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Isolation and Screening for Potential endosulfan-degrading bacteria from Soil

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Abstract

The urgent need for sustainable management of persistent organic pollutants, due to their negative impact on the environment, is of great concern to environmental stakeholders. In this study, the potential endosulfan-degrading (PED) bacteria were screened and identified from fresh soil samples collected from the Teaching and Research farm of Kwara State University, Nigeria. The bacterial strains were isolated using enrichment techniques and characterized through morphological, biochemical, and molecular analyses. Their potential for endosulfan utilization was assessed in an endosulfan-supplemented mineral salt medium. Three potential endosulfan-degrading (PED) bacterial strains isolated were Achromobacter xylosoxidans strain PED1, Pseudomonas alkylphenolica strain PED2, and Sphingomonas sp. Strain PED3 with the accession numbers MF973060, MF973061 and MF973062 respectively. The optical density (OD) values increased for all the isolates by the third day, and the highest OD values of 0.51 (PED1), 0.52 (PED2) and 0.59 (PED3) were recorded on the ninth day. The observed increase in population density of the isolates in the endosulfan-supplemented mineral salt medium suggests their capability to utilize the endosulfan compound. Further study is required to evaluate the suitability of these strains for bioremediation purposes.

Keywords: *Achromobacter xylosoxidans*, Endosulfan, PED, *Pseudomonas alkylphenolica*, *Sphingomonas* sp.

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INTRODUCTION

Endosulfan is a synthetic broad-spectrum pesticide compound that had been previously banned in most countries due to its adverse environmental effects (Tongu *et al*., 2023). It is a recalcitrant organochlorine pesticide (OCP) group and tends to persist in the soil media, due to its characteristics features such as hydrophobic nature, toxicity, and non-bioavailability. Endosulfan has a high octanol-water partition coefficient, thereby contributing to its persistence in the environment (Alarcon *et al*., 2023). It has been widely used for the control of pest infestation in agriculture (Zhou *et al*., 2024). Currently, there has been much concern about its toxicity, persistence and adverse ecological impact. Despite the ban imposed on endosulfan usage in Nigeria since 2007, recent reports confirmed its continuous usage for pest control (Atuanya and Aborisade, 2017; Oluwole-Banjo *et al*., 2022).

Like other OCPs, endosulfan has a tendency to bioaccumulate and biomagnify in the food chain within the ecosystem, posing a health risk to consumers (Sathishkumar *et al*., 2021). The residue of endosulfan has been detected in food products such as beans, cow milk, and even in surface water samples (Kelle *et al*., 2020; Odion, *et al*., 2021; Shaibu *et al*., 2021). These residues could pose a lot of health hazards not only to humans but also to the stability of ecosystems.

There are several ecological and public health issues related to pesticide residues in the environment. Persistent pesticide residues may pose an acute and chronic toxicity risk to the non-target organisms in the environment (Liu *et al*., 2023). They may also disrupt the ecological balance and reduce the resilience properties of ecosystems (Pathak *et al*., 2022). Pesticide residues can contaminate sources of drinking water, surface water and other aquatic habitats via leaching and flooding processes. This might result in a negative impact on aquatic-dependent organisms as well as humans. In the soil system, pesticide residue could impair beneficial soil microbial diversity and interfere with ecosystem function (Aborisade and Atuanya, 2022; Aborisade and Atuanya, 2023). Exposure to pesticide residue by humans through contaminated food, water, air, and work activities could lead to chronic diseases such as cancer, reproductive defects and neurological-related diseases (Sabarwal *et al*., 2018; Pedroso *et al*., 2022).

Microorganisms possess vast metabolic machinery that are crucial in the biodegradation of recalcitrant compounds and xenobiotics. This unique and rare feature contributes to the functional ecological role of microorganisms in recycling the elements, hence the engine room for the biogeochemical cycle in the ecosystem. Microorganisms have demonstrated the ability to metabolize various organic pollutants into less toxic by-products and thereby successfully utilized in the reclamation of polluted environments (Tarfeen *et al*., 2022).

Microbial isolation and characterization are fundamental microbiological techniques that can provide the basis for understanding the morphology, physiology and biochemical features of microbes (Tusher *et al*., 2021; Yang *et al*., 2023). By isolating and characterizing bacteria capable of degrading pollutants, researchers can identify microorganisms with the potential to remediate contaminated environments. The existing research has primarily focused on isolating and identifying pesticide-degrading bacteria in environments directly affected by pesticide contamination, such as agricultural fields (Sathishkumar *et al*., 2021) little attention has been given to investigating microbial communities in unaffected agricultural soils. Studying pollutant-degrading bacteria can provide insights into such organisms' ecological roles and interactions within contaminated ecosystems. Therefore, this study is on the screening for potential endosulfan-degrading bacteria.

MATERIALS AND METHODS

Study area
The study was conducted using soil samples collected from the research and teaching farm of Kwara State University, Malete. The research was conducted in the microbiology Laboratory of Kwara State University, Malete.

Chemicals Used
The Thiodan endosulfan formulation was procured from an agrochemical vendor in Ilorin, Kwara State, Nigeria. The reagents and chemicals were purchased from the Central Research Laboratory, Ilorin, Kwara State, Nigeria.

Soil Sample Collection and Pretreatment
The composite surface soil sample (0-15 cm layer) used for the analyses was collected from the cowpea farm of Teaching and Research farm of Kwara State University, Malete, Nigeria in September 2020 with total organic carbon content, phosphorus content, pH, and moisture content values of 29.0 %, 9.6 g/kg, 7.1 (in H_2O) and 18.3 % respectively (Aborisade *et al.*, 2021). This soil sample has no history of endosulfan application.

The visible fauna and plant debris were removed by hand-picking. The soil samples were homogenized and spread evenly in a shallow plastic tray. This allowed for acclimatization with moisture contents maintained at 20 % and incubated at room temperature in a dark cupboard for 2 weeks in the laboratory (Schroeder *et al*., 2021).
The acclimatized soil was then spiked with a 10 ppm endosulfan solution. This solution was thoroughly mixed to ensure even distribution of the pesticide. The spiked soil was then incubated for 1 week in a dark cupboard at room temperature to allow for microbial adaptation.

To further stimulate microbial adaptation and enhance the isolation of potential endosulfan-degrading bacteria, the pesticide-treated soil was spiked four more times at 1-week intervals over a 6-week period. The sample was stored in a sterile polythene bag at 4 ^oC for further study.

Isolation of potential endosulfan-degrading bacteria
The potential endosulfan-degrading bacterial groups were isolated using the International Standard Organization method 7827 (Aina *et al*., 2021). The isolation was performed through enrichment techniques in a mineral salt medium, "MSM" (4.0g NH_4Cl, 1.8g $K_4H_2SO_3$; 0.2g $MgSO_4$; 0.1g NaCl; 0.01g $FeSO_4$; 1L dH_2O; 15g/L agar) with endosulfan as the sole carbon source.

Five grams of pretreated soil was added to 95 ml of MSM in an Erlenmeyer flask. Endosulfan was supplemented as the sole carbon source. The initial endosulfan concentration in the enrichment medium was 0.75 mg/l, and more endosulfan solution was subsequently added to reach a final concentration of 7.5 mg/l. The flask was then placed on a benchtop shaker (MaxQ 4450 Model) at room temperature (27 ± 2 ^oC) on shaken at 100 rpm for one week.
From the enriched samples, further enrichment was carried out for three more weeks by transferring 5 ml of the culture broth into a sterile flask containing 95 ml of mineral salt medium (MSM) spiked with 3 mg/l endosulfan. The culture was then incubated at room temperature on a benchtop shaker at 100 rpm.

After four successful transfers, a loopful of the enriched culture broth was inoculated on MSM supplemented with agar (15g/L agar) and incubated at room temperature (27 ± 2 ^{o}C) for 48 h. Repeated sub-culturing was carried out on nutrient agar, and pure isolates were stocked on agar slants for further studies.

Screening for isolates' ability to utilize endosulfan.
The bacterial isolates were screened for their ability to utilize endosulfan based on their growth in mineral salt medium (MSM) supplemented with endosulfan (Aina *et al*., 2021).
The isolates from the stock culture were standardized using the 0.5 McFarland standard solution. Subsequently, 5 ml of the standardized isolates were each introduced into a flask containing 50 ml of MSM broth supplemented with endosulfan (7.5 mg/l). The MSM broth medium without endosulfan, inoculated with the respective isolates was used as the control. All set-ups were prepared in triplicate and incubated on a benchtop shaker (100rpm) at room temperature. The growth rate was assessed by measuring the optical density (OD 600nm) using a spectrophotometer (UV/Vis 721D Model) at 3-day intervals for fifteen days.

Identification of potential endosulfan-degrading bacterial isolates
The potential endosulfan-degrading bacterial isolates were characterized based on their morphological and biochemical characteristics using standard identification criteria (Cheesbrough, 2000). The features tentatively used for identification include Gram's staining, spore staining, motility test, catalase test, oxidase test, citrate utilization, starch hydrolysis, urease test, indole test, methyl red test and Voges-Proskauer test (Cheesbrough, 2000).
The isolates were further subjected to molecular characterization for proper identification.

Molecular characterization of potential endosulfan-degrading bacterial isolates
Molecular characterization of potential endosulfan (PED) degrading bacterial isolates was conducted by sequencing the 16S rRNA gene (Sting *et al*. 2019).
The nucleic acid contents of the pure overnight culture of PED isolate were extracted using the phenol-chloroform technique (Da-Silva *et al*., 2020). The universal primer (5′-GGACTACAGGGTATCTAAT - 3′ forward primer and 3′- GGACTACAGGGTATCTAAT - 5′) was used to amplify the highly variable gene fragment region of the 16S rRNA of the isolated bacteria.

The purified nucleotide sequence products were sequenced using the automated Sangers dideoxy method. The sequences were edited with the bioinformatics software Chromas. The homology of the 16S rRNA gene sequences was checked against the 16S rRNA gene sequences of other organisms on the Gen Bank database using the basic local alignment search tool for nucleotides (BLASTN) algorithm. The phylogenetic tree was constructed using the neighbour-joining method with Molecular Evolutionary Genetics Analysis software (MEGA version 11).

RESULTS

Characterization of potential endosulfan degrading bacterial isolates.
Morphological and biochemical characteristics of the potential endosulfan-degrading bacterial isolates (PED) are presented in Table 1 below.

The three isolates (PED1, PED2 and PED3) were Gram-negative rods (Table 1).
All the isolates showed positive results for the motility test except isolates PED3. The catalase and oxidase tests were positive for all the isolates but none of the isolates was positive for urease, indole, methyl red and Voges Proskauer (Table 1).

Two of the isolates (PED1 and PED2) were positive for the citrate utilization test and none of the isolates was observed to test positive for the starch hydrolysis test (Table 1).

Table 1: Morphological and biochemical characteristics of potential endosulfan degrading (PED) bacterial isolates.

Characteristics	Bacterial Isolates		
	PED1	**PED2**	**PED3**
Gram's reaction	-	-	-
Cell shape	rod	rod	rod
Endospore	absence	absence	absence
Motility	+	+	-
Catalase test	+	+	+
Oxidase test	+	+	+
Citrate utilization	+	+	-
Starch hydrolysis	-	-	-
Urease test	-	-	-
Indole test	-	-	-
Methyl red test	-	-	-
Voges Proskauer test	-	-	-
Probable Isolates	*Achromobacter* sp.	*Pseudomonas* sp.	*Sphingomonas* sp.

Keys: positive (+); negative (-); sp. means species

Molecular identification of potential endosulfan degrading bacterial species.
The amplified DNA of the potential endosulfan-degrading (PED) bacterial isolates is shown in Plate 1. The neighbour-joining phylogenetic tree of partial 16S rRNA gene sequence of potential endosulfan degrading (PED) bacterial isolates is shown in Figure 1.

The comparison of the sequenced results of the 16S rRNA gene of the isolates with previously published 16S rRNA gene of bacterial nucleotide on the National Center for Biotechnology Information (NCBI) database revealed that the isolates belong to the following genus; *Achromobacter* (PED1), *Pseudomonas* (PED2) and *Sphingomonas* (PED3); (Figure 1).

The isolate PED1 showed the highest percentage homology (99%) with the partial 16S rRNA gene sequence of *Achromobacter spanius* strain LMG5911 with accession number NR025686 and *A. xylosoxidans* strain NBRC15126 with accession number NR113733 respectively (Figure 1).
The isolate PED2 showed the highest percentage homology (99%) with the partial 16S rRNA gene sequence of *Pseudomonas alkylphenolica* strain KL28 with accession number NB145644 and 98% with *P. putida* strain ATCC12633 with accession number NR114479. The isolate PED2 formed a phylogenetic cluster with *P. alkylphenolica* (Figure 1).

The sequence from the isolate PED3 exhibited the highest gene sequence similarities of 97% with *Sphingomonas oligophenolica* strain S213 with accession number NR024685 and 96% with *S. soli* strain NBRC100801 with accession number NR113941. The isolate PED3 formed a phylogenetic cluster with *S. oligophenolica* (Figure 1).

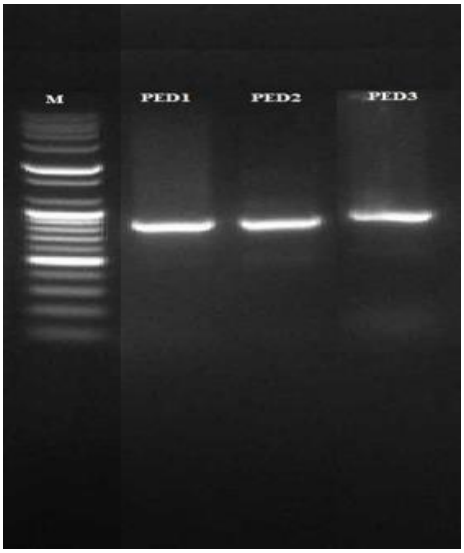

Plate 1: Amplified nucleic acid product (16S rDNA) of the bacterial isolates.
Key: PED means potential endosulfan degrader; M represents the molecular marker (1 Kb)

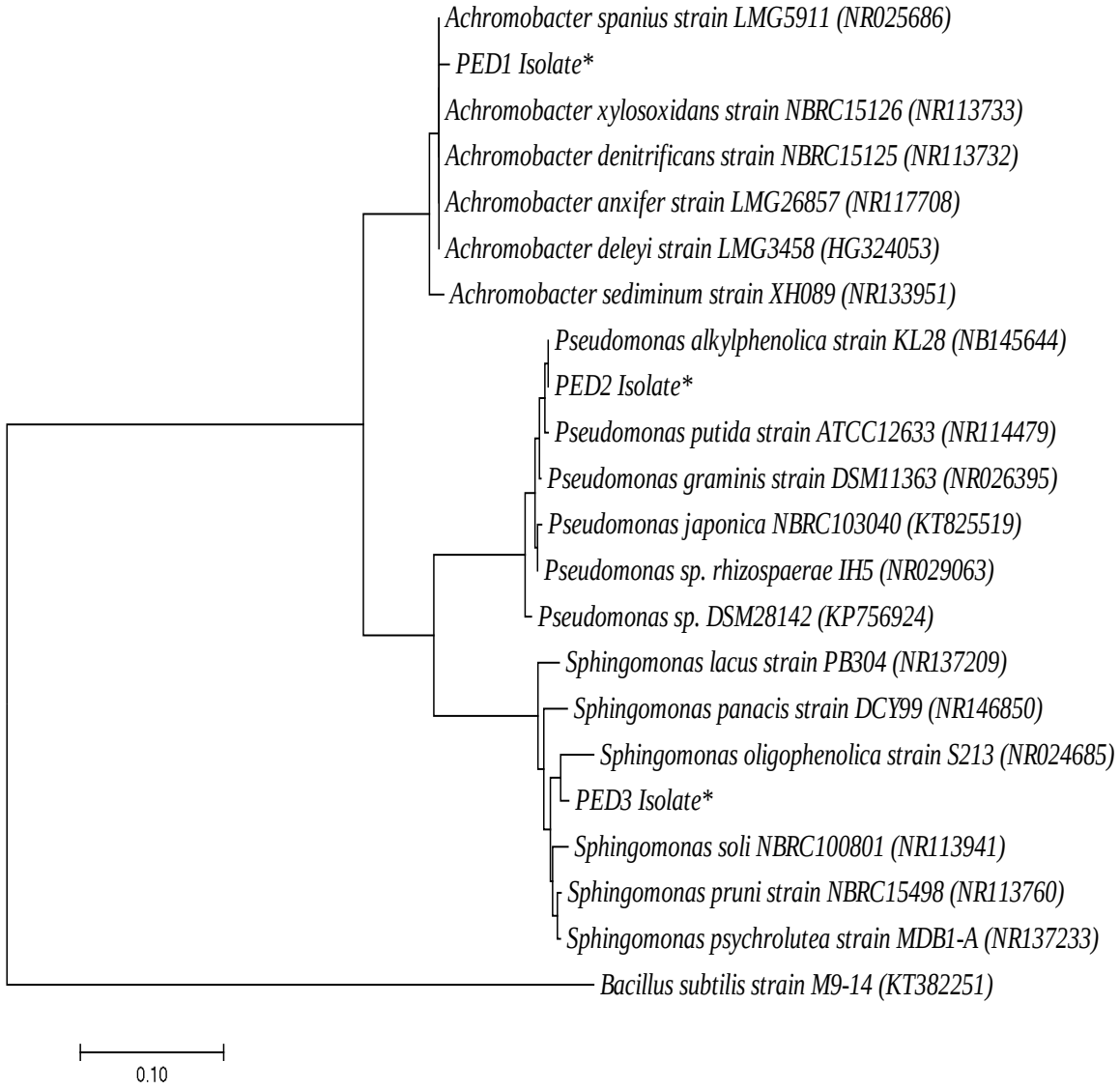

Figure 1: Phylogenetic relationship of potential endosulfan degrading bacterial isolates (PED) with reference bacterial strain by neighbor-joining tree of partial 16S rRNA gene sequence.
Key: * indicate the quarry isolate

Isolates growth rate in endosulfan-supplemented mineral salt medium.
The result for the potential endosulfan degrading (PED) bacterial isolates growth rate in endosulfan supplemented mineral salt medium is shown in Figure 1. The increases in the optical density (OD) values were observed for all the isolates from the third day to the ninth day (Figure 1). The highest OD values 0.51, 0.52 and 0.59, for PED1, PED2 and PED3 respectively were recorded for all the isolates on the ninth day (Figure 1).

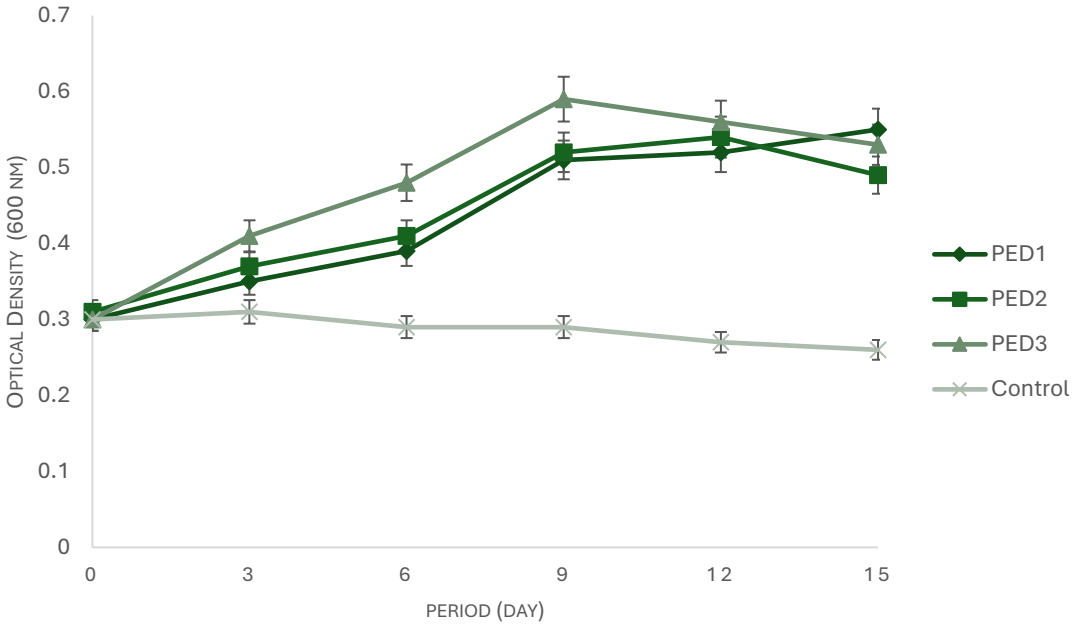

Figure 2: Growth rate of isolates in endosulfan enriched medium
Key: PED potential cypermethrin degrading bacteria

DISCUSSION

Soil bacteria were reported to possess diverse catabolic pathways for degradation of chemical pollutants and this potential can be exploited to design an effective bioremediation strategy. Hence, the indigenous bacterial strains capable of metabolizing endosulfan pesticides were isolated through an enrichment technique in a mineral salt medium (MSM) supplemented with endosulfan as the sole source of carbon. Each strain was characterized using standard morphological and biochemical techniques and subjected to molecular analysis for proper identification.

Morphological, biochemical and molecular characterization indicated that the isolates belong to *Achromobacter* (PED1), *Pseudomonas* (PED2) and *Sphingomonas* (PED3). Phylogenetic analysis constructed through the neighbour-joining method showed that the respective genus was the closest relative of the isolates.

Clarridge (2004) reported that gene sequences that shared percentage similarity values of ≥ 95% and ≥ 99% could be used to classify the prokaryotes into the same genus and species respectively. Sequence analysis revealed that PED1 and PED2 shared 99% homology with *Achromobacter xylosoxidans* strain NBRC15126 and *Pseudomonas alkylphenolica* strain KL28 respectively. Thus, isolates PED1 and PED2 were identified as *A. xylosoxidans* strain PED1 and *P. alkylphenolica* strain PED2. The percentage homology value of the 16S rRNA gene sequence of strain PED3 (97%) with their respective closest relative confirmed that these isolates belong to the genus *Sphingomonas* but could belong to another species different from those that were on the database. Thus, PED3 was identified as *Sphingomonas* sp. strain PED3.

The nucleotide sequences of the 16S rRNA gene of the isolates were deposited in the GenBank database under the submission code "SUB3052529" with the accession numbers MF973060 (PED1), MF973061 (PED2) and MF973062 (PED3).

The increases in population density of the PED isolates in endosulfan-supplemented mineral salt medium further indicate the potential ability of the isolates to degrade endosulfan. There is a strong positive correlation between the increases in optical density values and the population density of microbial cells (Meyers *et al*., 2018; Karamba and Ahmad, 2019). However, studies have shown the possibility of the strains of the bacteria metabolize different persistent organic pollutants. A strain of *Achromobacter xylosoxidans* isolated from polluted soil exhibited considerable biodegradation rates for a polyaromatic hydrocarbon compound (anthracene) in the contaminated soil (Muhammad *et al*., 2024). *Pseudomonas* species, known for their ability to thrive in minimal media and utilize diverse substrates, are recognized as crucial candidates for bioremediation applications due to their environmental resilience and metabolic versatility (Hassaine and Bordjiba, 2015; He *et al*., 2018; Mullaeva *et al*., 2022).

The screening of potential endosulfan-degrading bacteria represents a crucial step in developing efficient bioremediation strategies. Through the use of different screening methods and taking relevant factors into account, researchers can identify and select promising candidates for bioremediation purposes, thus aiding in creating eco-friendly environments and reducing human health risks associated with exposure to endosulfan contamination. Further studies are required to establish if PED strains are suitable for bioremediation of environments contaminated with endosulfan.

CONCLUSION

In this study, three potential endosulfan-degrading (PED) bacterial strains isolated were *Achromobacter xylosoxidans* strain PED1, *Pseudomonas alkylphenolica* strain PED2, and *Sphingomonas* sp. strain PED3 with the accession number MF973060, MF973061 and MF973062 respectively. The increased population density of the isolates in an endosulfan-supplemented mineral salt medium suggested that the isolates may have the capability to utilize an endosulfan compound. Further study is required to evaluate the suitability of these strains for bioremediation purposes.

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Conflict of interest: The authors declare no conflict of interest.

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