5.8S (5'-TGATACCACTTATCGCACTT-3' (Eurofins Scientific GmbH, Germany). Amplification reactions were performed in volumes of 50 μl with 200 ng DNA samples. The products were electrophoresed in 1 % agarose (Lonza Rockland, ME USA) gel in TAE buffer and analyzed. The

amplified ITS region was digested using restriction enzymes AluI, EcoRI, MspI (Bioenzyme, USA) and amplified ITS1 region was digested using restriction enzymes Hae III (Bioenzyme, USA). 15 μl of the DNA were restricted and the restriction products were analyzed by 3 % Agarose gel electrophoresis in TAE buffer.

Fig. 2 Schematic presentation of the MLEE profiles of UR6 with respect to other clinical isolate (AG83) and WHO reference strains. Lane 1 DD8, lane 2 AG83, lane 3 K27, lane 4 UR6. DD8 and K27 are WHO reference strains for *Leishmania donovani* and *L. tropica* respectively; AG83, UR6 were collected from confirmed Kala-azar patients

Hsp70 PCR amplification and Restriction digestion

The Hsp70 PCR amplification was performed with the primers HSP70sen (5'-GACGGTGCCTGCCTACTTCAA-3') and HSP70ant (5'-CCGCCCATGCTCTGGTACATC-3') (Eurofins Scientific GmbH, Germany) as previously reported (Montalvo et al. 2010). Then the digestion with Hae III was performed. Reactions were incubated 4 h at 37 °C and analyzed by electrophoresis in a 3 % Agarose gel.

Results

MLEE of UR6, AG83 and WHO recommended strains representing *L. donovani* (DD8), *L. tropica* (K27) were shown for GPI (Fig. 1a), NH (Fig. 1b), 6PGD (Fig. 1c) and PGM (Fig. 1d). UR6 (lane 2) showed same electrophoretic mobility as that of K27 (lane 1) but different from AG83 (lane 4) and DD8 (lane 3) for all enzymes studied except for MDH (panel MDH, Fig. 2). Here, UR6 showed single

Table 1 ELISA results^a of *Leishmania* isolates with species specific monoclonal antibodies

Stock code	Clinical form	Monoclonal antibodies		Species
		<i>L. donovani</i> Specific mAb D ₂	<i>L. tropica</i> Specific mAb Ø	
K27 (WHO reference strain)	CL ^b	1.06	5.24	<i>L. tropica</i>
UR6	VL ^c	1.25	3.23	Supposedly <i>L. tropica</i>
DD8 (WHO reference strain)	VL ^c	10.57	1.18	<i>L. donovani</i>
AG83	VL ^c	9.57	1.7	Supposedly <i>L. donovani</i>

^a Results shown express the positive/negative (P/N) ratio. If P/N = 3.0, it will be considered as positive

^b Cutaneous leishmaniasis

^c Visceral leishmaniasis

Fig. 3 RAPD-PCR amplification of 25 ng of total nucleic acid of clinical isolate UR6 along with other isolate and WHO strains using primer OPA3 (a); OPA4 (b); OPA9 (c). Lane 1 MW marker, lane 2 K27; lane 3 UR6, lane 4 DD8, lane 5 AG83. DD8 and K27 are WHO reference strains for *L. donovani* and *L. tropica* respectively; AG83 and UR6 were collected from Kala-azar patients

Fig. 4 The amplified ITS region of nuclear DNA of the clinical Isolates and the reference strains were digested using restriction enzyme *AluI*. Lane1, MW marker (100 bp); lane 2, K27; lane 3, UR6; lane 4, DD8, lane 5, AG83. DD8 and K27 are WHO reference strains for *L. donovani* and *L. tropica* respectively; AG83 and UR6 were collected from Kala-azar patients

Fig. 5 The amplified ITS 1 region of nuclear DNA of clinical isolates and reference strains were digested using restriction enzyme *HaeIII*. Lane 1 MW marker (100 bp), lane 2 K27, lane 3 UR6, lane 4 DD8, lane 5, AG83. DD8 and K27 are WHO reference strains for *L. donovani* and *L. tropica* respectively; AG83 and UR6 were collected from Kala-azar patients

prominent band (lane 4) while K27 (lane 3) showed triple bands. DD8 (lane 1) and AG83 (lane 2) showed double bands. GPI (Fig. 1a), 6PGD (Fig. 1c) and PGM (Fig. 1d) showed single band profile for all while NH (Fig. 1b), showed double bands for both K27 and DD8 and the mobilities of two bands are strikingly different. The fast moving band for DD8, AG83, UR6 and K27 are with same mobility. On the other hand, the slow moving band for DD8 and AG83 are of same type while K27 and UR6 showed similar pattern. Some of photographs of the isozymes have been given as supplementary material as Online Resource 1.

Fig. 6 The amplified hsp70 region of nuclear DNA of clinical Isolates and reference strains were digested with restriction enzyme *HaeIII*. Lane 1 MW marker (100 bp), lane 2 K27, lane 3 UR6, lane 4 DD8, lane 5 AG83. DD8 and K27 are WHO reference strains for *L. donovani* and *L. tropica* respectively; AG83 and UR6 were collected from Kala-azar patients

In Table 1, we summarized the ELISA results of responses of surface antigens prepared from UR6, AG83 and two WHO reference strains with species specific monoclonal antibodies. Surprisingly, UR6 showed negative reactivity to *L. donovani* specific mAb, LXXVIII 2E5-A8 while AG83 and DD8 reacted much well with the *L. donovani* specific mAb. On the other hand, its reactivity towards *L. tropica* specific monoclonal antibody, XCIV 1H2-A8 can be considered positive (Table 1) as the P/N value exceeds 3.0 (Abranches et al. 1991).

Figure 3a–c represented the RAPD-PCR patterns of UR6, AG83 and reference strains with OPA3, OPA4 and OPA9 respectively. In all gel run, UR6 (lane 3) showed similar pattern as that of *L. tropica* (lane 2) and different from AG83 (lane 5) and DD8 (lane 4) respectively.

We performed Restriction Fragment Length Polymorphism analyses of the ITS PCR products from the test samples and reference strains by digesting them with three restriction enzymes—*AluI*, *EcoRI* and *MspI*. Interestingly again, Fig. 4 represented the similar digestion pattern of UR6 (lane 3) to that of the *L. tropica* standard strain K27 (lane 2) but not with *L. donovani* standard strain DD8 (lane 4) or AG83 (lane 5). Our observation is further corroborated by employing ITS1 RFLP. This amplicon when subjected to restricted digestion with *HaeIII* produced different patterns that unambiguously differentiated *L. tropica* from *L. donovani* (Kumar et al. 2007; Khanra et al. 2012). In the present study, digestion products of ITS1

Table 2 Jaccard's similarity indices of different *Leishmania* sp. calculated from Isozyme study

	<i>L. donovani</i> (DD8)	AG83	UR6	<i>L. tropica</i> (K27)
<i>L. donovani</i> (DD8)	0	1	0.28	0.22
AG83	1	0	0.28	0.22
UR6	0.28	0.28	0	0.75
<i>L. tropica</i> (K27)	0.28	0.22	0.75	0

The Jaccard's Coefficient has values between 1, for identical Operational Taxonomic Unit (OTU) and 0 for OTUs with no similarity at all. For an average analysis with nine enzymes, if the coefficient falls between any values, 0.75–1, one arbitrarily assumes that the strains belong to the same species or species complex (Tibayrenc et al. 1993)

In the table matrix, same strain (e.g., DD8 vs. DD8; AG83 vs. AG83 and UR6 vs. UR6 respectively) is denoted by 0, because in case of single strain, the Jaccard's Similarity Coefficient cannot be determined

Table 3 Jaccard's similarity indices of different *Leishmania* sp. calculated from RAPD-PCR study

	<i>L. donovani</i> (DD8)	AG83	UR6	<i>L. tropica</i> (K27)
<i>L. donovani</i> (DD8)	0	1	0.29	0.29
AG83	1	0	0.29	0.29
UR6	0.29	0.29	0	0.80
<i>L. tropica</i> (K27)	0.29	0.29	0.29	0

The Jaccard's Coefficient has values between 1, for identical Operational Taxonomic Unit (OTU) and 0 for OTUs with no similarity at all. For an average analysis with nine enzymes, if the coefficient falls between any values, 0.75–1, one arbitrarily assumes that the strains belong to the same species or species complex (Tibayrenc et al. 1993)

In the table matrix, same strain (e.g., DD8 vs. DD8; AG83 vs. AG83 and UR6 vs. UR6 respectively) is denoted by 0, because in case of single strain, the Jaccard's Similarity Coefficient cannot be determined

amplicon of all parasite samples with the Hae III (Fig. 5) were performed. In this figure, the digestion profile of UR6 (lane 3) was matching that of K27 (lane 2).

Similar observation has been made with Hsp70 PCR RFLP. Figure 6 showed the Hsp70 RFLP (HaeIII digest) profile of the samples related to our study.

We calculated the relatedness among different clinical isolates studied here by Jaccard's Similarity Coefficient method (Sneath and Sokal 1973) using our data set generated from the Isozyme (Table 2) and RAPD PCR results (Table 3).

In short, the calculation for Jaccard's Coefficient was done with numbers of total bands and with numbers of primers or enzymes used in the study. The Jaccard Similarity Coefficient, S_j was defined as

$$S_j = \frac{a}{a + b + c}$$

where a = number of bands (DNA) in common, b = bands unique to the first or reference stock, c = bands unique to the second stock whose relatedness to the first stock needs to be ascertained. In simpler cases, where only relatedness or similarity between two stocks or clinical isolates were to be determined, the mean of all the S_j values for different enzymes or arbitrary primers were calculated. The

Jaccard's Similarity Coefficient has values between 1, for identical Operational Taxonomic Unit (OTU) and 0 for OTUs with no similarity at all. For an average analysis with 8–12 enzymes (for MLEE) as well as with 8–12 primers (for RAPD PCR), if the coefficient falls between any values, 0.75–1, one arbitrarily assumes the strains belong to the same species or species complex (Tibayrenc et al. 1993). Our MLEE and RAPD data showed 75 and 80 % homology of UR6 with K27 respectively while it revealed 28 and 29 % homology with DD8 as drawn from above mentioned techniques.

Discussion

Many sincere attempts have been made so far, to look into the biochemical and genetic characteristics of the different species of *Leishmania* and there is no single criterion generally accepted for species identification (Arora et al. 1998).

As mentioned earlier, UR6 has been used extensively by several workers in the field, since its stabilization at Indian Institute of Chemical Biology. For example, in 1990, it has been described as *L. donovani* by Das and Adhya (Das and Adhya 1990) but as some doubt arose regarding the species

status of the isolate, scientists started describing it as *Leishmania* strain (Bhattacharyya et al. 1993, 1996) without mentioning the species name. Subsequently, a report came out stating UR6 strain has an isozyme profile similar to *L. tropica* as tested in the laboratories of Professor J A Rioux, University of Paris, France and Dr R Killick-Kendrick of Imperial College, London (Manna 1998). The use of UR6 in immunoprophylaxis and immunotherapy against experimental visceral leishmaniasis was reported by the Indian authors mentioning it as ‘atypical’ (Mukhopadhyay et al. 2000) isolate. Another interesting observation was that UR6 when given through intra cardiac route failed to induce visceral infection, but when it was injected subcutaneously, UR6 induced a short-lived and localized self-healing skin lesion. These features match with that of *L. tropica*. Priming of peritoneal macrophages with UR6 in vitro induced superoxide (O_2^-) generation, whereas similar experiments with virulent AG83 inhibited O_2^- generation (Mukhopadhyay et al. 2000). The apparent co endemicity of *L. tropica* has been reported earlier also. In the voluminous work of Schnur et al. (Schnur et al. 1981) the authors typed several clinical isolates collected from Indian KA by the then emerging technique of Isozyme profiling with limited number of enzymes and reported one isolate that apparently corresponded to *L. tropica* but serious efforts has not been taken to screen by other molecular taxonomical techniques. Thus, lack of other sensitive and sophisticated molecular methods as well as lack of attitude to screen each and every isolate made the field of strain identification utterly neglected.

We have included AG83 in the present study for the facts that it is almost as old as UR6 and till date, both the isolates are in use for various research purposes; another point is that AG83 has published profile (Chatterjee et al. 1995; Manna et al. 2005) and it has always been regarded as *L. donovani* strain (Basu et al. 2005, 2006; Halder et al. 2010; Mukhopadhyay et al. 2011; Guha et al. 2013, 2014). So, it may act as another frame of reference besides DD8 and K27, the WHO reference strains for *L. donovani* and *L. tropica* respectively. AG83 has not become avirulent though it has been isolated long back but UR6 has been regarded as ‘avirulent’. Is UR6 avirulent *L. donovani* isolate or as it is *L. tropica*, it cannot visceralize? Our observations cleared the doubt for the first time with the help of polyphasic taxonomical methods that UR6 is *L. tropica*.

Conclusions

By using various molecular taxonomical techniques we observed that the isolate, UR6, a confirmed KA isolate, showed more relatedness to *L. tropica* than to *L. donovani*.

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Compliance with ethical standards

Conflict of interest None.

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