

# Extraction of *Trypanosoma cruzi* DNA from food: a contribution to the elucidation of acute Chagas disease outbreaks

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## ABSTRACT

**Introduction:** Before 2004, the occurrence of acute Chagas disease (ACD) by oral transmission associated with food was scarcely known or investigated. Originally sporadic and circumstantial, ACD occurrences have now become frequent in the Amazon region, with recently related outbreaks spreading to several Brazilian states. These cases are associated with the consumption of açai juice by waste reservoir animals or insect vectors infected with *Trypanosoma cruzi* in endemic areas. Although guidelines for processing the fruit to minimize contamination through microorganisms and parasites exist, açai-based products must be assessed for quality, for which the demand for appropriate methodologies must be met. **Methods:** Dilutions ranging from 5 to 1,000 *T. cruzi* CL Brener cells were mixed with 2mL of açai juice. Four extraction of *T. cruzi* DNA methods were used on the fruit, and the cetyltrimethyl ammonium bromide (CTAB) method was selected according to JRC, 2005. **Results:** DNA extraction by the CTAB method yielded satisfactory results with regard to purity and concentration for use in PCR. Overall, the methods employed proved that not only extraction efficiency but also high sensitivity in amplification was important. **Conclusions:** The method for *T. cruzi* detection in food is a powerful tool in the epidemiological investigation of outbreaks as it turns epidemiological evidence into supporting data that serve to confirm *T. cruzi* infection in the foods. It also facilitates food quality control and assessment of good manufacturing practices involving açai-based products.

**Keywords:** Chagas disease. *Trypanosoma cruzi*. *Euterpe* (açai). PCR detection. Food analysis.

## INTRODUCTION

In Brazil, both inside and outside the Amazon region, several cases of acute Chagas disease (ACD) have been recorded as an outbreak characterized by a group of individuals gathered in one place who, by ingesting the same type of food, became sick almost simultaneously with fever and general manifestations of a systemic infection<sup>(1)</sup>.

Until the year 2004, the occurrence of ACD by oral transmission, as associated with food consumption, constituted a scarcely known or investigated event. For the same reason, what was originally considered a sporadic and circumstantial occurrence has now become frequent in the Amazon region, which is associated with the occurrence of recent outbreaks in several Brazilian states<sup>(2)</sup>. Recent cases of ACD in Brazil are related to the consumption of açai juice where the contamination of the fruit itself or the pulp occurs through waste reservoir

animals or insect vectors infected with *Trypanosoma cruzi* in endemic areas<sup>(3) (4) (5)</sup>.

Açai (*Euterpe oleracea* Mart.) is a typical and popular berry fruit of the Amazon region in Northern Brazil, which in recent years has gained importance due to the health benefits associated with its phytochemical composition and antioxidant capacity.

Brazil is the world's largest producer, consumer, and exporter of açai. This fruit is marketed and consumed by the local people in the producing areas of Pará, Maranhão, Amapá, Rondônia, and Acre, which together comprise a distinct region considered endemic for the disease<sup>(6) (7)</sup>. With the increasing growth in commerce, not only has the consumption of the product spread across larger Brazilian cities, but it is now also being exported to America, Japan, China, and some European countries<sup>(7)</sup>.

The Ministry of Agriculture and Supply, through Normative Instruction number 1 of January 07, 2000, issued the technical regulation for setting the standards of identity and quality for fruit pulp, which includes açai pulp<sup>(8)</sup>. Pertinently, the pulp should be obtained from fruit that is fresh, sound, mature, and free of soil, dirt, parasites, and microorganisms that can render the product unfit for consumption<sup>(8)</sup>.

Although the guidelines for the processing of the fruit have been established with a view to minimize contamination

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through microorganisms and parasites, it is necessary to assess the quality of açai-based products, for which appropriate methodologies must be adopted<sup>(9)</sup>.

Several recommendations and incentives on research methodologies for the detection of *T. cruzi* in food exist<sup>(2) (10)</sup>; however, fully developed methods aimed at the detection of parasites in food are still lacking not only for furnishing a response to epidemiological investigations in cases of outbreaks, but also for controlling the quality of commercialized products.

Molecular methods for the quality control of foods are a reality, and as such, deserve special attention with regard to their availability for the regulation of products subject to sanitary surveillance.

Polymerase chain reaction (PCR) is widely used for the detection of food pathogens. Due to its high sensitivity and specificity, PCR-based methods have the advantage of providing rapid and precise identification of the pathogen and can therefore serve as a powerful tool in the hygienic and sanitary assessment of açai-based foods.

Polymerase chain reaction requires a high-quality amplifiable deoxyribonucleic acid (DNA). Therefore, the choice of the method for DNA extraction is of great importance and constitutes a pre-requisite for molecular analysis. DNA amplification by PCR is influenced by the structural integrity of the DNA and the presence of matrix co-purification inhibitors or extraction reagents, which may reduce PCR efficiency.

In foods, the problems most often linked to DNA extraction include contamination by phenols and polysaccharides, among others. Açai is composed of lipids, carbohydrates, fiber, vitamin E, proteins, minerals (Mn, Fe, Zn, Cu, and Cu), and antioxidant compounds such as anthocyanins, and phenolic compounds<sup>(11)</sup>, which pose a big challenge for DNA extraction from the parasites in such a complex food matrix.

In this context, the aim of this study was to compare the quantity and quality of *T. cruzi* DNA isolated from açai artificially contaminated with *T. cruzi* cells, whereby four different DNA extraction methods were applied, and spectrophotometric absorbance readings were used to confirm the quality of the extracted DNA by PCR amplification of the gp85 gene superfamily/sialidase.

## METHODS

### Staining

The stains used in the present work were for the identification of 1) *Leishmania (Leishmania (V.) braziliensis MHOM/BR/1975/M2903 – IOC-L 0566, Leishmania (L.) amazonensis IFLA/BR/1967/PH8 – IOC-L 575), Leishmania* Collection (CLIOC/FIOCRUZ), Instituto Oswaldo Cruz; 2) *Trypanosoma cruzi CL Brener, Instituto Nacional de Infectologia Evandro Chagas, Fundação Oswaldo Cruz (INI/FIOCRUZ)*; and 3) *Trypanosoma rangeli COLPROT 273, Protozoa* Collection (COLPROT/FIOCRUZ), Fundação Oswaldo Cruz.

### Artificial contamination of açai

Labeled açai medium, one kilogram of frozen fruit pulp was purchased from a supermarket in Rio de Janeiro, Brazil and thawed at 4°C. The pulp was then homogenized in a *Seward Stomacher® 400 Laboratory Blender* (Seward, UK) and distributed in 50-mL polypropylene vials. For each extraction method, five 30-mL glass bottle vials received 2mL of the homogenate of açai pulp and *T. cruzi* CL Brener with a cell suspension rate of 1,000 (Dilution 1), 100 (Dilution 2), 50 (Dilution 3), 10 (Dilution 4), and 5 (Dilution 5) parasite cells. This procedure was repeated three times, resulting in 15 glass bottles used per method. The homogenate of açai pulp and *T. cruzi* was subjected to a freeze-drying for about 20 hours for reducing the volume of the mixture and for obtaining a lyophilisate to allow efficient extraction.

### DNA extraction

Four different methods for genomic DNA extraction were tested as displayed in **Table 1**. Two of these were performed using cetyltrimethyl ammonium bromide (CTAB), as described by Cardarelli et al. 2005<sup>(12)</sup> and the Joint Research Centre 2005<sup>(13)</sup>, and the other two were performed using commercially available agents, namely DNAzol® (Invitrogen) and Nucleo Spin® (MACHEREY-NAGEL), strictly in accordance with the manufacturer's recommendations. The extractions were performed in triplicate for each method as indicated in **Table 1**.

### Assessment of concentration and purity of the extracted DNA

Deoxyribonucleic acid concentration was assessed by spectrophotometric measurement of the optical density (OD) at 260 nm using the GeneQuant™ equipment pro ribonucleic acid/deoxyribonucleic acid (RNA/DNA) calculator (Amersham Biosciences, UK). Ratios of the absorbance at A260/280 and A260/230 were determined to verify the purity of the preparations, which yielded samples that were found to be of acceptable quality and integrity.

### PCR for the detection of *Trypanosoma cruzi*

Standardization of the PCR for evaluating its specificity was performed using strains of *Leishmania chagasi*, *L. braziliensis*, *Trypanosoma rangeli*, and *T. cruzi*, where a pair of *T. cruzi*-specific primers were employed to amplify a telomeric region of the gp85/sialidase superfamily (Tc189Fw2 - 5'CCAACGCTCCGGGAAAAC-3' and Tc189RV3 - 5'GCGTCTTCTCAGTATGGACTT-3'), as described by Chiurillo et al.<sup>(14)</sup>.

After standardization, the same protocol was used for the detection of *T. cruzi* in different concentrations of cells mixed with açai. The negative and positive controls used for the PCR were *T. rangeli* and *T. cruzi*, respectively. The fragments amplified by PCR were separated by agarose gel electrophoresis (2%w/v) containing 0.3µg/mL ethidium bromide immersed in 1X tris-borate-ethylenediaminetetraacetic acid (TBE) buffer (90mM Tris-borate, 2mM EDTA pH 8, 4), visualized on the "UVP™ TM-20 Dual-Intensity transilluminator" (Cole-Parmer, US), and recorded in the image analyzer "Image Quant 300 Imager" (Amersham Biosciences, UK).

TABLE 1 - Summary of the DNA extraction methods used in this study.

Method	Description	Extraction/lysis buffer(volume)	Lysis incubation conditions	Final extract volume	Procedure references
CTAB	Selective precipitation of DNA using in-house reagents	2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl pH 8.0 (3.5mL)	60 min at 65°C	300µL DNase-free water	12
CTAB	Selective precipitation of DNA using in-house reagents	2% CTAB, 1.4M NaCl, proteinase K, RNase, 20mM EDTA 100mM Tris-HCl pH 8.0 (3.5mL)	60 min at 65°C	300µL DNase-free water	13
DNazol	DNazol® Reagent (Invitrogen)	Novel guanidine-detergent lysing solution(3.5mL)	Not necessary	300µL DNase-free water	Manufacturer's instructions
NucleoSpin	NucleoSpin® Food Kit (Machery-Nagel) Two washing steps	Buffer CF* with 0.4mg/mL proteinase K (1,100µL)	30 min at 65°C	Elution buffer CE*	Kit instructions

DNA: deoxyribonucleic acid; CTAB: cetyltrimethyl ammonium bromide, EDTA: ethylenediamine tetraacetic acid; CE: Elution Buffer CE ; CF: Lysis Buffer CF. \* Included with the kit.

### Validation of the selected DNA extraction method

Eighteen glass bottles containing about 2mL of açai were contaminated with 1,000 *T. cruzi* CL Brener cells and subjected to lyophilization. DNA was extracted according to the Join Research Centre (JRC) method, 2005, in 6 replicates, repeated for 3 consecutive days. After extraction, the concentration and purity of the extracted DNA were evaluated using the methods described above. The size of the extracted DNA was assessed by electrophoresis on 1% agarose gel. About 10µL of the DNA solution was deposited on an agarose gel in TBE 1X at 80 V for 1 hour.

## RESULTS

The DNA extracted using the NucleoSpin® method showed no reading in the spectrophotometer. The average concentration of the DNA extracted from açai infected with *T. cruzi* cells using the other three methods is shown in **Table 2**.

All three methods yielded sufficient quantities of DNA to allow PCR detection. Although the DNA extracted using the DNazol method produced a four times greater value (about 320ng/µL) than the other two methods using CTAB (about 75ng/µL), the eluate with the extracted DNA had a brownish color due to the inefficiency of the method in eliminating the pigments present in açai. This may have led to overestimation of the DNA concentration and/or may have inhibited the PCR.

The purity of the DNA extracted using the three methods after the removal of outliers using the Grubbs test is shown in **Table 2**.

The A260/A280 ratio is used to test for the presence of proteins and thus helps estimate the purity of nucleic acids. For pure DNA, the value must be between 1.8 and 2.0. The A260/A230 reflects contamination by substances such as carbohydrates, peptides, phenols, and aromatic compounds. In the case of pure samples, the ratio should be above 2<sup>(15)</sup>.

With respect to the A260/A280 ratios obtained, the method using CTAB, RNase, proteinase, and 70% ethanol washes was able to extract DNA with higher purity<sup>(13)</sup>. The DNA extracted using DNazol proved to exhibit a high protein contamination ratio, with values appreciably below 1.8.

With reference to the A260/A230 ratio, the method that came closest to the expected value was the CTAB method<sup>(13)</sup>. The use of DNazol resulted in values far below expectation, wherein the DNA extracted showed a high degree of chemical contamination. The specificity of PCR for *T. cruzi* DNA was established with all four strains belonging to the Trypanosomatidae family (**Figure 1**).

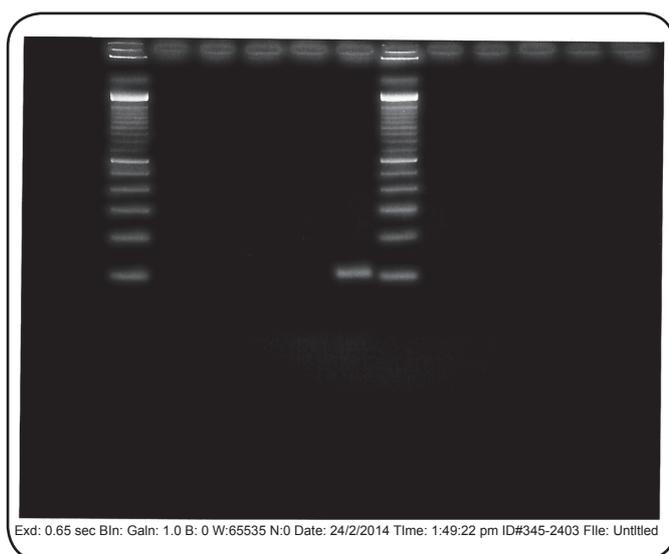
Five dilutions of the DNA extracted using the CTAB method<sup>(13)</sup> allowed PCR amplification; the results can be observed both in **Table 2** and in **Figure 2**.

The result of the validation of the 18 replicas (first day: 69.2, 66.0, 69.4, 72.4, 84.6, 84.4; second day: 78.2, 52.4, 61.0, 75.2, 54.0, 63.6; and third day: 73.4, 66.6, 76.0, 63.0, 87.2, 74.6) bears out an average of 70.6ng/µL, a standard deviation of 9.8ng/µL, and a coefficient of variation of 13.9%. No PCR inhibition was observed in the agarose gel, and amplicons of 100bp were generated in all the 18 replicates (**Figure 3**).

**TABLE 2 - Evaluation of the average standard deviation with concentrations and coefficient of variation (%), along with purity using absorbance readings at 230, 260, and 280nm of DNA extracted from açai contaminated with *Trypanosoma cruzi*.**

Extraction method	2mL açai with dilution	DNA conc. ng/µL (CV)	260:280nm ratio	260:230nm ratio	PCR inhibition
<b>CTAB<sup>(12)</sup></b>	1,000 cells	80 ± 7 (8.8%)	2.3 ± 0.2	2.7 ± 0.7	No
	100 cells	85 ± 1 (1.2%)	2.4 ± 0.1	3.2 ± 0.5	Yes
	50 cells	79 ± 10 (12.7%)	2.5 ± 0.4	3.1 ± 0.8	Yes
	10 cells	82 ± 11 (13.4%)	2.4 ± 0.1	3.6 ± 0.9	No
	5 cells	82 ± 17 (20.8%)	2.5 ± 0.5	2.8 ± 0.3	Yes
<b>CTAB<sup>(13)</sup></b>	1,000 cells	73 ± 18 (24.7%)	2.0 ± 0.2	2.6 ± 1.4	No
	100 cells	76 ± 14 (18.4%)	1.9 ± 0.1	3.0 ± 0.3	No
	50 cells	80 ± 20 (25.0%)	2.1 ± 0.1	2.4 ± 0.1	No
	10 cells	66 ± 1 (1.5%)	2.1 ± 0.2	2.8 ± 0.5	No
	5 cells	70 ± 6 (8.6%)	1.9 ± 0.0	2.6 ± 1.1	No
<b>DNAzol</b>	1,000 cells	302 ± 36 (12.0%)	1.5 ± 0.0	0.42 ± 0.0	Yes
	100 cells	331 ± 36 (11.0%)	1.5 ± 0.1	0.40 ± 0.0	Yes
	50 cells	338 ± 119 (35.2%)	1.3 ± 0.0	0.21 ± 0.0	Yes
	10 cells	314 ± 75 (24.0%)	1.3 ± 0.0	0.21 ± 0.0	Yes
	5 cells	349 ± 63 (18.0%)	1.4 ± 0.0	0.21 ± 0.0	Yes

DNA: deoxyribonucleic acid; CV: coefficient of variation; PCR: polymerase chain reaction; CTAB: cetyltrimethyl ammonium bromide.



**FIGURE 1 - Specificity of PCR for *Trypanosoma cruzi* established with four strains belonging to the Trypanosomatidae family. Lanes 1 and 7: contain a 100-bp size ladder (Gibco-BRL); Lane 2: H<sub>2</sub>O; Lane 3: *Leishmania amazonensis*; Lane 4: *Leishmania braziliensis*; Lane 5: *Trypanosoma rangeli*; Lane 6: *Trypanosoma cruzi* cL Brener. PCR: polymerase chain reaction.**

## DISCUSSION

To date, no studies evaluating a methodology for *T. cruzi* DNA extraction directly from food have been conducted. This

study was aimed at identifying an efficient method for the extraction of *T. cruzi* DNA from *T. cruzi*-contaminated açai. Although the Nucleo Spin method has been developed for various types of food such as chocolate-based products and fruit concentrates, among others, it was shown to not be adequate for the detection *T. cruzi* contamination in this study. DNA extraction by the CTAB method yielded satisfactory results with regard to purity and concentration for use in PCR.

The method employing the CTAB detergent for cell lysis is widely used for genetically modified organisms (GMO) analysis of raw or processed food; it is also commonly used for plants, especially in combination with other substances such as β-mercaptoethanol<sup>(16)(17)(18)</sup>. The major difference between the two methods using the CTAB detergent for cell lysis tested in this study was the use of two different enzymes, RNases and Proteinase K, and the 70% ethanol wash<sup>(13)</sup>, which confirms the high sensitivity of the method with amplification of up to 2.5 cells/mL of açai.

Contamination of food by *T. cruzi* may occur especially when triatomines deposit their droppings on the surface of infected food or food ingredients, or when these triatomines are crushed with foods, mainly during fruit juice processing, such as in the processing of açai juice.

Equally important in the epidemiology of foodborne acute Chagas disease (ACD) is the contamination of equipment and utensils, contamination during transport, and contamination in the food processing environment, where it typically occurs by either urine or anal secretions of marsupials as well as by feces of infected bugs caused due to human invasion of forests<sup>(5)(10)(19)</sup>.

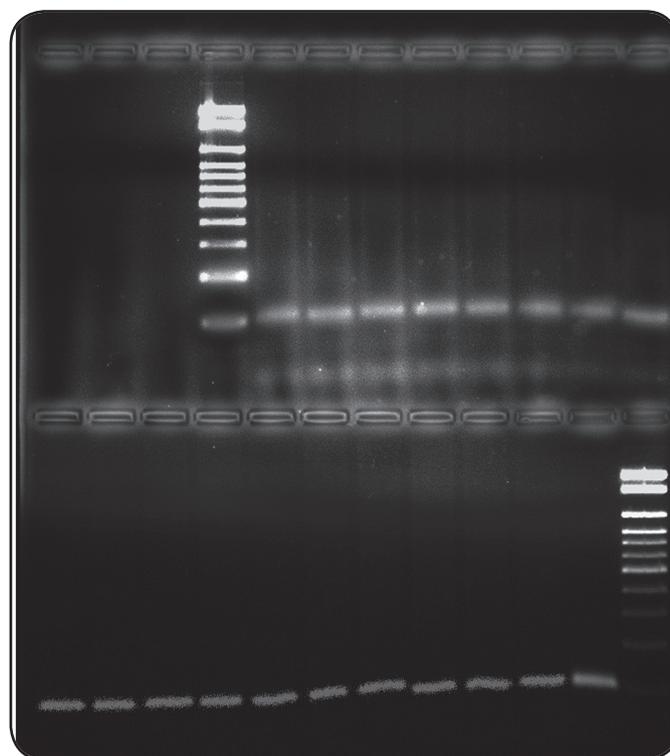


**FIGURE 2 - PCR amplification of the DNA extracted from the homogenate of açai with *Trypanosoma cruzi* cL Brener with a cell suspension rate of 1,000 to 5 parasite cells using 3 extraction methods. A) Lanes 1 to 5: DNAzol extraction method (no amplification); Lane 6: 100-bp size ladder (Gibco-BRL); Lanes 7 to 11: CTAB extraction method<sup>(12)</sup>; Lane 12: *Trypanosoma cruzi* cL Brener (positive control). B) Lanes 1 and 9: 100-bp size ladder (Gibco-BRL); Lanes 3 to 7: CTAB extraction method<sup>(13)</sup>. PCR: polymerase chain reaction; DNA: deoxyribonucleic acid; CTAB: cetyltrimethyl ammonium bromide.**

So far, it has not been possible to analyze the type of food involved in the outbreaks of orally transmitted Chagas disease to evidence the presence of the parasite. However, as quoted above, PANAFTOSA<sup>(19)</sup> proposes hypotheses that point to an explanation for the several outbreaks that have occurred in recent years in Northern Brazil, which were associated with the intake of possibly *T. cruzi*-contaminated açai juice.

The recorded cases of ACD in the outbreaks are related to the consumption of handmade açai juice.

In Latin America, around 100 million individuals suffer from Chagas disease acquired by various types of transmission. Additionally, the estimated number of people infected with *T. cruzi* in non-endemic countries for the disease is >300,000 in the



**FIGURE 3 - Agarose gel electrophoresis of all the 18 amplified products obtained from the validation assay with primers Tc189Fw2 and Tc189Rv3. Top - Lane 1: H2O; Lane 2: *Trypanosoma rangeli* (negative control); Lane 3: 100-bp size ladder (Gibco-BRL); Lanes 4 to 10: homogenate of açai with *Trypanosoma cruzi* CL Brener with a 100-cell suspension. Bottom - Lanes 1 to 10: homogenate of açai with *Trypanosoma cruzi* CL Brener with a 100-cell suspension; Lane 11: *T. cruzi* cL Brener (positive control); Lane 12: 100-bp size ladder.**

United States; >5,500 in Canada; >80,000 in Europe and Western Pacific Regions; >3,000 in Japan; and >1,500 in Australia<sup>(20)</sup>. Migratory movements and the subsequent transmission by blood transfusion and organ transplantation have been suggested as the cause of the spread of *T. cruzi* around the World.

In Brazil, 2 million individuals are infected with *T. cruzi*<sup>(21)</sup>. Considering the high consumption of açai by tourists visiting the country, plus Brazilians themselves traveling to the above-mentioned non-endemic areas of the world, the oral transmission is assumed to be contributing to the increase in overseas cases. This assumption is rooted in the fact that, having acquired the asymptomatic disease, or even the manifestation of symptoms similar to those of other types of illnesses, visitors to Brazil are likely to return to their countries where blood banks may not be equipped with the proper framework required to provide an effective diagnosis of the disease.

Barbosa-Labello et al, in 2010<sup>(22)</sup>, used animal models to show that, in addition to surviving in açai pulp by different incubation periods and under different low temperatures treatments, *T. cruzi* was also able to preserve its virulence in mice. Regarded to be of great epidemiological importance, this finding discards the use of cooling processes and conventional freezing during long periods, which have been previously used as control methods

for orally transmitted ACD. This finding also reinforces the need to use bleaching as for the conservation of açai.

In consideration of the above findings, safety measures must be taken at all stages of the commercial or craft supply chain during the processing of açai. The development of large-scale detection and isolation methods for *T. cruzi* in food products is essential so that epidemiological evidence may serve as supporting data to certify that the food in question is actually contaminated with the parasite. Concurrently, despite the fact that DNA identification in foods itself does not constitute a methodology to assess the viability of the parasite, it can become an assessment tool to ensure proper application of good manufacturing practices.

In the case of açai, these tests will allow the identification of possible contamination during the production chain. This procedure is of great importance since oral transmission aggravates an epidemic of the disease, and disposes off refrigeration and food freezing processes as control methods for the oral transmission of ACD, thus inducing a new reflection, strongly related to underdevelopment<sup>(3)</sup>.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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