PCR-Based Diagnosis of *Neoscytalidium dimidiatum* Infection Using Internal Transcribed Spacer 1 Region of Ribosomal DNA Primers

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ABSTRACT

Objective: To develop *N. dimidiatum*-specific single PCR-based identification with DNA sequences of nuclear ribosomal internal transcribed spacer (ITS) 1 region primers to facilitate the rapid and accurate detection of *N. dimidiatum*.

Methods: *N. dimidiatum*-specific PCR primers were designed based on the sequence of the internal transcribed spacer 1 region, which is located between 18S and 5.8S nuclear rDNA. Fungal DNA extracted from common causative species for superficial fungal infection including: 2 strains of *N. dimidiatum*, 9 species of dermatophyte (DMP) and 25 species of non-dermatophyte (NDM) colonies grown on culture plates were used for PCR analysis. Also, 30 clinical specimens collected from 30 patients clinically diagnosed with fungal nail and feet infection who attended Dermatology clinic Siriraj Hospital during October 2015 to November 2015 were used for PCR assay.

Results: Using *N. dimidiatum*-specific PCR primers, the PCR product was amplified from two standard strains of *N. dimidiatum*, and there was no amplification from other DMP or NDM species. Regarding sensitivity as lower limit of detection, this PCR method was able to detect 10 pg of *N. dimidiatum* DNA with ethidium bromide staining and could detect *N. dimidiatum* in clinical samples.

Conclusion: This newly developed *N. dimidiatum*-specific PCR identification system is rapid, sensitive, and specific. This diagnostic method will facilitate early and accurate diagnosis and accelerate appropriate treatment in patients with *N. dimidiatum* infection.

Keywords: *Neoscytalidium dimidiatum; N. dimidiatum* infection; *N.dimidiatum*-specific primers; PCR-based diagnosis; nondermatophytes (Siriraj Med J 2018;70: 28-35)

INTRODUCTION

Neoscytalidium dimidiatum is an important causative organism in nondermatophyte (NDM) skin and nail infection worldwide, but especially in the West Indies, South America, West Africa, and Asia.[1-4] NDM onychomycosis has been recently reported to be increasing.[5] The clinical characteristics of superficial fungal skin infection and NDM onychomycosis were reported to be clinically indistinguishable from dermatophyte (DMP) onychomycosis, except that fungal skin infection above the feet patients was not commonly detected in patients with NDM onychomycosis.[1,2,6,7] Mycological culture remains an essential method for isolating causative fungi. Moreover, drug resistance were commonly detected in

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NDM infection, especially in *N. dimidiatum* infection. Standard systemic antifungal treatment used for DMP infections had variable effect on NDM infections.[8] As such, the development of an accurate mycological laboratory technique that enables identification, diagnosis, and proper management of NDM infections is essential.

Diagnosis of *N. dimidiatum* infection is generally made by fungal slide culture, which is always timewasting and requires specially trained personnel. Molecular biological techniques have recently been developed that facilitate the precise and rapid identification of DMPs and NDMs.[4,9,10] Restriction fragment length polymorphism targeting (PCR-RFLP) of the hypervariable V4 domain of the small ribosomal subunit 18S gene was reportedly used to identify *N. dimidiatum*.[4,10] However, species-specific single PCR-based identification for *N. dimidiatum* has not been reported. This PCR method was established to identify different types of fungi by single PCR reaction using a unique amplicon. Accordingly, the aim of this study was to develop *N. dimidiatum*-specific single PCR-based identification with DNA sequences of nuclear ribosomal internal transcribed spacer (ITS) 1 region primers to facilitate the rapid and accurate detection of *N. dimidiatum* infection.

MATERIALS AND METHODS

Primer design

The primer sequence of *N. dimidiatum* was designed using MEGA4 alignment software (http://www.megasoftware.net/download_form) and Primer Express software (Applied Biosystems, Foster City, CA, USA) based on upstream and downstream sequences of the ITS1 region of the ribosomal DNA gene (rDNA) of *N. dimidiatum* in the DDBJ/EMBL/GenBank (accession no. AB490817.1). Oligonucleotides used in this study were designed based on comparisons with sequences of ITS1 rDNA data of DMPs and NDMs [accession numbers: AB042608.1 (*Microsporum duboisii*); AB042607.1 (*M. distortum*); AB042606.1 and AB042605.1 (*M. ferrugineum*); AB049929.1 (*M. gallinae*); AB017178.2 (*M. audouinii*); AB017180.1 (*M. cookie*); AB017177 (*M. gypseum*); AB017179 (*M. canis*); AB017181.1 (*Epidermophyton floccosum*); AB017176.1 (*Trichophyton verrucosum*); AB017174.1 (*T. violaceum*); AB017173.1 (*T. schoenleinii*); AB017172.1 (*T. tonsurans*); AB011453.1 (*T. rubrum*); AB011463 (*T. mentagrophytes*); AJ223852.1 (*Aspergillus niger*); Y14003.1 (*Candida albicans*); LT594689.1 (*Exophiala jeanselmei*); AM412643.1 (*Fusarium solani*); AJ853776.1 (*Scopulariopsis brevicaulis*); and, AB011466 (*Arthroderma vanbreuseghemii*)].

Base sequences specific to *N. dimidiatum* were analyzed with primer 3 output and BLAST® blastn suite software (https://blast.ncbi.nlm.nih.gov) to develop primers. *N. dimidiatum*-specific primers NeoF1 (5'-TGTACCCACCTCTGTTGCTTTG-3') and NeoR1 (3'-TCGATGCCAGAACCAAGAGATC-'5) were made by BEX Co., Ltd. (Tokyo, Japan). The size of the amplified product was estimated to be 173bp.

Preparation of fungal DNA from strains

DMP and NDM fungal strains were used in this study (Table 1). All of these standard strains were maintained in the TIMM culture collection of Teikyo University Institute of Medical Mycology, and most of them were isolated from clinical cases or environment in Japan and part of them were obtained from international culture collections. All fungal strains were grown on SDA at 28°C for 5-14 days. Small amounts of fungal colony on SDA were placed in lysis buffer (200 mMTris-HCl, pH 8.0, with 25 mM EDTA, 0.5%, w/v, sodium dodecyl sulfate, and 250 mMNaCl) and ground with a conical grinder. Then samples were incubated at 100°C for 15 min and mixed with 100 µl of 3.0 M sodium acetate. Samples were maintained at -20°C for 10 min and then centrifuged at 10,000 x g at 4°C for 10 min. Supernatants were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and then extracted once with chloroform. DNA was precipitated with an equal volume of isopropanol, washed with 150 µl of 70% ethanol, dried, and suspended in 50 µl of TE buffer (pH 8.0). Aliquots of 1µl of the resulting solutions were then used as templates for the PCR.

Preparation of fungal DNA from clinical specimens

Twenty nail specimens and 10 skin specimens from 30 patients clinically diagnosed with fungal nail and feet infection (respectively) were used for PCR assay. Clinical samples were collected at Department of Dermatology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand during the study period October 2015 to November 2015. The protocol for this study was approved by the Siriraj Institutional Review Board (Si 090/2016). Written informed consent was waived, given the nature of the study and the associated anonymity of patient and specimen data.

A portion of each sample was placed on a slide and microscopically studied after clearing in 20% potassium hydroxide. For culture, part of each sample was inoculated in SDA with and without cyclohexamine. Cultures were then incubated at 27°C and examined every 4 days for 6 weeks. Identification of microorganisms was based

TABLE 1. Results of PCR analysis with ITS1 universal and *N. dimidiatum*-specific primer pairs for standard strains

No.	Fungal species	Strain	PCR with ITS1 primers	PCR with *N. dimidiatum* primers
1	*Neoscytalidium dimidiatum*	NBRC110913	+	+
		NBRC110914	+	+
2	*Acremonium curvulum*	NBRC32242	+	-
3	*Alternaria alternata*	NBRC4026	+	-
		NBRC6587	+	-
4	*Arthroderma benhamiae*	SM103(+)	+	-
		SM104(-)	+	-
5	*Arthroderma vanbreuseghemii*	TIMM2789	+	-
6	*Aspergillus flavus*	NBRC4186	+	-
		JCM2061	+	-
7	*Aspergillus fumigatus*	NBRC4057	+	-
		TIMM0108	+	-
8	*Aspergillus niger*	JCM10254	+	-
		NBRC4066	+	-
9	*Candida albicans*	ATCC10231	+	-
		TIMM1768	+	-
10	*Candida glabrata*	ATCC90030	+	-
		NBRC0005	+	-
11	*Candida parapsilosis*	ATCC22019	+	-
		NBRC1396	+	-
12	*Candida tropicalis*	ATCC750	+	-
		NBRC0006	+	-
13	*Chaetomium globosum*	TSY-0369	+	-
14	*Cryptococcus neoformans*	TIMM3147	+	-
		TIMM1835	+	-
15	*Epidermophyton flocosum*	NBRC9045	+	-
		KMU8915	+	-
16	*Exophiala jeanselmei*	NBRC6857	+	-
		TSY-0396	+	-
17	*Fonseceae pedrosoi*	NBRC6071	+	-
		TSY-0394	+	-
18	*Fusarium oxysporum*	NBRC7152	+	-
		TSY-0351	+	-
19	*Fusarium solani*	NBRC5232	+	-
		TSY-0403	+	-
20	*Fusarium verticillioides*	TSY-0219	+	-
21	*Histoplasma capsulatum*	TIMM1846	+	-
22	*Hoetae werneckii*	TIMM4094	+	-
23	*Microsporum canis*	NBRC7863	+	-
		TIMM4092	+	-

TABLE 1. Results of PCR analysis with ITS1 universal and *N. dimidiatum*-specific primer pairs for standard strains (Continued)

No.	Fungal species	Strain	PCR with ITS1 primers	PCR with *N. dimidiatum* primers
24	*Microsporum gypseum*	NBRC5948	+	-
		KMU8642	+	-
25	*Paecilomyces variotii*	NBRC4855	+	-
		TIMM4066	+	-
26	*Penicillium marneffei*	TIMM6206	+	-
27	*Pseudallescherichia boydii*	TIMM0886	+	-
		TIMM0887	+	-
28	*Rhizopus oryzae*	IFM40515	+	-
		IFM62923	+	-
29	*Rhodotorula minuta*	JCM3777	+	-
		TIMM6222	+	-
30	*Scopulariopsis brevicaulis*	NBRC4843	+	-
		TSY-0668	+	-
31	*Scytalidium lignicola*	NBRC104988	+	-
32	*Trichophyton mentagrophytes*	NBRC5466	+	-
		TIMM1189	+	-
33	*Trichophyton rubrum*	NBRC5467	+	-
		TIMM2659	+	-
34	*Trichophyton tonsurans*	NBRC5928	+	-
		KMU8913	+	-
35	*Trichosporon asahii*	NBRC1200	+	-
		TIMM1318	+	-

(+), detected; (-), not detected

on the observation and description of macroscopic and microscopic characteristics of colonies.

QIAamp® DNA Micro Kit (Qiagen GmbH, Hilden, Germany) was used for DNA extraction from nail and skin samples. One µl of DNA solution was used as a template for PCR and sequencing analysis. DNA extraction from *N. dimidiatum* colonies on culture plate and water were used as positive and negative controls, respectively.

Polymerase chain reaction (PCR)

TaKaRa Taq™ (Takara Bio Inc., Tokyo, Japan) was used for PCR. PCR master mix was prepared using 5 µL of 10X PCR buffer, 4 µL of 2.5mMdNTP mix, 0.25 µL of Taq DNA Polymerase, and 38.8 µL of distilled water. One µL of DNA solution and 0.5 µL of each 30 pM specific primer were added to the mixture, which was then placed in a TaKaRa PCR Thermal Cycler (Takara Bio Inc., Tokyo, Japan). Each mixture was heated to 94°C for 4 min and PCR was performed under the following conditions: 94°C, 30 sec; 60°C, 1 min; 72°C, 1 min; 30 cycles. Thermal cycles were terminated by polymerization at 72°C for 10 min. Amplification products were visualized on 1.2% agarose gel containing ethidium bromide. Universal ITS1-specific primers (18SF1: 5'-AGGTTTCCGTAGGTGAACCT-3', 58SR1: 5'-TTCGCTGCGTTCTTCATCGA-3') previously reported by Makimura, et al. were used to confirm DNA detection.[11]

DNA sequencing and analysis

Both strands of each PCR product were directly sequenced using a DNA Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with primers NeoF1 and NeoR1 and an automatic sequencer (Genetic Analyzer 310; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequences were compared with the ITS1 sequence of *N. dimidiatum* using GENETYX-MAC 10 software (https://www.genetyx.co.jp/) to verify a 100% match.

RESULTS

Primer Specificity and Sensitivity

The primer specificity and sensitivity (lower limit of detection) of *N. dimidiatum*-specific PCR for standard DMP and NDM strains were evaluated using specific primers (Table 1). Two strains of *N. dimidiatum* demonstrated band at 173 bp, and DNA sequencing revealed a 100% match with ITS1 region of *N. dimidiatum*. No expression was identified for any other DMPs or NDMs. Fig 1 shows PCR products amplified with universal ITS1 primers 18SF1 and 58SR1 (Fig 1A) and *N. dimidiatum*-specific primers NeoF1 and NeoR1 (Fig1B). DNA of all fungi was amplified successfully with universal ITS1 primers and only *N. dimidiatum* DNA was detected using *N. dimidiatum*-specific primers.

Dilution technique was used to evaluate the lower limit of detection of primers to detect DNA with ethidium bromide staining. *N. dimidiatum*-specific PCR was able to detect 10 pg of *N. dimidiatum* genomic DNA with ethidium bromide staining (Fig 2).

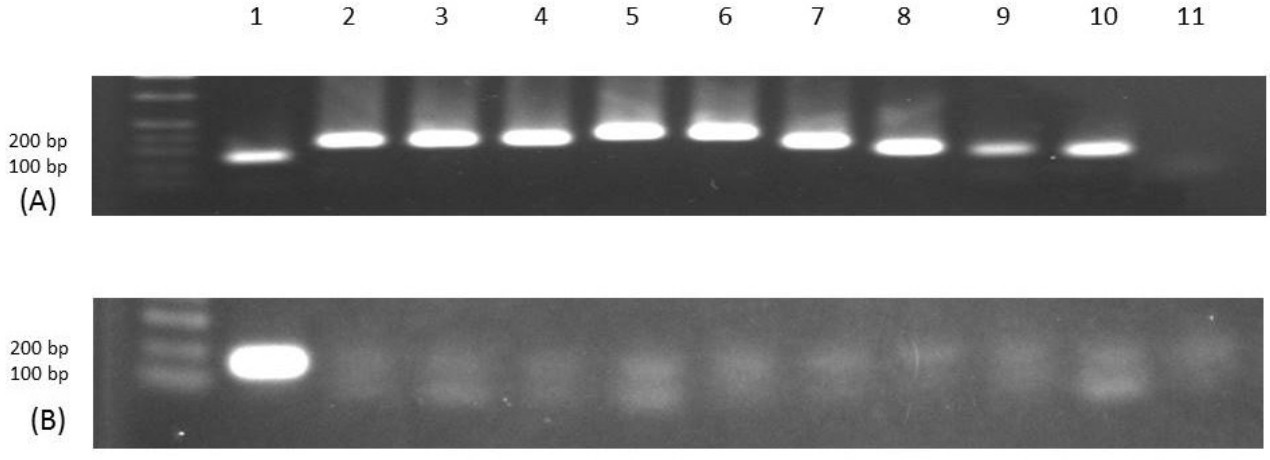

Fig 1. Primer Specificity of *N. dimidiatum*-specific primers: gel electrophoresis images of PCR using ITS1 primers (A); and, *N. dimidiatum*-specific primers (B). M, DNA molecular weight marker (BIONEXUS, USA): (1) *N. dimidiatum*; (2) *T. rubrum*; (3) *T. mentagrophytes*; (4) *T. tonsurans*; (5) *E. floccosum*; (6) *M. canis*; (7) *M. gypseum*; (8) *Aspergillusflavus*; (9) *Fusariumsolani*; (10) *C. albicans*; (11) water (negative control)

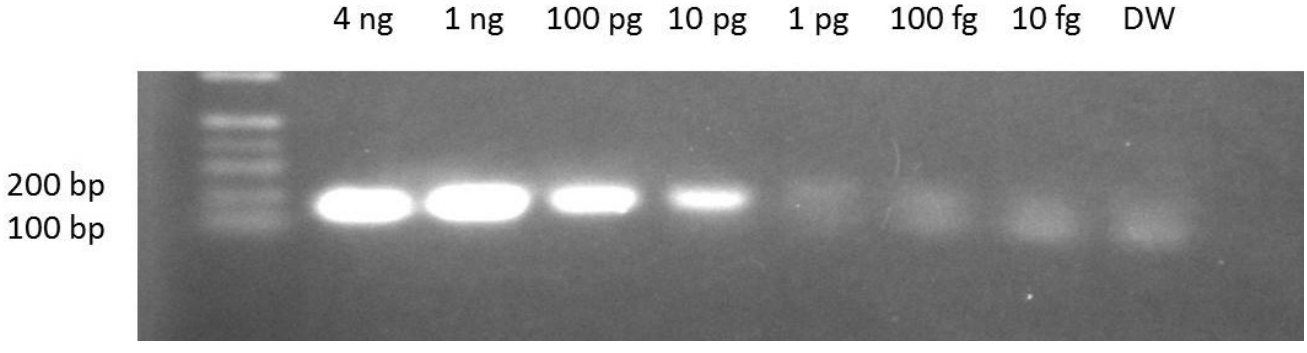

Fig 2. Lower limit of detection of *N. dimidiatum*-specific primers with dilution of *N. dimidiatum* DNA solutions

PCR detection of N. dimidiatum DNA from clinical specimens

DNA extraction and PCR reaction were performed in all clinical samples. *N. dimidiatum*-specific primers and ITS primers were used in PCR reaction (Table 2). *N. dimidiatum* was found from culture in 8 nail and 5 skin samples that demonstrated positive bands with both ITS1 and *N. dimidiatum*-specific primers. DMPs and NDMs were detected from culture in 10 nail and 4 skin samples that all showed ITS1 single bands. There were 4 DMP samples (2 nail and 2 skin) and 3 NDM nail samples that demonstrated single band with *N. dimidiatum*-specific primers. Three samples did not detect fungus from culture, with one sample expressing single bands with ITS1 and *N. dimidiatum*-specific primers. Compared with fungal culture as gold standard, sensitivity and specificity of *N. dimidiatum*-specific primers were 100% and 53%, respectively. Positive predictive value was 62% and negative predictive value was 13%.

TABLE 2. Detection of fungi from clinical samples.

Patient No.	Specimen	Microscopy	Culture	PCR with ITS1 primers	PCR with *N. dimidiatum* primers
1	Nail	+	*N. dimidiatum*	+	+
2	Nail	+	*N. dimidiatum*	+	+
3	Nail	+	*N. dimidiatum*	+	+
4	Nail	+	*N. dimidiatum*	+	+
5	Nail	+	*N. dimidiatum*	+	+
6	Nail	+	*N. dimidiatum*	+	+
7	Nail	+	*N. dimidiatum*	+	+
8	Nail	+	*N. dimidiatum*	+	+
9	Nail	+	*T. rubrum*	+	+
10	Nail	+	*T. rubrum*	+	-
11	Nail	+	*T. mentagrophytes*	+	+
12	Nail	+	*T. mentagrophytes*	+	-
13	Nail	+	*Fusarium* spp.	+	-
14	Nail	+	*Fusarium* spp.	+	-
15	Nail	+	*S. brevicaulis*	+	+
16	Nail	+	*S. brevicaulis*	+	-
17	Nail	+	*A. niger*	+	+
18	Nail	+	*Penicillium* spp.	+	+
19	Nail	+	No growth	+	+
20	Nail	+	No growth	-	-
21	Skin	+	*N. dimidiatum*	+	+
22	Skin	+	*N. dimidiatum*	+	+
23	Skin	+	*N. dimidiatum*	+	+
24	Skin	+	*N. dimidiatum*	+	+
25	Skin	+	*N. dimidiatum*	+	+
26	Skin	+	*T. rubrum*	+	+
27	Skin	+	*T. mentagrophytes*	+	+
28	Skin	+	*T. mentagrophytes*	+	-
29	Skin	+	*Fusarium* spp.	+	-
30	Skin	+	No growth	-	-

(+), detected; (-), not detected

DISCUSSION

N. dimidiatum infection is an important health problem in Thailand and many other tropical countries. Previous studies from Thailand reported that 24.1%-51.6% of onychomycosis cases were NDM onychomycosis, and *N. dimidiatum* was the most common pathogen.[1,12] NDM infection is more difficult to treat and more resistant to several drugs than DMP infection.[8] *N. dimidiatum* is not responsive to most systematic antifungal therapies.[4] Therefore, species identification is essential for both accurate diagnosis and appropriate management. Microscopic examination cannot normally differentiate hyphae between DMPs and

N. dimidiatum. Culture remains the standard method for species identification. However, culture requires at least a week to obtain typical macroscopic and microscopic characteristics. In some cases, subculture is required for exact identification. These culture-related processes delay diagnosis and proper management for a week.[4] In this study, PCR identification with *N. dimidiatum*-specific primers based on ITS1 region required approximately 3 hours to provide accurate identification in patients with superficial skin and nail infection. Moreover, this method is objective and does not require specially-trained personnel to morphologically identify the fungus. This method will facilitate early and accurate diagnosis and proper management in clinical practice.

A previous study reported PCR-RFLP method for discriminating between DMPs and *N. dimidiatum* targeted on subunit 18S rDNA. Also PCR-RFLP demonstrated accurate and rapid result, although this technique used two primer sets and two enzymes for *N. dimidiatum* identification.[4] Although fast and accurate, this 2 enzyme-2 primer technique is limited by the fact that it cannot be performed in most standard laboratories. The present study demonstrated molecular diagnosis using a single PCR method with one primer set that may be prepared in a wide range of clinics and hospitals.

In this study, culture of 7 clinical samples revealed DMPs and other NDMs apart from *N. dimidiatum* within positive band that were acquired using *N. dimidiatum*-specific primers. This may be the result of mixed infection or contamination. Mixed infection of DMP and NDM onychomycosis is increasingly reported using molecular analysis, as antibiotics and cyclohexamine in traditional culture may inhibit NDM growth.[13] Identification of mixed infection would be useful for the prediction of clinical course and prognosis, and would lead to proper management.[14] However, diagnosis criteria for mixed infection are still debated and need to exclude contamination. Further study is required to elucidate this mixed infection.

NDMs have been identified in both soil and plants, so infection or colonization may be acquired by direct contact.[1] Previous study reported that NDMs, such as *Acremonium* spp., *Aspergillus* spp., *Fusarium* spp., and *Scopulariopsis* spp., may colonize in an abnormal nail unit and may be clinically indistinguishable from onychomycosis.[15] However, no prior reports described *N. dimidiatum* colonization in a nail unit. Currently, NDM onychomycosis diagnosis is based on diagnostic criteria that require at least 3 of the following: positive microscopic examination, positive mycological culture, at least two consistent isolations from repeated sampling, dermatophyte exclusion, and histology. To ensure that NDMs are the true pathogens and not contaminants, serial sampling at multiple time points is recommended to confirm the repeated presence of NDMs with or without DMP infection.[6] Molecular techniques will be a powerful and valuable tool for diagnosing NDM onichomycosis.

Fungal hyphae were microscopically detected in all clinical samples in this study, but 3 samples did not detect fungus from culture, with one sample expressing single bands with ITS1 and *N. dimidiatum*-specific primers. Positive microscopic examination would support that this finding was not due to contamination. It may be explained by the low sensitivity of traditional culture or an uneven distribution of fungus in clinical samples.

This study has some mentionable limitations. First, this study was a pilot study that used specific primers to detect fungus in clinical samples, so the sample size was relatively small. Further studies with a larger sample size should be performed to validate our results. Second, NDM detection could not be distinguished from true pathogen or colonization in the nail unit. Clinical characteristics, repeated sample examinations, and evaluation of nail histology may be useful to confirm diagnosis and to guide appropriate treatment.

In conclusion, this newly developed *N. dimidiatum*-specific PCR identification system is rapid, sensitive, and specific. This diagnostic method will facilitate early and accurate diagnosis and accelerate appropriate treatment in patients with *N. dimidiatum* infection.

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Conflict of interest declaration

The authors hereby declare no personal or professional conflicts of interest regarding any aspect of this study.

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