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ORIGINAL ARTICLE

A method of direct PCR without DNA extraction for rapid detection of begomoviruses infecting jute and mesta

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Significance and Impact of the Study: Identification of begomoviruses by serology is not suitable due to difficulty in preparing high titre and specific antisera. Begomoviruses are routinely detected by PCR-based techniques using universal or specific primers. However, it is a prerequisite to isolate pure DNA from the samples before PCR. DNA extraction from some plants such as jute, mesta is very difficult due to the presence of mucilage and other impurities. Therefore, we have developed a method of direct PCR without DNA extraction for detection of begomoviruses from these crops. It is the first report of a direct PCR method in jute and mesta.

Keywords
direct PCR, jute, lysis buffer, mesta.

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Abstract

We have developed a simple method of direct PCR (dPCR) without time-consuming procedures of DNA extraction by directly using the leaf bits for rapid detection of begomoviruses in jute and mesta. The leaf bits were treated with a lysis buffer for 35 min, and the lysate was used as PCR template. Different components and their concentration in lysis buffer systems were optimized and the optimal buffer system composed of 20 mmol l^{-1} tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8·0), 1·5 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA) (pH 8·0), 1·4 mol l^{-1} NaCl and 200 μg/mL Proteinase K. Further, 3% PVP (w/v) and β-marcaptoethanol (1% v/v) were additionally added into the buffer in case of jute. Under optimized PCR conditions, both viral DNA as well as plant (jute and mesta) genomic DNA were amplified from the lysate. dPCR required fewer reagents and less incubation time reducing both time and cost of detection.

Introduction

In recent years, the whitefly transmitted begomoviruses which belong to the family *Geminiviridae* are emerging as a major threat to a number of economically important crops particularly in the tropical and subtropical world (Wyatt and Brown 1996). Moreover, evolution of newer begomoviruses through variation and the occurrence of mixed infection with different begomoviruses have aggravated the disease spectrum and the ability of these begomoviruses to infect new hosts poses an additional threat to the cultivation of several crops (Varma and Malathi 2003; Ghosh *et al.* 2009). In addition to several vegetable crops, the two most important bast fibre crops namely jute and mesta are also infected by begomoviruses. Jute is mainly grown in the South-East Asian countries like India, Bangladesh, Nepal, China, Indonesia, Thailand, Myanmar and few South American countries, and the fibre is used in making sacks, ropes, bags, carpets, shoes, geotextiles, jewellery, home decorations, etc. (Kundu 1956). It is of two kinds namely, *tossa* jute (*Corchorus olitorius*) and white jute (*Corchorus capsularis*). Mesta is also of two types, viz. HS mesta (*Hibiscus sabdariffa*) and HC mesta (*Hibiscus cannabinus*). HS mesta is widely grown in the tropics of Central America, India, Africa, Brazil, Australia, Hawaii, Florida, Caribbean islands and Philippines. The major HC mesta-growing countries are India, China, Brazil, Cuba, Vietnam, Indonesia and Thailand (IJSG 2010). Mesta has valuable industrial use because of its bast fibre, medicinal value and application in paper industry (Mahadevan *et al.* 2009). Both *tossa* jute (*C. olitorius*) and white jute (*C. capsularis*) are infected by *Corchorus golden*

mosaic virus (CoGMV), a begomovirus, although white jute is affected most (Ghosh *et al.* 2008, 2010). Both kinds of mesta viz. HS mesta and HC mesta are severely affected by another devastating begomovirus named *Mesta yellow vein mosaic virus* (Chatterjee *et al.* 2005; Chatterjee and Ghosh 2007). Mesta yellow vein mosaic virus and Corchorus golden mosaic virus (CoGMV) both are bipartite begomoviruses and they are detected by DNA-A- or DNA-B-specific primers through PCR which requires isolation of good quality DNA in large quantity (Chatterjee *et al.* 2005; Ghosh *et al.* 2009). Unlike from animal tissues, DNA extraction from plant tissues is generally difficult due to the presence of a rigid cell wall surrounding the plant cells. Furthermore, presence of mucilage makes DNA isolation from jute and mesta leaves more difficult because the mucilage often binds with other secondary metabolites and coprecipitate with DNA during isolation (Ghosh *et al.* 2009). To address this problem, we developed a modified CTAB method with improved salt concentration and simple sodium acetate treatment (Biswas *et al.* 2013) which yielded good quantity of high-quality DNA from jute seed, but the method is time-consuming and expensive. Therefore, in the present investigation, we have developed a method of direct PCR (dPCR) which obviates the procedure of DNA extraction before PCR by directly using the lysate of leaf bits as PCR template. This method would enable rapid detection of *Corchorus golden mosaic virus* and *Mesta yellow vein mosaic virus* from jute and mesta, respectively and the cost of detection per sample would also be much lower.

Results and discussion

In the present study, two begomoviruses viz. *Corchorus golden mosaic virus* from jute (white jute as well as *tossa* jute) and *Mesta yellow vein mosaic virus* from mesta (both HS mesta and HC mesta) were detected by duplex direct PCR without prior DNA isolation. Leaf bits from infected plants were subjected to lysis, and the lysate was directly used as template in PCR.

Standardizing lysis of leaf bits

Different components and their concentration in lysis buffer systems were optimized with major modifications of cell lysis method. The finally acquired optimal buffer system composed of 20 mmol l^{-1} tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8·0), 1·5 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA) (pH 8·0), 1·4 mol l^{-1} NaCl and 200 μg/mL Proteinase K, 3% PVP (w/v) and β-mercaptoethanol (1% v/v). PVP and β-mercaptoethanol were added to eliminate mucilage and secondary metabolites (Biswas *et al.* 2013).

Standardization of PCR conditions

In the duplex d-PCR method, reaction components and reaction cycling parameters were so standardized which enabled simultaneous amplification of both viral and genomic DNA. The concentrations of the main ingredients, such as primers, $MgCl_2$, Taq DNA polymerase, dNTPs and PCR conditions including annealing temperature and amplification cycles were examined and optimized. In the present study, infected jute or mesta sample generated two different amplicons of both viral DNA-A fragment and plant genomic DNA, but in case of healthy control, only SSR primer was amplified which indicated the efficiency of described method.

dPCR detection of CoGMV and mesta yellow vein mosaic virus

CoGMV, a begomovirus could be detected from white jute (*C. capsularis*) and *tossa* jute (*C. olitorius*) leaves, showing mosaic symptoms using the lysate as template in dPCR with DNA-A-specific primer which generated an amplicon of 2667 bp and 1158 bp, respectively (Fig. 1), and jute-specific SSR was amplified at 250 bp. All the jute leaf samples showing yellow mosaic symptom were amplified, but in case of asymptomatic healthy looking plants, no virus-specific amplification was obtained and only the SSR primer generated amplicon of 250 bp. Similarly, *Mesta yellow vein mosaic virus* was detected from infected mesta leaves by dPCR. In case of HS mesta (*H. sabdariffa*), DNA-A-specific primer showed amplification of

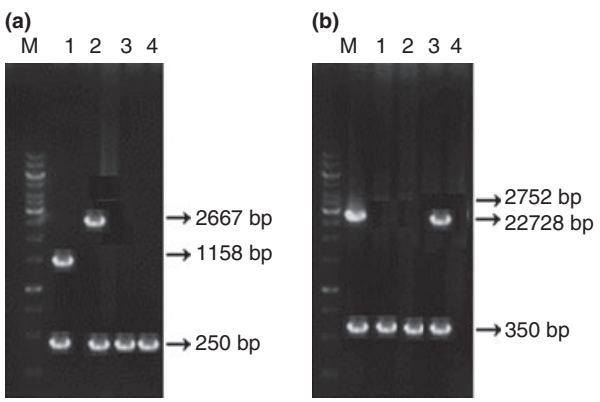

Figure 1 (a) Direct PCR (dPCR) amplification of CoGMV and jute genomic DNA in *Corchorus capsularis* and *Corchorus olitorius*. Lane 1: CoGMV-infected *C. olitorius*, lane 2: CoGMV-infected *C. capsularis*, lane 3: healthy *C. olitorius*, lane 4: healthy *C. capsularis*. Lane M: 1-kb ladder. (b) Amplification of mesta yellow vein mosaic virus and mesta genomic DNA in *Hibiscus sabdariffa* and *Hibiscus cannabinus*. Lane 1: virus-infected *H. sabdariffa*, lane 2: healthy *H. sabdariffa*, lane 3: healthy *H. cannabinus*, lane 4: virus-infected *H. cannabinus*. Lane M: 1-kb ladder.

2752-bp PCR product from all the symptomatic samples, whereas HC mesta (*H. cannabinus*) gave 2728-bp amplicon. No begomovirus-specific amplification was obtained from healthy samples, only Hibiscus-specific SSR generated amplicon at 350 bp (Fig. 1). Duplex dPCR of infected samples caused amplification of both viral DNA as well as plant (jute/mesta) genomic DNA, but in case of asymptomatic healthy samples, only plant genomic DNA was amplified. The present direct PCR method was highly efficient in amplifying viral DNA as well as genomic DNA in all the four selected plant species. Of 30 samples tested in each species, the number of dPCR positive samples varied from 25 to 30 (Table 1), which were further confirmed to be same by conventional method (DNA isolation by CTAB method followed by PCR).It implies that dPCR-negative samples were actually noninfected.

Detection limit of begomoviruses

DNA could be amplified from the lysate prepared from 1 mg tissue up to 1 : 3500 dilution in case of *C. capsularis* and *H. sabdariffa* (Fig. 2). In case of *H. cannabinus* and *C. olitorus* detection, limit was 1 : 4000 and 1 : 4500, respectively (Fig. 3). *Tomato yellow leaf curl Thailand virus* could be detected by a direct PCR method in 1 : 20 000 diluted sap from 10 mg tissue (Leamkhang *et al.* 2005). As only 1 mg tissue was used in the present method, its detection limit appears to be about twofold higher than this method.

Five each PCR-positive products of *C. olitorius, C capsularis*, *H. Sabdariffa* and *H. cannabinus* samples were cloned and sequenced. All the five sequences of each plant species were found identical among themselves. The BLASTn analysis of the nucleotide sequence revealed that the present CoGMV sample had 100% homology with *Corchorus golden mosaic virus* segment DNA-A, complete sequence (Accession no. DQ641688). BioEdit software (version 7) and ORF finder of NCBI suggested the complete segment of DNA-A, which revealed that complete CDS had four genes in the complementary sense viz. AC1, AC2, AC3, AC4 and AV1 gene in the viral sense. In case of *C. olitorius* infected sample, it showed 100% similarity with *Jute yellow mosaic virus* Bahraich isolate with nonfunctional replication initiator protein (AC1) gene, partial sequence; and coat protein (AV1) gene, partial cds (Accession no: EU68668). The concerned gene translated replication initiation protein product. In case of HS mesta (*H. Sabdariffa*), BLASTn analysis of the nucleotide sequence revealed that the present infected sample had 100% homology with *Mesta yellow vein mosaic virus* -[India: Barrackpore2:2006] segment DNA- A, complete sequence (Accession: EF428256). BioEdit software (version 7) and ORF finder of NCBI suggested the complete segment of DNA-A revealed that complete CDS had seven genes viz. AV1, AC5, AV2, AC3, AC2, AC1 and AC4. Infected *H. cannabinus* samples showed 100% identity with *Mesta yellow vein mosaic virus* isolate Barrackpore segment DNA-A, complete sequence (Accession no: EF373060) which encodes precoat protein, coat protein, replication initiator protein, transcription activator protein, replication enhancer protein and transcriptional regulator protein.

Table 1 PCR amplification efficiency of direct PCR protocol in different plant species

Plant species and cultivar	Primer	Product size (bp)	PCR efficiency (no. of PCR-positive samples/no. of samples tested)
Corchorus capsularis Cultivar JRC 212	DNA-A/ MJM561	2667/ 250	26/30
C. olitorius Cultivar JRO 524	DNA-A/ MJM561	1158/ 250	30/30
Hibiscus sabdariffa Cultivar AMV5	DNA-A/ MJM609	2752/ 350	25/30
H. cannabinus Cultivar HC583	DNA-A/ MJM609	2728/ 350	28/30

PCR has become the most popular technique with application in medical, agricultural, biotechnological, phyllogenetical and basic research (Gindro *et al.* 2005). For research concerning disease diagnostics, plant diversity analysis, marker-assisted selection, genetic purity testing, etc., PCR generally requires prior purification of good quality DNA and it involves many steps making the whole process time-consuming and expensive. To overcome these preparatory steps from PCR-based amplification, some direct PCR methods have been developed (Bellstedt *et al.* 2010; Sharma *et al.* 2012) mainly to amplify plant genomic DNA. Direct PCR being simple and rapid has been very useful in detecting different bacteria including *E. coli* from untreated environmental samples (Olive 1989; Fode-Vaughan *et al.* 2001, 2003). However, there are fewer instances of detecting plant pathogens by direct PCR from infected plants. *Tomato yellow leaf curl Thailand virus* was detected by capturing virus particles to the surface of PCR tube without DNA extraction; however, the method required tissue grinding in phosphate buffer (Leamkhang *et al.* 2005). Tissue blotting PCR assay (also known as print capture PCR) was used for detecting plum pox virus (PPV) from infected plants without grinding the sample (Olmos *et al.* 1996).

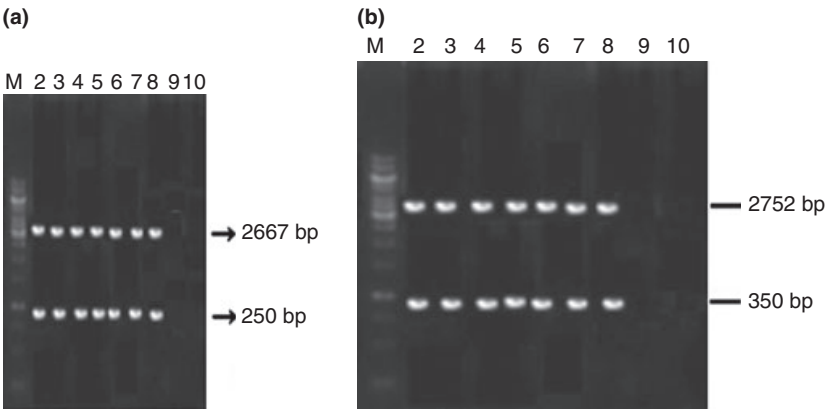

Figure 2 (a) Detection limit of CoGMV in *Corchorus capsularis* by direct PCR (dPCR). (b) Detection limit of *Mesta yellow vein mosaic virus* in *Hibiscus sabdariffa* by direct PCR. Lane M: 1-kb ladder, lanes 2–10 (different dilutions of lysate) dilutions 1 : 500, 1 : 1000, 1 : 1500, 1 : 2000, 1 : 2500, 1 : 3000, 1 : 3500, 1 : 4000 and 1 : 4500, respectively.

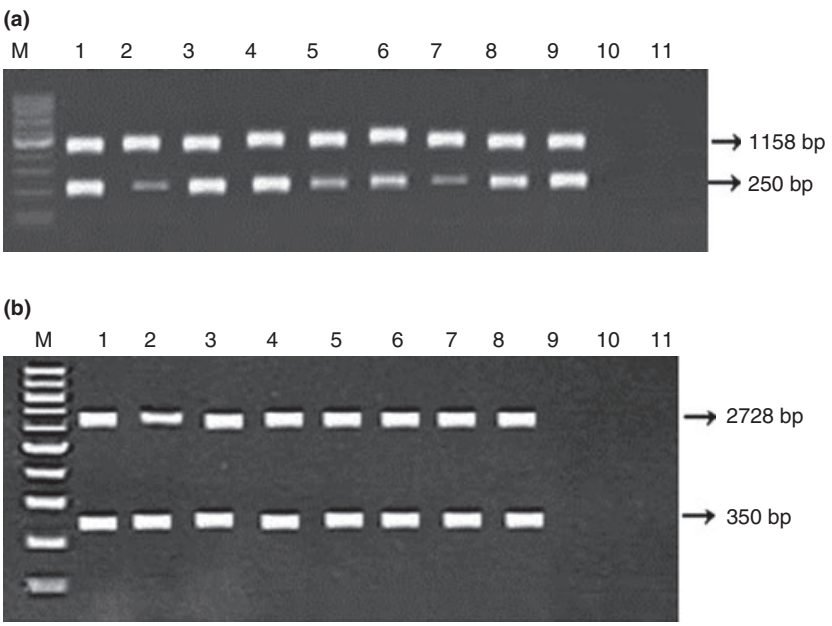

Figure 3 (a) Detection limit of CoGMV in *Corchorus olitorius* by direct PCR (dPCR). (b) Detection limit of *Mesta yellow vein mosaic virus* in *Hibiscus cannabinus* by direct PCR. Lane M: 100-bp ladder, lanes 1–11 (different dilutions of lysate) dilutions 1 : 500, 1 : 1000, 1 : 1500, 1 : 2000, 1 : 2500, 1 : 3000, 1 : 3500, 1 : 4000, 1 : 4500, 1 : 5000 and 1 : 5500, respectively.

But, the method required extraction of DNA from blotting paper or nylon membrane. Fungal plant pathogen, *Botrytis cinerea* was detected from immature grape berries by PCR amplification of *B. cinerea*-specific sequence without DNA isolation step (Gindro *et al.* 2005). Subgroup III geminivirus isolates were detected by PCR in leaf extracts without DNA extraction wherein the leaf tissue was ground in Tris-EDTA buffer (Wyatt and Brown 1996). Banana bunchy top virus, zucchini yellow mosaic potyvirus and lettuce necrotic yellows rhabdovirus were detected by a direct PCR method using Tris-HCl, KCl and EDTA-based buffer for virus release wherein only the viral nucleic acid was amplified (Thomson and Dietzgen 1995). However, in the present method in addition to Tris-HCl, NaCl and EDTA, PVP, β-marcaptoethanol and Proteinase were used to handle high mucilage content and other impurities, and unlike the previous method, it amplified both viral DNA as well as plant genomic DNA.

Most of the direct PCR methods (Klimyak *et al.*1993; Steiner *et al.* 1995; Thomson and Henry 1995) suggested earlier for amplification of plant genomic DNA involved tissue grinding. The method of direct PCR described by Bellstedt *et al.* 2010 required grinding of plant samples in a grinding buffer used in ELISA followed by dilution in another buffer for direct amplification of nuclear and plastid DNA which makes it cumbersome and time-consuming. But the present method did not require tissue grinding, instead leaf bits were directly put in a lysis buffer and then the lysate was used as PCR template. In the direct PCR method developed by Sharma *et al.* 2012 for plant genetic analysis, small amount of plant tissue was directly used as PCR template. But the process of template preparation was too lengthy which took 90–120 min including

30–60 min fixing in absolute alcohol followed by 60 min drying at room temperature. However, in the present method, template preparation took only 40 min (alcohol wash for 5 min and treatment in lysis buffer for 35 min).

By this direct PCR method, begomovirus DNA-A as well plant genomic (jute/mesta) DNA could be amplified from the lysate taken from infected leaf tissues. The cost of treating one sample in this method was less than $0·1, and all steps could be completed on a thermal cycler in a 96-well format. Thus, the present method proved to be a low cost, efficient, rapid and reliable technique for detection of begomoviruses viz. *Corchorus golden mosaic virus* and *Mesta yellow vein mosaic virus* from jute and mesta, respectively.

Materials and methods

Plant material

Naturally infected white jute (*C. capsularis*), *tossa* jute (*C. olitorius*) and mesta (*H. sabdariffa, H. cannabinus)* plants were used in the present study. The samples were collected in August 2012 from the experimental field of the Central Research Institute for Jute and Allied Fibres (CRIJAF), Kolkata, India (22·45ºN, 88·26ºE; 3·14 m above msl). Samples were collected from infected plants showing mosaic symptoms as well as from asymptomatic healthy plants. The samples were stored at −80°C.

Sample preparation for jute cell lysis

Different components and their concentration in lysis buffer systems were optimized using cell lysis method (Li *et al.* 2011) with major modifications. At first, leaf samples (both infected as well as healthy) were cut into small pieces (less than 3 mm). Tissue bits were washed with 95% ethanol for 5 min. Two to three leaf bits were placed in 1·5 ml Eppendorf tube and 200 μl of lysis buffer was added and incubated at 65°C for 35 min. The optimized buffer system composed of 20 mmol l^{-1} tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8·0), 1·5 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA) (pH 8·0), 1·4 mol l^{-1} NaCl and 200 μg/mL Proteinase K. Further, 3% PVP (w/v) and β-marcaptoethanol (1% v/v) were additionally added into the buffer. Incubated sample was centrifuged at 12 000 × ***g*** for 15 min, and the lysate was carefully taken out. Lysate was used for PCR amplification.

Sample preparation for mesta cell lysis

In case of mesta, very small amount of tissue was taken and the leaf samples were cut in to bits less than 1·5 mm. Composition of lysis buffer was 30 mmol l^{-1} tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8·0), 2·5 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA) (pH 8·0), 1·4 mol l^{-1} NaCl and 200 μg/mL Proteinase K. 5 mol l^{-1} of sodium acetate was added in buffer which helped to remove secondary metabolites. 300 μl of pre-heated (65°C) lysis buffer was added to the sample and initially incubated at 65°C for 5 min followed by incubation at 60°C for 20 min. The incubated sample was centrifuged at 11 000 × ***g*** for 15 min.

Duplex PCR conditions for amplification genomic and viral DNA

The lysate extracted by the above-said method was used as template in duplex PCR for amplification of genomic component (DNA-A) of begomovirus. To ensure that the present method could amplify both viral and jute/mesta genomic DNA, we used two sets of primers viz. begomovirus DNA-A-specific primer pair, JM FL-A F: 5′ CGTACG AATGCTGGGACCTCC 3′ JM FL-A R: 5′CCATGGGGCC TCACGTTTCATC3′ (Ghosh *et al.* 2009) and jute (MJM-561, Biswas *et al.* 2013) or *Hibiscus* (MJM 609, Satya *et al.* 2012)-specific SSR primer were used for amplification. Duplex PCR assay was performed in 30-μl reaction mixture containing 5 μl of template lysate, 3 unit of Taq polymerase (New England Biolabs, Ipswich, MA), 25 mmol l^{-1} $MgCl_2$ (1 μl), 10 mmol l^{-1} dNTPs (0·5 μl), 0·5 μ mol l^{-1} of each of the forward and reverse primer (1 μl) and 10× reaction buffer (2·5 μl). The amplification was carried out using a thermal cycler (Bio-Rad Laboratories, Hercules, CA). Before adding Taq polymerase, the other reagents were mixed and the mixture was subject to 94°C for 5 min for an initial denaturation. After such hot-start, Taq polymerase was added to the reaction mixture and amplification was carried out for 30 cycles. The PCR cycles were programmed as 30 s each for denaturation at 94°C, annealing at 56°C and chain extension at 72°C. A final extension cycle at 72°C for 7 min was carried out to ensure the completion of amplification of all the target templates. Amplified products were visualized by agarose gel electrophoresis in the presence of ethidium bromide.

Testing the sensitivity of the dPCR method

To detect the sensitivity of dPCR method, the lysate from 1 mg infected leaf bit was diluted in different concentrations viz. 1 : 500, 1 : 1000, 1 : 2500, 1 : 3000, 1 : 3500 and 1 : 4000. The differentially diluted lysates were used as templates in PCR under same PCR conditions. For PCR amplification along with begomovirus DNA-A-specific primer, jute/mesta-specific SSR marker was used in duplex PCR. PCR protocol and amplification programme remained same.

Sequencing

The 2667-bp amplicon from five CoGMV-infected white jute (*C. capsularis)* samples, 1158-bp amplicon from *tossa* jute (*C. olitorius),* 2752-bp amplicon from HS mesta (*H. sabdariffa)* samples and 2728-bp amplicom of HC mesta (*H. cannabinus)* samples were eluted through PCR clean-up system (Promega, Madison, WI) and cloned. Then, the clones were sequenced.

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Conflict of interest

There is no conflict of interest to declare.

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