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Isolation and Characterization of 2,4-Dichlorophenoxyacetic Acid Degrading Bacteria from Rice Cultivated Soil in Kura Local Government Area of Kano State. Nigeria

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Abstract
2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used pre-/post emergent systemic herbicide that controls broad-leaved weeds and other vegetation on rangelands purposely used for increasing agricultural yield, soil productivity, products quality, minimizing losses of agricultural products caused by crop pest. However, excessive use of this herbicide results in both soil and water pollution which is detrimental to humans, animals and the environment. Aboriginal bacteria normally occurring in low density in soil have been shown to degrade pesticides and other chemicals, such microbes have possibility for use in bioremediation of polluted environments. Isolation of 2,4-D degrading bacteria was accomplished through incubation experiments in mineral salt medium supplemented with 2,4-D. Aboriginal bacterial strain capable of using 2,4-dichlorophenoxyacetic acid as the sole carbon and energy source was isolated from Rice cultivated soil with a long-term history of herbicide use in Kura L.G.A, Kano State. The strain named K2-BUK-BCH was recognized as *Pseudomonas plecoglossicida based* on its 16S rRNA sequence analysis, morphological, and biochemical features. The degradation characteristics of strain K2-BUK-BCH were evaluated using one factor at a time approach (OFAT). The degradation conditions for 2,4-D were as follows: pH 8.0, 35°C, 200µgL^{-1}inoculum size, and 2,4-D concentration of 0.72gL^{-1}. The degradation potential of 2,4-D was determine using high-performance liquid chromatography (HPLC). Up to 96.68% of the 2,4-D was degraded under optimal conditions after 6 days of incubation. *Pseudomonas plecoglossicida* sp was reported for the first time as able to degrade 2,4-D in Kano State, Nigeria. The isolated strain represents a great potential for bioremediation.

Nomenclature and units

°C	Temperature
gL^{-1}	Concentration
Hrs	Time
nm	Optical density

1.0 Introduction

Population explosion and climate change requires improved and extensive agricultural activities in order to meet up with food requirements (Tripathi *et al*., 2019). This brought about the use of herbicides for control of weed and improved yield of crops all over the world. This continuous and excessive use of herbicides had resulted in severe consequences to the environment including pollution and destruction of certain beneficial plants. While many of them were banned but studies have shown that they are still in use especially in low developed countries (Rani *et al*., 2021). Most of the herbicides have half lives ranging from days to years and some up to decades. The herbicide or its metabolites can accumulate in the food chain leading to serious health problem to humans and other animals (Kaur *et al*., 2019).

2,4-dichlorophenoxy acetic acid (2,4-D) is one of the herbicides introduced since 1945 and extensively being used since then. It is extensively used largely because of its efficiency in controlling weeds and added advantage of mimicking auxin thereby improving plant growth (Bernat *et al.,* 2018). In fact more than 95% of virtually all herbicide formulations contain 2,4-D as one of their ingredients. The herbicide has been extensively used to control weeds in farmlands and residential areas. It has a half life ranging from few days to up to a year. Because of the comparatively high water solubility and low soil-absorption coefficient of the free acid of 2,4-D, it often contaminates the environment when it enters streams, rivers, or lakes and drainage of agricultural lands. 2,4-D has been detected in air, water, and even in the blood of children living close to agricultural fields (Islam et al., 2018). The World Health Organization (WHO) has classified 2,4-D as a hormonal herbicide with level II toxicity. The herbicide is carcinogenic, hepatogenic and can induce cell death through apoptosis. The carcinogenicity is mainly mediated through the generation of free radicals when bound to membrane phospholipids. It also affects the central nervous system, leading to convulsions (Bernat *et al*., 2018). To help clean the environment for better human and animal health and also to maintain ecological balance, various methods were employed to reduce the negative impact of herbicides in the environment (Thakkar & Vincent, 2021). However, the use of microorganisms appeared to be one of the cheapest, effective and with less dangerous by products. This makes the search for indigenous bacteria and other microorganisms a viable option in the quest to clean the environment. Bacteria that has the potential of degrading 2,4-D have been reported to belong to a number of genera, including *Achromobacter, Alcaligenes, Arthrobacter, corynebacterium, Flavobacterium, Pseudomonas and Streptomyces* (Carboneras *et al*., 2017). Kura local government, Kano state, Nigeria is extensively known for rice cultivation throughout the season because of its easy accessibility to water irrigation system. The soil in this area had extensive contact with the herbicide 2,4-D for over three decades, therefore, the possibility of isolating a bacteria capable of degrading this compound with high efficiency is very high. Therefore, the aim of this study is to isolate and characterized bacteria capable of degrading 2,4-dichlororphenoxyacetic acid from three different rice cultivated soils in Kano State, Nigeria.

2.0 Materials and Methods

Materials: the materials used in this research are (brand of HPLC used), PCR machine brand, gel scanner brand, DNA isolation kit, Electron microscope, Spectrophotometer.

Reagents: polymerase used, agarose, 2, 4-D, Mineral Salt Medium, Nutrient Agar, LB Broth, Methylated spirit, $1.74K_2HPO_4$, $0.68KH_2PO_4$, 0.1 $MgSO_47H_2O$, 4NaCL, $0.03FeSO_4.7H2O$, $1.0NH_4NO_3$, 0.02 $CaCL.2H_2O$. All the reagents listed are of good purity.

2.1 Study site

Soils used for isolation and screening of 2,4-D-resistance bacteria were collected from Rice cultivated area in Kura L.G.A (11º46’17’’N latitude, and 8 º25’49”E longitude), Kano State. These soils has a long term exposure to 2,4-D.

2.2 Methods

Isolation of 2,4-D Degrading Bacteria

Five gram of the soil was inoculated into (250 mL) Erlenmeyer flask containing 50 mL mineral salt medium. The media was autoclave at 121ºC for 15mins prior to the addition of

sterilized 2,4-D. Iron sulfate was added to the medium to avoid formation of precipitates after autoclaving).Which was supplemented with 0.72 gL^{-1} 2,4-Das the sole energy and carbon source. The flasks were incubated at 30 °C for 96 h in a rotary shaker at 120rpm. Then, 5mL of the culture media showing degradation of 2,4-D was transferred to 50mL flask containing fresh MSM supplemented with 0.72gL^{-1} 2,4-D and further incubated for 96 h. Subsequent rounds of enrichment were done and the final enrichment cultures were serially diluted and spread on cultured MSM plates containing 0.72 gL^{-1} 2,4-D. After incubation at 30 °C for 4 days, colonies showing different morphologies were selected for further analysis of their degradation potentials. The extent of degradation for the selected strain was measured by quantifying the amount of 2,4-D remaining in the culture by high-performance liquid chromatography (HPLC). One bacterial strain isolated from Kura rice farm, coded as K2-BUK-BCH, shows high optical density indicating high utilization of 2,4-D as energy and carbon source. This strain was used for further analysis. Isolate was stored at 4˚C refrigerator prior to the analysis.

2.3 Identification and Characterization of Strain

Isolate K2-BUK-CH was identified on the basis of its 16S rRNA gene sequence analysis and biochemically. Biochemical and morphological analysis were performed according to Manual of Systematic Bacteriology . Samples were set up for scanning electron microscopy. Genomic DNA was extracted using bacterial DNA Kit. The 16SrRNA genes were amplified using the universal primers F:5-'TGGAGAGTTTGATCCTGGCT CAG-3′ and R:5′-TACCGCGGCTGCTGGCAC-3′ microbial DNA extraction was done at the Instrumental laboratory department of Biochemistry Bayero University Kano (BUK), Kano. The DNA was isolated using Prepease DNA isolation kit according to the manufacturer's instruction. Identification of the sequence was done by Blasting in the NCBI database to find the neighboring strains with valid published prokaryotic names. Different 16S rRNA gene sequences from GenBank were aligned using CLUSTALW version 2.0 (Larkin *et al.*, 2007). A phylogenetic tree was design using MEGA6.0 software (Tamura *et al.*, 2011). A bootstrap analysis based on 1000 replicates was used to place on the tree.

2.4 Characterization of 2,4-D-Degradation Bacteria

Isolate K2-BUK-BCH was cultured in Luria-Broth (LB) medium fortified with 0.72 gL^{-1} 2,4-D. The cells were centrifuge and harvested at 6,000 g for 5 min, and then washed with sterilized MSM. Flasks were placed in a rotary shaker at 120 rpm. For controls, un-inoculated media were used and tested in the same manner above. Culture samples were pulled out at 76-h to measure cell growth at an optical density of 600nm. the effects of initial 2,4-D concentration was examined at different concentrations (0.36gL^{-1}, 0.72 gL^{-1}, and 1.44 gL^{-1}), culture temperature 25 - 45 °C at 5°C interval, inoculum size 100 - 500µgL^{-1} at 100µgL^{-1}interval, medium pH 5.5 - 8.5 with 0.5 interval, and incubation time (interval from 24-120h) on the growth and 2,4-D degradation. All experiments were conducted in triplicates. Cell growth was measured by optical density measurement of the sample at 600nm using spectrophotometer. The degradation rate was access by quantifying the amount of 2,4-D remaining in the culture medium by HPLC. A standard curve of 2,4-D was used to calculate the percentage of degradation.

2.5 Statistical Analysis:

Graphs and tables were used for data presentation. Characterization of bacterial isolate at different time periods were represented using bar charts, graphs and soil physicochemical was testedusing one way anova with replications ($p < 0.05$). The statistical package used was genstat.

3.0 Results

3.1 Screening and Isolation of 2,4-D-Degrading Bacteria

Three (3) different bacteria were isolated by selecting colonies with differing features. All the isolates were able to use 2,4-D as

the sole energy and carbon source. Out of the 3 isolates, 1 shows highest resistance to 2,4-D (1.44 g/L) during 3 days of incubation at 30°C. Sequence analyses showed that the genomes of these strains contained the conserved sequence of a class I *tfdA* gene (Han *et al.*, 2014). K2-BUK-BCH strain showed greater resistance and can degrade 2,4-D best among these 2,4-D-degrading isolates. Therefore, K2-BUK-BCH was selected for further analysis.

3.2 Identification of Strain K2-BUK-BCH

Morphological appearance of the bacterial cell and biochemical tests confirms, K2-BUK-BCH is a motile, has rod-shaped Gram-negative bacterium with oxidase and catalase activities. The isolate showed no reaction to tests for glucose and citrate and indole utilization. The 16S rRNA analysis of the isolate K2-BUK-BCH was clustered with bacterial 16S rRNA sequences deposited in the GenBank.The 16S rRNA gene sequence of strain K2-BUK-BCH showed similarity of 100% to that of *Pseudomonas plecoglossicida* (Fig. 1).*P. plecoglossicida*is a new species assigned in 2011. An herbicide resistance type strain, was isolated from herbicide exposed Soil in Jordan Valley (Khalil, 2003).

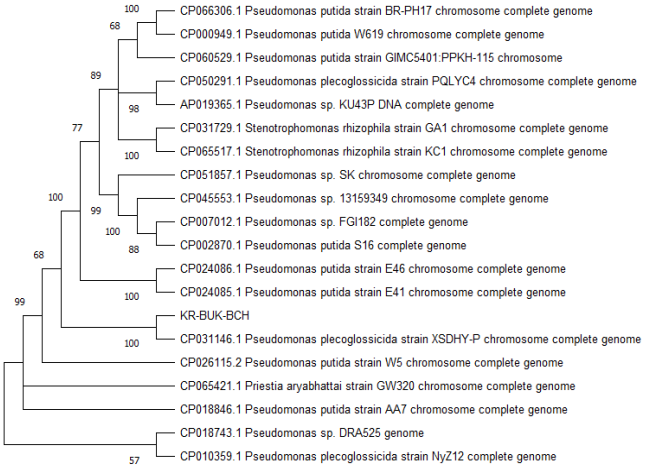


Figure 1. Phylogenetic Analysis of K2-BUK-BCH and other related strains.

3.3 Effect of Environmental Factors on the Growth of 2,4-D Degrading Bacteria

Strain K2-BUK-BCH degrade 2,4-D rapidly at low substrate level ($0.35gL^{-1}$), and indicate nearly absolute degradation of ($0.72gL^{-1}$) 2,4-D in 3 days (Table 1). At $0.72gL^{-1}$ 2,4-D concentration, K2-BUK-BCH degrade up to 96.68% 2,4-D after 7 days of incubation. The effects of other factors in the degradation of 2,4-D by K2-BUK-BCH was also assessed. Out of six pH ranges, pH 7.5 was found to be stable for 2,4-D degradation by K2-BUK-BCH (Figure 2).Temperature also present a key factor influencing degradation. Degradation was around 96.68% at the temperature of (35 °C) by K2-BUK-BCH (Figure 3), the optimum inoculum size was found to be $200\mu gL^{-1}$ (Figure 4). The effect of incubation time was also determined between the time intervals 24-120h and maximum growth was achieved at 96h after which a decline in the growth was experienced (Fig. 5).

$$(\%Degradation\ of\ KR-BUK-BCH) = \frac{0.72-0.222}{0.72} \times 100\% = 96.68\%$$

Table 1: Effect of substrate concentration on the growth of 2,4-D resistance bacteria

2,4-D Concentration	OD (600nm)
$0.35gL^{-1}$	0.205^{c}
$0.72\ gL^{-1}$	0.247^{a}
$1.44\ gL^{-1}$	0.206^{b}

a,b,c indicate a significant difference in the growth rate at three different concentrations with ($0.72\ gL^{-1}$) having the highest

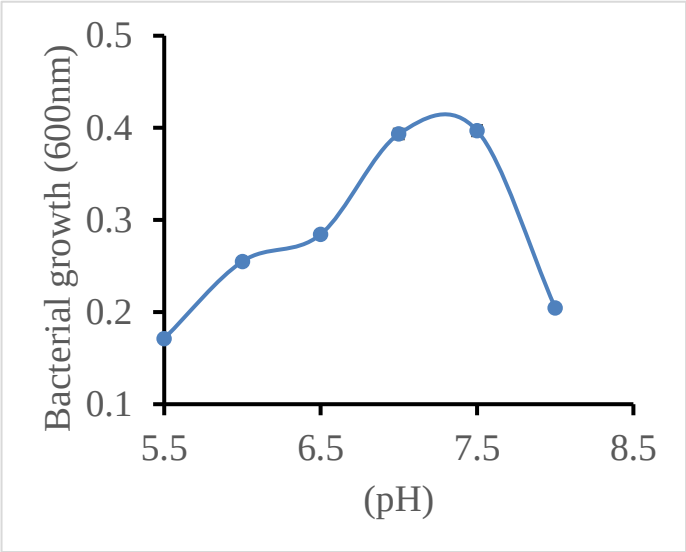


Figure2. Effect of pH on the growth of 2,4-D degrading bacteria

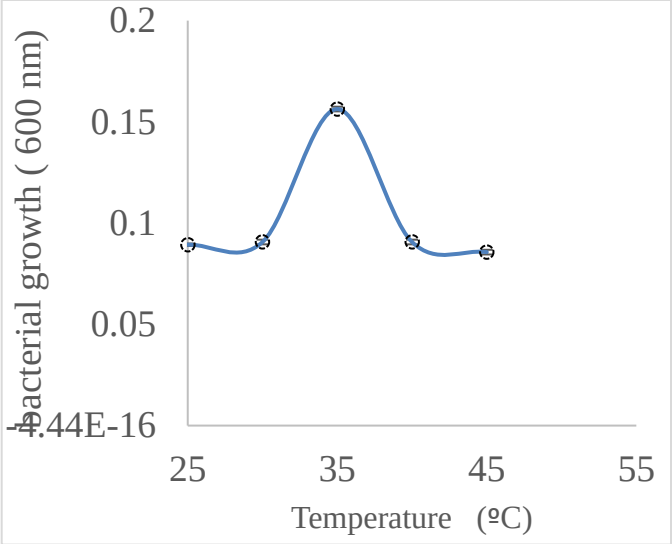


Figure 3.Effect of Temperature on the Growth of 2,4-D Degrading Bacteria

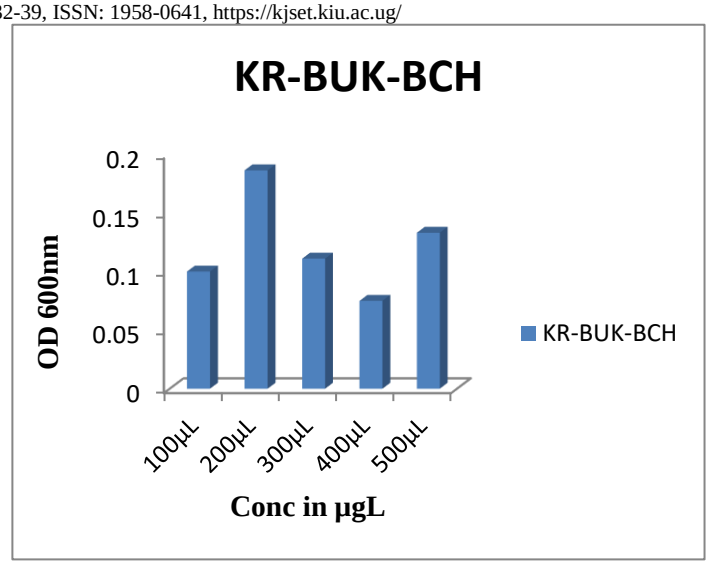


Figure 4. Effect of Inoculum Size on the Growth of 2,4-D Degrading bacteria

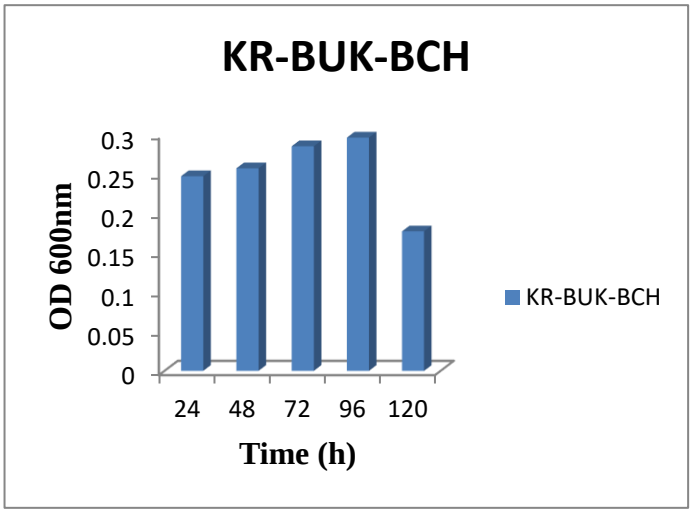
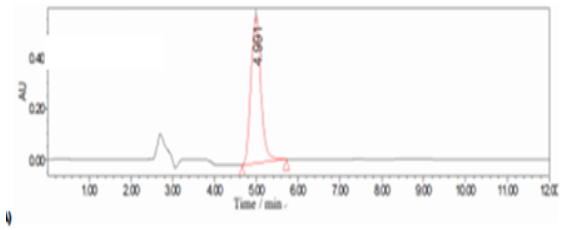
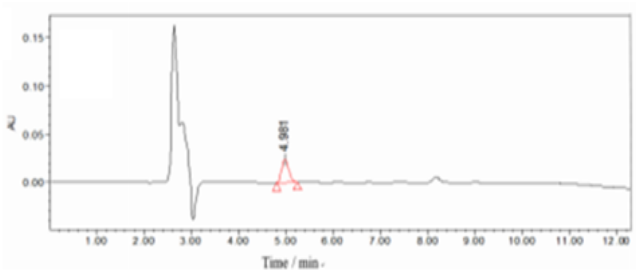


Figure 5. Effect of Incubation Time on the Growth of 2,4-d Resistance Bacteria



A



(D)

Figure 6. HPLC chromatogram for 2,4-D degradation
(Standard) HPLC Chromotogram
B (Chromotogram of sample)

3.4 Percentage Degradation: The percentage degradation was calculated using the formula

$$(\%degradation) = \frac{initialconc - finalconc}{initialconc} \times 100\%$$

$$(\%Degradation\ of\ KR - BUK - BCH) = \frac{0.72-0.222}{0.72} \times 100\% = 96.68\%$$

4.0 Discussions

To advance crop yields and avoid the menace of economic loss brought about by weeds, it becomes necessary to make use of herbicides in the soil, e.g., 2,4-D, nevertheless, improper storage practices and utilization can cause 2,4-D being spread all over the environment. Succeeding the dispersal, their excess can build up in aboriginal plant and aquatic organisms, where they can seriously affect their metabolism. Even though they are delivered into the environment encompassing phenoxy acids salts or esters, these products instantly oxidizes to their analogous anionic when they get to the environment (Bernat *et al*., 2018). 2,4-D residues can be consume by soil particles and migrated to terrestrial and water habitats (surface and groundwater) through runoff and in the soil profile (Angın & Güneş, 2021). Consequently, there is a need to recognize methods that enable immediate removal of these pollutants from the environment; of these, nature-based approaches proposes the great solution, as these are economical and less likely to be harmful to the ecosystem.In this study, An aboriginal 2,4-D utilizing bacteria were isolated from a contaminated Rice cultivation area using mineral salt medium supplemented with 2,4-D in Kura L.G.A, Kano State, Nigeria. The isolate was identified as *P. Plecoglossicida* following its 16S rRNA gene analysis and its biochemical features. In the recent studies, many varieties of 2,4-D-utilizing bacteria were isolated from 2,4-D-contaminated sites (Serbent *et al*., 2019; Zhang *et al*., 2020). Microbes that have been reported to be able to utilize 2,4-D belong to a number of genera, such as *Alcaligenes,Achromobacter,corynebacterium,Flavobacterium,Arthrobacter,Streptomyces* and *Pseudomonas* (Gangola *et al*., 2022; Magnoli *et al*., 2020). The degradation potential of strain K2-BUK-BCH was higher than those reported for *Sphingomonasagrestis* strain 58-1 and also three isolates; *Burkholderiacepacia*DS-1, *Pseudomonas* sp. DS-2, and *Sphingomonaspaucimobilis*DS-3 (Cycoń *et al*., 2010; Shimojo *et al*., 2009).

The effect of temperature on growth of bacteria was access in the MSM medium containing 2,4-D as the sole source carbon between the temperature ranges 25 - 45°C, The optimal temperature for the growth of K2-BUK-BCH was 35°C, (Figure 3). Physicochemical properties such as humidity, temperature, and moisture content affect the rate of degradation of herbicides in soil, degradation of chemical pesticides rises with increase in temperature between 10 to 45ºC (Zhang *et al*., 2020).

pH is another important parameter to be considered in bioremediation because it affect the microbial activity. Strain K2-BUK-BCH isolates shows greater activity at the pH range of 7-8 with maximum at 8 as shown in (Figure 2). The soil from the farm is acidic with pH of (6.14 ± 0.43) which is not suitable for the isolate obtained in this current study. The acidity of the soil could be attributed to the heavily use of chemical fertilizer in such farms (Zhang *et al*., 2020).

The inoculum size was also another important parameter for the growth of 2,4-D resistant bacteria. The growth pattern of the 2,4-D resistant bacteria culture was monitored at different inoculum concentrations ranging from 100 - 500µgL^{-1}. Over the period of time the turbidity of the medium for isolate K2-BUK-BCH was higher at 200µgL^{-1} compared to the rest of the concentrations (Figure 4). *Pseudomonas plecoglossicida* might be a suitable candidate for bioremediation. Because it is an aboriginal

organism, it is more likely to survive than any other foreign bacteria when introduced into contaminated soil and water environments in Kura Local Government, Kano State, Nigeria..

5.0 Conclusions

Isolation of 2,4-dichlorophenoxy acetic acid degrading bacteria was achieved through incubation with MSM media a total of three bacterial strains with ability to utilize 2,4-D as sole source of carbon and energy out of which one isolate shows higher resistance to 2,4-D exposure and were selected and identified both morphologically and molecularly as *Pseudomonas Pleccoglossicida* and characterization of the isolate was done using one factor at a time approach (OFAT) to find the best conditions at which the degradation was carried out. Soil from the farm was found to be acidic. There were high residues of 2,4-D discovered from the soil. This hike interest on the appropriate amount of 2,4-D that should be use to control weed. Also, the high levels of residues discovered from the study causes threat to the human and other organism due to the herbicide adverse effects on environment. This result therefore, raises interest on the appropriate field rate at which the 2,4-D should be use as well as its effects. Even though the use of herbicides is paramount as it offers less expensive and best way of weed control, their application should be considered due to the detrimental effects they cause to the untargeted organisms. These finding are good discrimination for the degradation activity that there are such biodegrades in Kura rice cultivation farm.

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Declaration of conflict of interest

The authors declare that there is no conflict of interest

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