

Optimized DNA extraction from sea and lagoon water samples to detect cyanobacteria, the genus *Microcystis* and the *mcyA* gene by endpointPCR

Extracción optimizada de ADN en muestras de agua de mar y dulce para la detección de cianobacterias, el género *Microcystis* y el gen *mcyA* mediante PCR punto final

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ABSTRACT

Monitoring cyanobacteria in aquatic ecosystems is essential for assessing the risk of blooms and cyanotoxin production, given their negative impacts on public health and the environment. The objective of this study was to optimize a DNA extraction protocol for molecular detection of cyanobacteria and cyanotoxin genes in marine and freshwater environmental samples using PCR. Samples were collected from two bodies of water in Sonora (Tóbari Bay and Nainari Lagoon), Mexico, and a modified extraction protocol was used, followed by purification with a commercial kit. This led to the successful extraction of environmental DNA; subsequently, molecular detection of cyanobacteria of the genus *Microcystis* and the *mcyA* gene was carried out using PCR using specific oligonucleotides. The results showed the presence of cyanobacteria in both samples; however, neither *Microcystis* nor the *mcyA* gene was detected in marine water. These findings highlight the importance of optimizing nucleic acid extraction protocols to obtain quality DNA from complex environmental matrices, which is critical for strengthening molecular surveillance strategies for cyanobacteria and their toxins in aquatic ecosystems.

KEY WORDS: DNA extraction, Molecular detection, Environmental sample, Cyanobacteria, *Microcystis*, *mcyA* gene.

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RESUMEN

El monitoreo de cianobacterias en ecosistemas acuáticos es esencial para evaluar el riesgo de floraciones y la producción de cianotoxinas, debido a sus impactos negativos sobre la salud pública y el ambiente. El objetivo del estudio fue optimizar un protocolo de extracción de ADN para detección molecular de cianobacterias y genes de cianotoxinas en muestras ambientales marinas y de agua dulce, mediante PCR. Se recolectaron muestras en dos cuerpos de agua de Sonora (La Bahía del Tóbari y la Laguna del Nainari), México, y se utilizó un protocolo modificado de extracción, seguido de purificación con un kit comercial. Posterior a la extracción exitosa de ADN ambiental, con el uso de oligonucleótidos específicos, se realizó la detección molecular de cianobacterias, del género *Microcystis* y del gen *mcyA* mediante PCR punto final. Los resultados mostraron la presencia de cianobacterias en ambas muestras; sin embargo, no se detectó la presencia de *Microcystis* en agua marina, ni al gen *mcyA* en ambas muestras. Estos hallazgos destacan la importancia de optimizar los protocolos de extracción de ácidos nucleicos para obtener ADN de calidad a partir de matrices ambientales complejas, lo cual es fundamental para fortalecer la vigilancia molecular de cianobacterias y sus toxinas en ecosistemas acuáticos.

PALABRAS CLAVE : Extracción de ADN, Detección molecular, Muestra ambiental, Cianobacterias, *Microcystis*, Gen *mcyA*.

Introduction

The ocean is the habitat of a wide variety of microorganisms, which constitute a natural capital that harbors an immense genetic and physiological diversity, still largely unexplored. Before the 1990s, knowledge about bacterial diversity was limited to phenotypic tests and numerical taxonomy of bacteria cultured in growth media, achieving the isolation of 0.001 % to 0.1 % of marine bacteria (Lee *et al.*, 2009). This led to most of the microbial community remaining unstudied; however, with the emergence of culture-independent molecular biology techniques, such as 16S rRNA gene-based identification and metagenomics, the understanding of uncultivable bacteria has been revolutionized (Morales-Sandoval *et al.*, 2021). These tools have allowed advances in the knowledge of microbial physiology and diversity, revealing the complexity and adaptability of microorganisms in the marine environment (Laakmann *et al.*, 2020).

Cyanobacteria play a significant role in the oxygen, carbon, and nitrogen cycles, and are characterized by possessing a wide diversity of adaptations (photosynthesis, akinetes, heterocysts, or aerotopes), which allow them to adapt and inhabit freshwater, marine, terrestrial, as well as extreme habitats (Kaštovský, 2024). However, when natural conditions are considered together with anthropogenic activities, a phenomenon called cyanobacterial bloom may arise, which is characterized by an increase in cyanobacterial biomass over periods ranging from a few days to 2 weeks (Moreira *et al.*, 2022). Among the most common genera are: i) *Microcystis*, characterized by its colonial and spherical cells that inhabit both running water, such as rivers and streams, as well as lentic environments, such as ponds and lakes; ii) *Anabaena*, with filamentous cell trichomes and the presence of heterocysts that act as central sites for nitrogen fixation; iii) *Nostoc*, described as a genus with isopolar, uniseriate, and unbranched filaments, possessing differentiated chains of akinetes and terminal and intercalary heterocysts, embedded in mucilaginous material to form colonies; iv) *Cylindrospermopsis*, of filamentous and motile morphology, usually found in freshwater systems such as lakes and rivers, as well as artificial reservoirs such as ponds and canals; and v) *Planktothrix*, composed of filamentous and motile cells that are found in eutrophic lakes (Singh *et al.*, 2020; Massey *et al.*, 2022; Thawabteh *et al.*, 2023).

Cyanobacterial blooms have raised environmental concern due to their negative effects, such as the emission of unpleasant odors, alterations in water color, and, more alarmingly, the release of toxic compounds known as cyanotoxins, which represent a risk to human and animal health (Moreira *et al.*, 2014). These toxins can be classified according to their chemical structure, as cyclic peptides, alkaloids, and polyketides; or based on the main organs or functions they affect in mammals, such as hepatotoxins, neurotoxins, dermatotoxins, and cytotoxins, with microcystins, nodularins, and anatoxins, among others, being some of those present in cyanobacteria (Nugumanova *et al.*, 2023). Among the acute health effects caused by common cyanobacterial toxins, the United States Environmental Protection Agency (EPA) reports the appearance of abdominal pain, headache, nausea, vomiting, diarrhea, and respiratory difficulty, among other symptoms (EPA, 2025). However, when toxins accumulate, their ingestion or exposure (usually through the consumption of shellfish) leads to poisoning, and the resulting syndrome will depend on the toxin causing it; for example, paralytic shellfish poisoning syndrome is caused by saxitoxin (EPA, 2025). Therefore, it is essential to understand the danger implied by the presence of cyanobacterial blooms and to assess their risk by monitoring aquatic ecosystems through methods that allow a rapid response, both to identify the species responsible for the blooms and to detect the cyanotoxins that are produced or released.

In this sense, molecular methods are fundamental for the detection and monitoring of cyanobacteria in marine ecosystems (Moreira *et al.*, 2022; Saleem *et al.*, 2023), among them the polymerase chain reaction (PCR). Endpoint PCR emerges as a strategy based on the amplification of specific sequences of the genomic DNA of the organism under study, which allows qualitative analyses, such as determining the presence or absence of cyanobacteria in the analyzed sample. However, working with these molecular methods, and especially with environmental samples, poses significant challenges. The possible cyanotoxicity of samples detected by these methods is one of the limitations for determining the danger of their presence, since the detection of genes related to the production of such toxins does not indicate their actual production, as in the case

of indicated presence with genetic inactivation (Moreira *et al.*, 2022). In addition, the extraction of nucleic acids from environmental samples for PCR represents a challenge, for example, salinity, organic matter, and the coexistence of diverse microbial communities can affect both the efficiency of extraction and the quality of the recovered genetic material, as well as the performance of PCR, since these matrices often introduce inhibitors that compromise cell lysis, as well as the sensitivity and specificity of PCR (Klein *et al.*, 1997; Wilson, 1997; van der Loos & Nijland, 2021). Therefore, standardized molecular biology protocols must be established for obtaining efficient samples that allow the detection of cyanobacteria and genes related to cyanotoxin production. In this article, a strategy is presented to address the problems of nucleic acid extraction from environmental samples for PCR, with the purpose of contributing to the development of robust strategies for the monitoring of cyanobacteria in freshwater and saline water bodies.

Material and methods

Collection of environmental samples

The seawater sample was collected on August 9, 2024, in Bahía del Tóbari, Sonora, Mexico (coordinates: 27.097598, -109.997521), while the freshwater sample was collected on November 11, 2024, in Laguna del Nainari, Ciudad Obregón, Sonora, Mexico (coordinates: 27.4989886, -109.9706528). First, the photic zone was measured using a Secchi disk, and then the sample was collected with a 2-inch rigid PVC tube with a check valve. One liter of water was deposited into a sterile, wide-mouth, transparent 1 L polypropylene bottle.

Physicochemical parameters were measured at the middle of the photic zone, including dissolved oxygen and temperature, using a portable YSI Pro20 dissolved oxygen meter. Salinity was determined with an Aquafauna BIO-MARINE refractometer (model ABMTC), and pH was measured with a HANNA pH meter (model HI98108, pHep+) (Furtado *et al.*, 2009; García, 2009; Samanez *et al.*, 2014) (Furtado *et al.*, 2009; García, 2009; Samanez *et al.*, 2014). Both samples were preserved on ice during transport to the Laboratory of Microbial Resource Biotechnology (LBRM) at the Instituto Tecnológico de Sonora, where they were processed for DNA extraction and microscopic observation.

DNA extraction from environmental samples

DNA extraction from environmental samples was performed following the protocol described by Raeder & Broda (1985) with modifications. To a 2 mL microtube containing biomass obtained from 50 mL of centrifuged culture, 500 µL of extraction buffer (Tris HCl pH 8.5, 200 mM; NaCl, 200 mM; EDTA, 25 mM; SDS, 0.5 %) was added, resuspended, transferred to a 2 mL Eppendorf microtube, and mixed vigorously using a vortex mixer at maximum speed for 30 s. Then, 350 µL of phenol was added and homogenized vigorously with the vortex at maximum speed for 30 s. Subsequently, 150 µL of chloroform was added and mixed vigorously with the vortex for 30 s. The resulting suspension was centrifuged at 13,000 rpm for 30 min. The aqueous phase (upper layer) was transferred to a new 2 mL microtube, 4 µL of RNase A (20 mg/mL; RNase A RB0743, BIO

BASICS INC.) was added according to the manufacturer's specifications, and the mixture was incubated at 60 °C for 10 minutes.

Then, 1 volume of chloroform was added and mixed vigorously with the vortex for 30 s. The suspension was centrifuged at 13,000 rpm (15,890 rcf) for 10 min, and the aqueous phase was transferred to a new 2 mL microtube. The DNA purification process was then completed by adding 210 µL of absolute ethanol, followed by vigorous vortexing for 30 s. For each sample, a spin column was placed in a collection tube, both provided by the ISOLATE II Genomic DNA Kit (Bioline). The entire sample was applied to the column and centrifuged at 11,000 rcf for 1 min. The filtrate was discarded, and the silica membrane of the spin column was washed by adding 500 µL of Wash Buffer GW1, centrifuging at 11,000 rcf for 1 min, discarding the liquid, and reusing the collection tube. Subsequently, 600 µL of Wash Buffer GW2 was added to the spin column, centrifuged at 11,000 rcf for 1 min, and the filtrate was discarded. To dry the silica membrane, residual ethanol was removed by centrifugation at 11,000 rcf for 1 min.

Once dry, the spin column was placed in a 1.5 mL microtube, and 50 µL of Elution Buffer G preheated to 70 °C was added directly onto the silica membrane, incubated at room temperature for 1 min, and centrifuged at 11,000 rcf. The DNA eluted with Elution Buffer G in the 1.5 mL microtube was collected, the spin column discarded, and the DNA stored at -80 °C for later use. The integrity of the extracted DNA was assessed by electrophoresis on a 1 % agarose gel (MCD LAB) at 100 V for 1 h. DNA quality and concentration were determined using a microvolume spectrophotometer (Thermo Scientific NanoDrop 2000C).

Detection of cyanobacteria, *Microcystis*, and the *mcyA* gene by endpoint PCR

Endpoint PCR was performed using the PCR Master Mix 9PIM750 kit (Promega), with the oligonucleotides listed in Table 1, and 5 ng of DNA in a final volume of 25 µL. Amplification was carried out under the following conditions: initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for the 16S rRNA gene of cyanobacteria and *mcyA*, and at 54 °C for the 16S rRNA gene of *Microcystis* for 40 seconds, and elongation at 72 °C for 30 seconds. A final elongation step was performed at 72 °C for 5 minutes. The presence and integrity of the PCR products were analyzed by electrophoresis on a 1 % agarose gel.

Table 1. Primers used for amplification of the 16S rRNA gene from cyanobacteria, the 16S rRNA gene from *Microcystis*, and the *mcyA* gene.

Test	Primer	Sequence	Source
Cyanobacteria 16S rRNA	CYAN738F	ATACCCWGTAGTCCTAGC	(Valério <i>et al.</i> , 2009)
	CYAN1281R	GCAATTACTAGCGATTCTCC	
<i>Microcystis</i> 16S rRNA	MICR 184F	GCCGCRAGGTGAAAMCTAA	(Martins & Vasconcelos, 2011)
	MICR 431R	AATCCAAARACCTTCCTCCC	
<i>mcyA</i> gene	<i>mcyA</i> -Cd-1F	AAAATTAAAAGCCGTATCAAA	(Sabart <i>et al.</i> , 2015)
	<i>mcyA</i> -Cd-1R	AAAAGTGTTTTATTAGCGGCTCAT	

Source: Own elaboration based on Chávez-Luzanía *et al.* (2024).

Amplicon sequencing

The obtained amplicons were purified using the commercial EZ-10 Spin Column DNA Gel Extraction Minipreps Kit (BIO BASIC) and sequenced by Sanger technology at the Genomic Services Laboratory of Cinvestav, Irapuato, Guanajuato. The sequences obtained were processed using FinchTV v1.4.0, aligned in CLC Sequence Viewer 8, and compared in EZBioCloud (<https://www.ezbiocloud.net/>).

Results and discussion

DNA extraction

The fluorescence observed in the agarose gel revealed good integrity of the DNA extracted from the environmental samples (Figure 1). The extracted DNA was evaluated using a spectrophotometer, obtaining for the seawater sample a concentration of 31.3 ng of DNA/ μ L, and the absorbance ratio values 260/280 and 260/230 were 1.86 and 1.74, respectively. The freshwater sample showed a concentration of 8.76 ng of DNA/ μ L, and the absorbance ratio values 260/280 and 260/230 were 2.26 and 1.16, respectively, which are acceptable values for DNA extracted from environmental samples ($A_{260}/A_{280} \geq 1.8$) (Lucena-Aguilar *et al.*, 2016). In this way, the combination of both methods allowed obtaining the required concentration through phenol-chloroform extraction and, in turn, the necessary purity through the use of the kit column; this is fundamental given the presence of contaminants and inhibitors inherent to the environmental matrix.

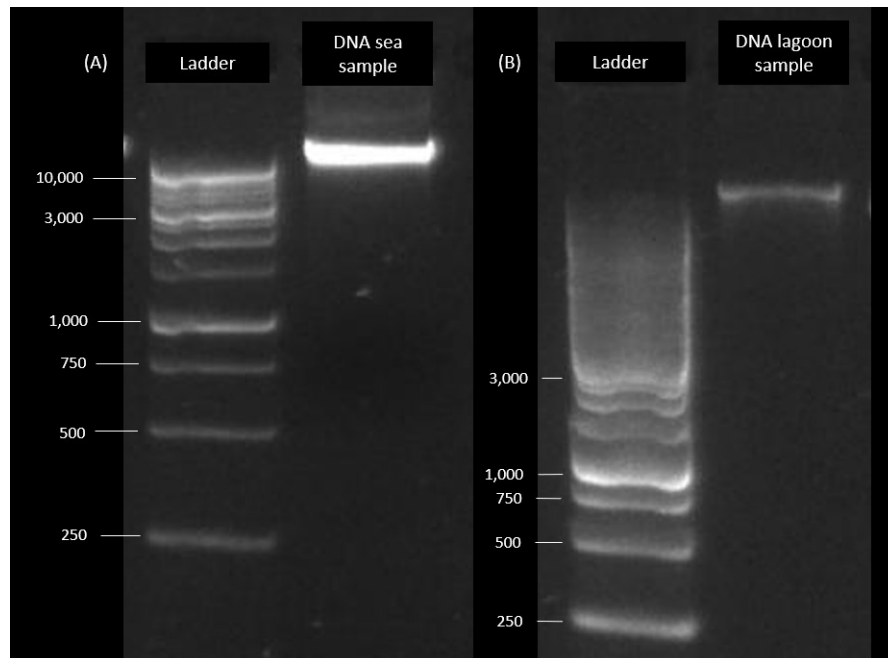


Figure 1. DNA extraction from an environmental sample, (A) is the extraction of DNA sea sample, and (B) is the extraction of DNA lagoon sample.

Source: Own elaboration.

Detection of cyanobacteria, *Microcystis*, and the *mcyA* gene by endpoint PCR

The electrophoresis of the PCR products from the seawater sample showed amplification with the oligonucleotides for the 16S rRNA gene of cyanobacteria (~550 bp), indicating the presence of cyanobacteria in the environmental sample; however, no amplification was observed with the oligonucleotides for the 16S rRNA gene of *Microcystis* (~220 bp) and the *mcyA* gene (~290 bp), thus ruling out the presence of the genus *Microcystis* and the potential production of microcystins (Figure 2A). The electrophoresis of the PCR products from the water sample of Laguna del Náinari showed amplification with the oligonucleotides for the 16S rRNA gene of cyanobacteria and the 16S rRNA gene of *Microcystis*, indicating the presence of cyanobacteria and the genus *Microcystis* in that environmental sample; however, no amplification of the *mcyA* gene was observed, thereby ruling out the potential production of microcystins (Figure 2B). Chávez-Luzanía *et al.* (2024) reported that the use of these oligonucleotides allows amplification from 1 ng of DNA per reaction for the 16S rRNA gene of cyanobacteria and the 16S rRNA gene of *Microcystis*, and from 10 ng of DNA for the *mcyA* gene. However, the amplification observed in the positive control with 5 ng of DNA confirms a lower detection limit than previously reported. In this context, the non-detection of the *mcyA* gene in the environmental samples is associated with the minimal presence of cyanobacteria producing this gene or, alternatively, with its absence.

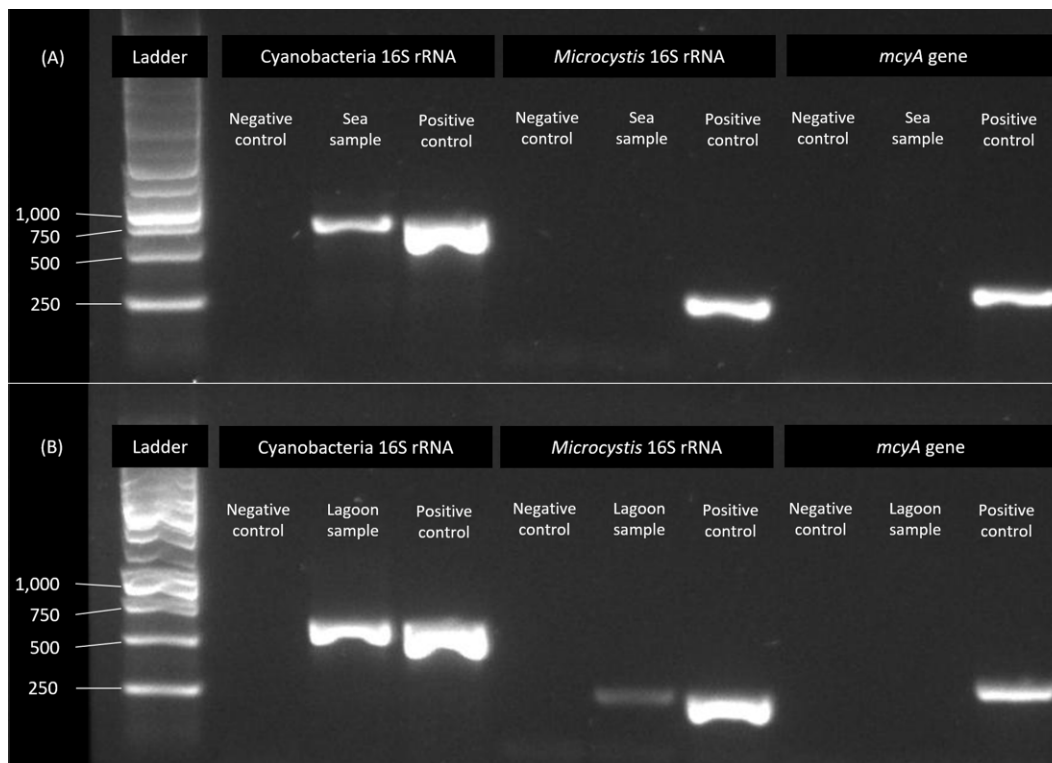


Figure 2. PCR of an environmental sample with 5 ng of DNA. The sea sample (A) and the lagoon sample (B). The positive control: microcystin-producing *Microcystis aeruginosa*.

Source: Own elaboration.

The sequences obtained from the PCR products showed, in the case of the 16S rRNA gene of cyanobacteria from the seawater sample, a 97.60 % similarity with the genus *Prochlorothrix*. This value is below the accepted thresholds for taxonomic assignment at the genus level (>98.65–99 % for the 16S rRNA gene) (Kim *et al.*, 2014). However, the sequence can be considered phylogenetically related to *Prochlorothrix*, due to microscopic observation, which revealed the presence of straight, unbranched, and immobile trichomes (Velichko *et al.*, 2016). On the other hand, the sequence from the freshwater sample showed 98.75 % similarity with the genus *Cylindrospermopsis*. The sequence obtained from the 16S rRNA gene of *Microcystis* showed 99.13 % similarity with *Microcystis aeruginosa*, characterized by its colonial morphology of spherical cells (Krstic & Aleksovski, 2016). The taxonomic affiliation based on the sequencing of the cyanobacterial amplicons was confirmed by the microscopic observation of these genera (Figure 3).

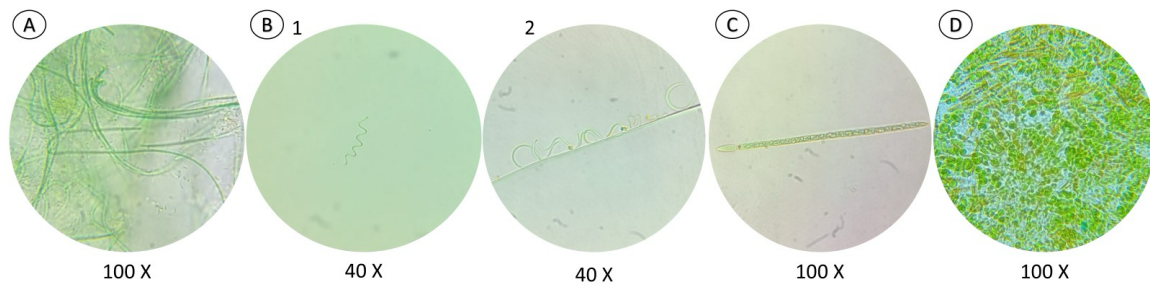


Figure 3. Microscopic observation of cyanobacteria isolated from environmental samples. (A) *Prochlorothrix* filaments observed from seawater at 100×, showing their characteristic unbranched morphology. (B) *Cylindrospermopsis* isolated from freshwater, displayed at 40× (1–2), highlighting terminal heterocytes and elongated filaments; and *Raphidiopsis* filaments at 100×. (C). Colonies of *Microcystis aeruginosa* at 100×, recognizable by their spherical cells arranged in mucilaginous aggregates.

Conclusion

The optimized protocol enabled DNA extraction for the detection of cyanobacteria in marine and freshwater environmental samples through endpoint PCR and sequencing on the Sanger platform. Through the amplification and sequencing of amplicons, complemented by microscopic observation, the presence of the genus *Prochlorothrix* was identified in seawater, as well as *Cylindrospermopsis* (using oligonucleotides targeting the 16S rRNA gene of cyanobacteria) and *Microcystis aeruginosa* (using oligonucleotides specific for the 16S rRNA gene of *Microcystis*) in the freshwater sample. Finally, the *mcyA* gene was not detected in the analyzed samples, suggesting that the cyanobacteria present cannot produce microcystins.

Author's contribution

Conceptualization of the work, SdISV, FIPC; methodology development, SdISV, JAI, PHMS; software management, JAI, PHMS; experimental validation, JAI, PHMS; results analysis, JAI, MEOU; data management, JAI, MEOU, PHMS; manuscript writing and preparation, JAI, MEOU,

PHMS, SdISV; writing, review, and editing, JAI, PHMS, FIPC, SdISV; project administration, SdISV; funding acquisition, SdISV. All authors of this manuscript have read and approved the published version.

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Conflict of interest

The authors declare that they have no conflict of interest.

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