
Comparative evaluation of IS6110 PCR via conventional methods in rapid diagnosis of new and previously treated cases of extrapulmonary tuberculosis

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ÖZET

Yeni ve önceden tedavi edilmiş ekstrapulmoner tüberkülozlu hastaların hızlı tanısında IS6110 ile konvansiyonel yöntemlerin karşılaştırılması değerlendirilmesi

Gelişmekte olan ülkelerde ekstrapulmoner tüberküloz (EPTB) tanısı önemli bir problemdir. EPTB’de, az sayıda basil içerme özelliği, yetersiz miktarda örnek gibi birçok sorun bulunmaktadır. Bütün bu kısıtlamalar, konvansiyonel bakteriyolojik tekniklerin EPTB tanısına düşük katkısına neden olmaktadır. Nükleik asit amplifikasyon yöntemleri, mikobakteriyel DNA’nın saptanması amacıyla geliştirilen hızlı ve duyarlı tekniklerdir. *Mycobacterium tuberculosis complex*’in spesifik genomunda yer alan “insertion sequence” IS6110’a ait 123bp’nin DNA fragmanı, EPTB’nin hızlı tanısı amacıyla polimeraz zincir reaksiyonu (PCR) ile çoğaltıldı. Bu çalışmada, yeni ve önceden tedavi edilmiş EPTB’li hastaların hızlı tanısında IS6110 PCR ile konvansiyonel yöntemler karşılaştırıldı. EPTB şüpheli hastalardan 450 örnek toplandı ve *Mycobacteria* için Zeihl Neelson (ZN) boyama ve *M. tuberculosis* için BACTEC kültürü yapıldı. Bütün örnekler ayrıca, *M. tuberculosis complex*’in insertion element IS6110’un 123bp fragmanını hedefleyen primerlerle PCR amplifikasyonu ile IS6110 için çalışıldı. Testler arasında duyarlılık bakımından anlamlı fark saptandı. Dört yüz elli örnek içinde, 60 (%13.4)’ünde ZN boyamada ARB pozitif, 202 (%45)’inde BACTEC kültürü ve 283 (%63)’ünde *M. tuberculosis complex* için IS6110 PCR pozitifliği ($p < 0.05$). Bununla birlikte, testler arasında spesifite bakımından anlamlı fark yoktu ($p > 0.05$). IS6110 PCR’nin hem yeni hem de önceden tedavi edilmiş hastalarda, yayma mikroskopi ve BACTEC kültüründen daha duyarlı olduğunu bulduk. IS6110 PCR, yeni ve önceden tedavi edilmiş EPTB’li hastaların tanısında kullanışlı olabilir. Şüpheli EPTB’li hastaların tedavi kararında fayda sağlayabilir.

Anahtar Kelimeler: Tüberküloz, ekstrapulmoner tüberküloz, polimeraz zincir reaksiyonu, IS6110.

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SUMMARY

Comparative evaluation of IS6110 PCR via conventional methods in rapid diagnosis of new and previously treated cases of extrapulmonary tuberculosis

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In developing countries the diagnosis of extrapulmonary tuberculosis (EPTB) is a major burning challenge. EPTB encounters many problems like pauci-bacillary nature, inadequate specimen volume. All the limitations reflect in the poor contribution of conventional bacteriological technique in the establishment of diagnosis of EPTB. Nucleic acid amplification methods are rapid and sensitive has modified strategies for the detection of mycobacterial DNA. A fragment of DNA of 123bp belonging to insertion sequence IS6110 based on specific gene of Mycobacterium tuberculosis complex was amplified by polymerase chain reaction (PCR) for the rapid diagnosis of EPTB. The present study was to comparative evaluation of IS6110 PCR via conventional methods in the rapid diagnosis of new and Previously treated cases of extra pulmonary tuberculosis. Four hundred fifty specimens were collected from suspected cases of EPTB were processed for Mycobacteria by Ziehl Neelson (ZN) staining and BACTEC culture for M. tuberculosis. All the specimens were also processed for IS6110 based PCR amplification with primers targeting 123 bp fragment of insertion element IS6110 of M. tuberculosis complex. We found significant difference was seen in sensitivities of different tests. Of these 450 specimens, 60 (13.4%) were positive for AFB by ZN staining, 202 (45%) for BACTEC culture and IS6110 PCR were positive for M. tuberculosis complex in 283 (63%) specimens ($p < 0.05$). However, there was no significant difference ($p < 0.05$) as far as specificity of different tests. We found that IS6110 PCR has higher sensitivity than smear microscopy and BACTEC culture in both cases of new cases as well as in previously treated cases. IS6110 PCR can be highly useful in diagnosis of new and treated cases of EPTB. It may facilitate therapeutic decisions for those with suspected of EPTB.

Key Words: Tuberculosis, extrapulmonary tuberculosis, polymerase chain reaction, IS6110.

Tuberculosis (TB) continues to be a major global public health problem. Incidence of extrapulmonary tuberculosis (EPTB) is on increasing worldwide as well as in India (1,2). EPTB comprises 20% of all TB cases in India (3). Diagnosis of EPTB in different clinical presentations has been always as challenge. Smear microscopy and culture lack of sensitivity in EPTB case and culture (solid and liquid media) also takes at least two to four weeks for grow of mycobacteria. A study has reported smear positive is around 10-37% of the patients and mycobacterial culture is positive in variable proportional 12-80% in different biological specimens (3). Studies from many laboratories around the global were using primers most commonly targeting the IS6110 insertion element (4-9). The detection of the IS6110 insertion element present in form of multiple copies to detect of *Mycobacterium tuberculosis* complex but not other mycobacterial species (9-11). Polymerase chain reaction (PCR) using IS6110 insertion sequences as the target, has potential to conquer limitation of conventional method and to established as rapid, sensitive tech-

nique for detecting DNA of *M. tuberculosis* in different clinical specimens from respiratory and non respiratory sites (10,12,13). Author has provided results 61.5% sensitivity for smear negative samples up to 87.8% specificity on *M. tuberculosis* culture (5). Various studies were revealed that sensitivity of PCR in these ranges 42-93% (14-17). Various studies showed that PCR can be used for the detection of pulmonary TB cases in routine laboratories (18-20). Few studies shown data on detection of *M. tuberculosis* in extrapulmonary tuberculosis cases but results were not defined on basis of new and previously treated of EPTB. The aim of this study was to comparative evaluation of IS6110 PCR vs. conventional methods in the diagnosis of new and previously treated cases of extrapulmonary tuberculosis cases at tertiary care hospitals.

MATERIALS and METHODS

Study Design

The study was performed prospectively in a blinded manner.

Study Population

Study setting was Referral Medical Institutions at Northern India. Indoor and Outdoor, Department of Pulmonary Medicine, Chhatrapati Shahu Ji Maharaj Medical University (IP, Lucknow, India (Erstwhile King George Medical University) and Mycobacteriology Laboratory, Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India.

Clinical Specimens and Data Collection

2-5 mL of specimens was collected from 450 specimens, non-repeated specimens from suspected cases of extrapulmonary tuberculosis. The specimens were included as Lymph Node Aspirate and Cold Abscesses, Pleural fluid, C.S.F, Synovial Fluid, Ascetic Fluid, Urine, Gastric Aspirate, Pus, Bone Marrow, Wound and Pus swab and Others specimens (biopsies tissues). All specimens were kept in ice box and transported Mycobacteriology Laboratory, Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India for smear examination by ZN Staining, BACTEC Culture and PCR test. All patients were signed with due informed consent of the patients from indoor and outward wards of Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University, Lucknow, India and Mycobacteriology Laboratory, Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India during Jan 2009 to Dec 2010. The clinical history regarding, present and past history of antitubercular treatment (ATT); family history of tuberculosis and any other associated disease were taken in prescribed Performa.

Microbiological Analysis of Extra Pulmonary Specimens

Specimens was divided in to two part one part was kept at -20 for PCR till processing and another part was processed for mycobacterial smear preparation and BACTEC culture. Smears were stained with Ziehl Neelsen (ZN) method and examined for acid-fast bacilli (AFB) (21). BACTEC vials were incubated and interpreted as per Becton Dickinson (BD, Sparks, MD, USA) manual instructions (22). NAP (p-nitro- α -acetylaminobeta-hydroxy propiophenone) (Becton Dickinson, Sparks, MD, USA), identification was done to differentiate *M. tuberculosis* form non tuberculous mycobacteria (22). A decrease or unchanged growth index (GI) in nap vial indicated presence of *M. tuberculosis* complex (MTBC), while an increase in GI indicated the presence of *Mycobacterium* other than tuberculosis (MOTT). Standard H₃₇ Rv strain of *M. tuberculosis* complex was used as positive control.

Extraction of DNA

Extraction of DNA was done by the CTAB (cetyl-trimethyl-ammonium bromide) -phenol chloroform extraction method (23). Specimens were centrifuged at 10.000 rpm for 10 min. The supernatant was discarded and the pellet suspended in 567 μ L of TE (Tris EDTA, pH 7.4) buffer, 30 μ L 10% SDS (sodium dodecyl sulfate) and 3 μ L proteinase K (20 mg/mL), mixed and incubated at 37°C for 1 hour. After incubation, 100 μ L of 5 M NaCl and 80 μ L of high-salt CTAB buffer (containing 4 M NaCl, 1.8% CTAB was added and mixed followed by incubation at 65°C for 10 min. An approximate equal volume (0.7-0.8 μ L) of chloroform-isoamyl alcohol (24.1) was added, mixed thoroughly and centrifuged for 4-5 min in a microcentrifuge at 12.000 rpm. The aqueous viscous supernatant was carefully decanted and transferred to a new tube. An equal volume of phenol: chloroform-isoamyl alcohol (1:1) was added followed by a 5 min spin at 12.000 rpm. The supernatant was separated and then mixed with 0.6 volume of isopropanol to get a precipitate. The precipitated nucleic acids were washed with 75% ethanol, dried and re-suspended in 100 μ L of TE buffer.

Primer and IS6110 PCR

The amplification reaction was performed in a final volume of 20 μ L. the reaction mixture contained 10 μ L Pyrostart Fast PCR Master Mix 2X (dNTP, Taq polymerase with MgCl₂, Fermentas, India), 1 μ L (10 pmole) of each primer, 3 μ L water (nuclease free) and 5 μ L of extracted DNA. The oligonucleotide primers used were IS1 and IS2, are: 5'-CCT GCG AGC GTA GGC GTC GG3' and 5' CTC GTC CAG CGC CGC TTC GG 3' respectively (SBS Gentech Co. Ltd) (24). These primers amplified a target fragment at 123 base pairs (bp) from the insertion, *M. tuberculosis* sequence element IS6110. The PCR amplification was done in thermal cycler (MJ Research, PTC-100, GMI, Inc, USA), which involved 40 cycles of denaturation at 94°C for 2 minute, annealing of primers at 68°C for 2 minute, and primer extension at 72°C for 1 minute. The amplified products were separated on 2% agarose gels, visualized on a UV-light transilluminator (Bangalore Genei, Bangalore, India). The presence of 123bp fragment indicate as positive test as *M. tuberculosis* complex. The positive controls included the DNA of H37Rv strain. Negative control included PCR grade water (Figure 1).

Statistical Analysis

Data were analyzed using SPSS 15.0 (Statistical Package for the Social Sciences, Chicago, IL, USA) for

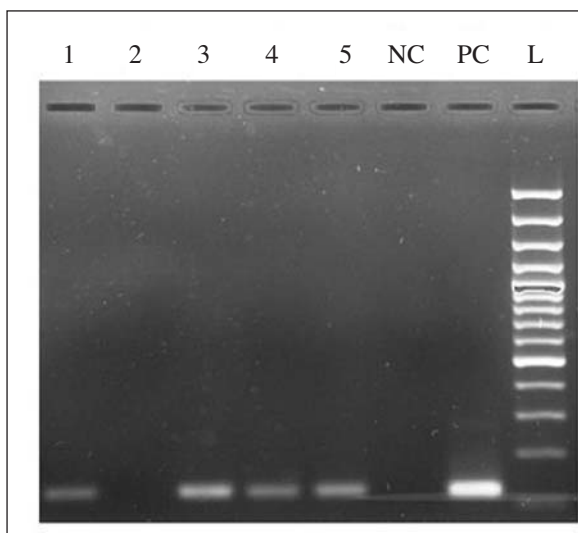


Figure 1. PCR based detection of *M. tuberculosis* targeting IS6110. Electrophoresis separation of the amplicon into 2% agarose gel is documented across Lanes 1-5. The presence of a 123 bp amplicon in the Lanes 1,3,4,5 indicated the presence of the target while the absence of the amplicon in the Lane 2 pointed towards the absence of the target. Lane NC was negative control and Lane PC was positive control (H37Rv). Ladder 100bp was well shown.

Windows. The significance of difference was taken as significance value ($p < 0.05$). Sensitivity was calculated as $[Tp / (Tp + Fn)] \times 100$; specificity was calculated as $[Tn / (Tn + Fp)] \times 100$; Tp = total number of positives; Tn = total number of negatives; Fp = total number of false positive, Fn = total number of false negative; respectively.

RESULTS

Specimen's Characterization of Extrapulmonary Tuberculosis Cases

During the two year study period, 470 clinical specimens were strong clinical suspicion of extrapulmonary tuberculosis were subjected from tertiary care hospitals and all mention test were performed. Out of these, 20

specimens found to be contaminated in BACTEC culture. 450 specimens of results were used in the study. Out of 450 specimens, 153 (34%) lymph node aspirate and cold abscesses, 58 (12.8%) pleural fluid, 44 (9.7%) cerebrum spinal fluid (CSF), 48 (10.7%) urine, 31 (6.8%) ascetic fluid, 26 (5.8%) pus, 22 (4.9%) wound and pus swab, 16 (3.5%) gastric aspirate, 10 (2.2%) bone marrow, 10 (2.2%) synovial fluid and 30 (6.7%) others specimens (biopsies tissues). Out of 450 patients, 320 (71.1%) patients were males and 130 (28.9%) females. The mean age of all patients was 39.8 ± 16.1 years. Patients 25-44 years of age accounted for 45% of the total cases. Out of 450 cases, 328 (72.8%) were new cases and 122 (22.2%) were previously treated cases of EPTB.

Detection Rate of *M. tuberculosis* by IS6110 PCR, BACTEC Culture and ZN Smear Microscopy According to New Cases and Previously Treated Cases

All specimens were collected from suspected case of extra pulmonary tuberculosis were found to be AFB positive were 60 (13.4%). On the basis of cases, we found that sensitivity of AFB staining on EPTB were 37 (11.2%) in new cases and 23 (18.8%) in previously treated cases. The sensitivity of AFB staining was higher in comparison to previously treated cases. Overall detection rate of *M. tuberculosis* by AFB Staining was 60 (13.4%). The detection of *M. tuberculosis* by BACTEC culture was 202 (45%). Results of BACTEC culture according to cases, 151 (46.03%) were in new cases and 51 (41.8%) were in previously treated cases. We found that sensitivity of BACTEC culture was higher in new cases. All culture isolates obtained were confirmed as mycobacteria with biochemical tests mentioned. Using IS 6110 PCR, 283 (61.8%) were positive for IS6110 PCR for *M. tuberculosis*. 203 (61.8%) were positive in new cases and 80 (65.5%) were positive in previously treated cases. We found that sensitivity of IS6110 PCR was higher in previously treated cases. Overall comparison of tests, IS6110 PCR was found to have much hig-

Table 1. Detection by *Mycobacterium tuberculosis* by IS6110 PCR via others conventional tests according to new cases and previously treated cases of extrapulmonary tubercular cases (450).

Cases, %	ZN staining (450)	Detection rate (%)	
		BACTEC culture (450)	IS6110-PCR (450)
New cases (328, 72.8%)	37 (11.2%)	151 (46.03%)	203 (61.8%)
Previously treated cases (122, 22.2%)	23 (18.8%)	51 (41.8%)	80 (65.5%)
Total (450, 100%)	60	202	283
Overall sensitivity	13.4%	45%	63%

Table 2. Results of 50 sputum collected from healthy patients.

Test performed	Test performed	Results		
		Positive	Negative	Specificity
ZN Smear	50	0	50	100%
BACTEC culture	50	0	50	100%
IS6110 PCR	50	1	49	98%

Table 3. Comparison of sensitivity of PCR test via others conventional tests according to new cases and previously treated cases.

Test (450)	New cases (328)				Previously treated cases (122)			
	PCR results			Sensitivity (%)	PCR results			Sensitivity (%)
	No.	Positive	Negative		No.	Positive	Negative	
Smear positive	37	37	0	100%	23	23	0	100%
Smear negative	291	164	127	56.3%	99	57	42	57.5%
BACTEC positive	151	147	4	97.3%	51	50	1	98.1%
BACTEC negative	177	58	119	32.7%	71	30	41	43.6%
Smear positive BACTEC positive	23	23	0	100%	18	18	0	100%
Smear negative BACTEC positive	128	117	11	91.4%	37	35	2	94.5%
Smear positive BACTEC negative	14	6	8	42.8%	5	3	2	60%
Smear negative BACTEC negative	163	57	106	34.9%	62	24	38	38.7%

her sensitivity of 61.8% (Table 1). Among 50 healthy controls, all the samples were negative for smear as well as culture, while only 1 out of these 50 was PCR positive for *M. tuberculosis*. Specificity was found to be 100% by ZN Staining and BACTEC culture but IS6110 PCR was showed specificity of 98% (Table 2).

Comparison of Sensitivity of IS6110 PCR Test Via Others Conventional Tests According to New Cases and Previously Treated Cases

IS6110 PCR test was found to be much more sensitive than ZN staining and BACTEC culture results individually as well as in combination are shown in (Table 3). IS6110 PCR test was found to be much more sensitive than ZN staining and BACTEC culture ($p < 0.05$). We have compared of sensitivity of IS6110 PCR test via others conventional tests according to new cases and previously treated cases. Of these new cases 328, 37 specimens were positive with AFB and PCR had posi-

ve (100%), 127 (56.3%) were PCR positive with smear negative specimens, 147 (97.3%) specimens were BACTEC positive and PCR has positive for *M. tuberculosis*, 23 (100%) specimens were both positive with AFB smear and BACTEC culture but PCR had positive in 23 (100%), 128 specimens were negative with AFB smear and BACTEC culture was positive but PCR had positive in 117 (91.4%), 14 specimens were positive with AFB smear but BACTEC culture was negative but PCR was positive in 6 (42.8%). In previously treated cases 122, 23 patients were positive with AFB and PCR had positive (100%), 57 (57.5%) were PCR positive with smear negative specimens, 50 (98.1%) specimens were BACTEC positive and PCR has positive, 37 specimen were negative with AFB smear and BACTEC culture was positive but PCR had positives in 35 (94.5%), 5 specimens were AFB smear positive and BACTEC culture were negative but only PCR had positive in 3 (60%). In 225 specimens negative by conven-

tional tests used, PCR test was able to detect 57/163 (34.9%) positives in new cases and 24/68 (38.7%) positives in previously treated cases. these were not likely to represent false positive results as PCR repeat was positive in these specimens and its belonged to highly suspected of EPTB who responded to antitubercular treatment. We found that sensitivity of PCR with ZN staining was not different in new cases and previously treated cases. Our results shown the sensitivities of PCR were higher in previously treated cases as comparison to new cases (Table 3). Studies have reported average detection time for *M. tuberculosis* was 13.37 days in EPTB cases (25). Our result shown average detection time for *M. tuberculosis* was 14.76 days by BACTEC and less than one day by PCR test.

DISCUSSION

Tuberculosis (TB) is a major public health dilemma in India. India is the highest TB burden country accounting for one fifth of the global incidence. Global annual incidence estimate is 9.4 million cases out of which it is estimated that 1.98 million cases are from India (26). In India, EPTB comprises 20% of all TB cases. Its prevalence in the country varies between 8.3-13.1% in different districts according to cohort analysis by Central TB Division, Ministry of Health and Family Welfare in 2002 (27,28). The diagnosis of extrapulmonary tuberculosis is till now challenging for diagnostic routine laborites. Numeric reasons are showing that, lack of adequate specimens amounts or volumes; distribute of the specimens for different diagnostic tests (histology/cytology, biochemical analysis, microbiology, and PCR), non-uniform distribution of microorganisms; paucibacillary nature of the specimens; presence of inhibitors that undermine the performance of nucleic acid amplification-based techniques; and the lack of an efficient sample processing technique universally applicable on all types of extrapulmonary samples (29). The poor performance of conventional *M. tuberculosis* detection techniques, based on microscopic examination of Ziehl-Neelsen stained and culture of *M. tuberculosis* (LJ Medium and BACTEC Radiometric culture) are still in widespread use for diagnostic purposes, still though they fail to provide the required sensitivity and specificity. The PCR test would be particularly useful in the diagnosis of EPTB where conventional microbiological techniques for *M. tuberculosis* are showing poor performance of sensitivity. The specificity, sensitivity and speed of PCR test in diagnosis of *M. tuberculosis* infection shown in this study should encourage the use of this method in routine diagnosis of EPTB.

Previously studies shown the success of microscopy is highly variable from 22% to 96% and most authors rate it at round 60% (30-32). Our results shown that sensitivity of smear microscopy was 13.7% and specificity was 100%. The sensitivity of microscopy depends on the clinical presentation and more than 10.000 bacilli per milliliter are necessary for secure microscopic positivity (33). Our studies shown that conventional bacteriological technique were positive in 202 (45%) specimens, where as IS6110 PCR showed that 283 (63%) specimens were positive for *M. tuberculosis*. The difference was found that to be statistical significant ($p < 0.05$). Several studies have been reported on PCR to detect *M. tuberculosis* (34-39). The detection of the IS6110 insertion element present in multiple copies to detect *M. tuberculosis* complex, but not other mycobacterial species (9,40). This fact is increased sensitivity afforded by the detection of the IS6110 insertion sequence considerably improves the yield for the detection of mycobacterial DNA in EPTB cases. Studies reported form documented increased positivity by PCR targeting IS6110 in specimens of EPTB. Sekar et al. showed 63% of positivity, Negi et al. showed that 73%, Tiwari et al. showed 62% of positivity rate among EPTB (12,41,42). But we found that IS6110 PCR positivity was 63% among new and previously treated cases of EPTB. The overall sensitivity of PCR for detecting EPTB greatly exceeded the sensitivity of the acid-fast smear (13.5%) and culture (45%) techniques alone, and the sensitivity of smear and culture used together (72%). PCR sensitivity was considerably better than the conventional methods in all the clinical specimens of EPTB examined. PCR test detected *M. tuberculosis* is less than one day, compared to average 12.89 to 13.37 days by radiometric BACTEC technique in pulmonary TB and EPTB cases, as supported by earlier studies (25,43). Our studies had shown 14.76 days by radiometric BACTEC technique and PCR detected for *M. tuberculosis* was less than one day for EPTB cases. Negi et al. shown that 62.5% were PCR positive with smear negative specimens, 97.5% specimens were BACTEC positive and PCR has positive, 97.8% specimen were negative with AFB smear and BACTEC culture was positive but PCR had positives (41). Therefore our study results on the basis of cases shown that 56.3% were PCR positive with smear negative specimens in new cases and but 57.5% were in previously treated cases. 97.3% specimens were BACTEC positive and PCR has positive in new cases and but 98.1% in previously treated cases. 91.4% specimens were negative with AFB smear and BACTEC culture was positive but PCR had positives in new cases and 94.5% were in previously treated cases. We found that ZN smear examina-

tion and PCR results were positive but BACTEC culture was negative; these could be the presence of nonviable mycobacteria in the sample as patients were receiving antitubercular treatment. IS6110 PCR test is higher sensitivity than microscopy and the culture and could help in therapeutic decision for patients with clinical suspicion of EPTB.

CONCLUSION

IS6110 PCR test for DNA specific *M. tuberculosis* may be hopes of a rapid and accurate diagnostic test for EPTB and it will help where conventional diagnosis fails and provisional diagnosis of tuberculosis is made on the basis of clinical presentation and histology/cytology examination without evidence of AFB. IS6110 PCR may be great potential to improve the clinician vision for the early diagnosis, treatment and prevention of EPTB.

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CONFLICT of INTEREST

None declared.

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