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Assessment of Phosphorus Mobilizing Capacity of *Bacillus* spp. Isolated from Mangrove Rhizospheric Sediment and its Potential Application in Aquaculture

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ABSTRACT

Background: Phosphatase producing bacteria (PPB) plays a major role in mineralising organic P into inorganic form. Though application of bacterial biofertilizers are practiced in agriculture, their application is very limited in aquaculture.

Methods: The PPB isolates were screened and isolated from rhizospheric sediment of mangrove, *Avicennia marina* of Ennore creek, Tamil Nadu. Their P transformation potential and the possibilities of their application in aquaculture to mineralise organic P to inorganic P were studied.

Result: Twenty PPB isolates were screened in the study site and their Alkaline Phosphatase (ALP) activity was in the range of 13.6-20.4 µmol.ml^{-1}.hr^{-1}. Out of this, five *Bacillus* sp isolates were selected to assess their P transformation potential at various salinity. The P mobilizing potential of these isolates were compared with the commercial PPB following a microcosm study for a period of 14 days. During the study period, there is a significant increase in phosphorus in water as well as ALP activity and available phosphorus concentrations in sediments were observed between control and treatment tanks. Among the treatment groups, *B. subtilis* treatment tanks showed maximum P in water *i.e*. 1.60 mg.L^{-1}, followed by *B. altitudinis* -1.49 mg.L^{-1}; *B. pumilus* -1.47 mg.L^{-1}; the soil ALP activity was in the order of commercial P products > *B. pumilus* > *B. subtilis* > *B. paramycoides* > *B. altitudinis* > *B. aryabhattai*. In terms of available phosphorus content in sediments on 14th day, there is no significant difference observed between *B. pumilus* and commercial product with respect to available P content of sediment.

Key words: Alkaline phosphate activity, *Bacillus* sp., Mangroves, Phosphatase producing bacteria.

INTRODUCTION

Phosphorus is one of the major nutrients that limit the productivity in aquatic ecosystems. Microbial activity plays a major role in mobilization of Phosphorus (P) and brings about a number of transformations of soil P, which include: mineralizing organic compounds with release of inorganic phosphate and increasing the solubility of inorganic compounds of P (Alexander, 1983; Swain *et al*., 2012). Phosphatase producing bacteria (PPB) are the heterotrophic bacteria that produce extracellular enzymes like alkaline phosphatase that can mineralize organic phosphates into inorganic form, which is then available to plants (George *et al*., 2002) or exchanged into water as soluble P. Alkaline phosphatase is a hydrolyse enzyme that is responsible for removing P from 5' and 3' phosphate groups from many types of molecules including DNA, RNA, proteins *etc*. and produce inorganic phosphate (Sitdhipol *et al*., 2012). Several studies reports that bacterial species of *Bacillus, Pseudomonas, Vibrio, Azobacter, Aereobacter, Rhizobium, Agrobacterium, Enterococcus, Micrococcus, Achromobacter, Flavobacterium, Paenibacillus* exhibit phosphatase activity (Behera *et al*., 2017; Olenska *et al*., 2020; Audipudi *et al*., 2012; Kathiresan and Selvam 2006; Sreevidya and Gopalakrishnan 2017; Chandran *et al*., 2021). There are many studies carried out to screen PPB from a variety of ecosystem ranging from forests to agricultural lands in search of P mineralising and solubilising microbes for their application as biofertilizers in agriculture (Kalayu 2019; Aruna and Jain 2021; Alori *et al*., 2017). Recent researches are focusing on the screening of salt tolerant microbes in warrant of their application in salt affected land to increase the soil fertility (Grover *et al*., 2011; Zhang *et al*., 2018; Pooja and Rajesh 2015; Egamberdieva *et al*., 2019). Very few studies have been carried out in aquatic ecosystems that to in mangrove areas. As mangroves are the transitional ecotone, they harbour halophilic and saline tolerant bacteria which could be used as biofertilizer in salt affected inland agriculture lands or low saline or coastal aquaculture (Arora *et al*., 2021; Sahay *et al*., 2018; Egamberdieva *et al*., 2019). The application of salinity-tolerant PPB as

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biofertilizer will be an effective and economical means of improving the P availability or soil fertility.

Biofertilizer technology has shown promising applications in integrated nutrient management in the agriculture sector. Their relative application in aquaculture is very limited particularly in inland saline areas. Addition of extraneous phosphorus fertilizers in aquaculture ponds is still in practice to increase the primary productivity, in spite of background phosphorus level due to its non-availability. Application of native bacterial microbiota as biofertilizer would be helpful to boost the soil fertility as well as the productivity of water. Thus, the present study was carried out to screen the potential PPB isolates from mangrove rhizospheric sediments and to assess their nutrient transformation potential and the possibilities of their application in aquaculture to mineralise organic P to inorganic P.

MATERIALS AND METHODS

Isolation of phosphatase producing bacteria

The rhizospheric sediments of *Avicennia marina* from Ennore region were collected aseptically by carefully removing the soil adhering to 2-3 mm thickness around the individual roots. Serially dilutes of soil samples were plated on Phenolphthalein phosphate agar (Himedia) for selective screening of phosphatase enzyme producing bacteria. (Abhijith *et al*., 2017). Upon exposure to ammonia vapour, if the colonies turn into pink as a result of increase in pH, they were picked up and pure colonies were obtained by sub-cultured by streak plating method. The study was carried out Dr MGR Fisheries college and Research Institute, TNJFU, Ponneri for the period of 2018-2022.

Genomic DNA isolation, 16S rDNA sequencing, RAPD amplification of 16S rDNA

The genomic DNA was extracted from the bacterial isolates (using QIAamp genomic DNA kits (Qiagen, Germany) as per manufacturer's protocol. PCR amplification of DNA using the universal 16S rDNA primer of Weisburg *et al*. (1991). Nucleotide sequencing (forward and reverse) was done by commercial sequencing services (Eurofins, India). The edited gene sequence was compared against the GenBank database of the National Center for Biotechnology Information (NCBI) by using the BLAST (Basic Local Alignment Search Tool) program (http://blast. ncbi .nlm.nih.gov) and submitted to GenBank. Partial 16S rRNA gene sequences of *Bacillus* isolates have been archived in GenBank and the accession number is given in Table 1.

Alkaline phosphatase assay

The bacterial cultures grown in broth (48h) were used for the assessment. The Alkaline phosphatase activity (mineralization) of isolated strains was measured by using para-nitrophenyl phosphate (*p*-NPP) as organic P substrate. When *p*-NPP loses its phosphate group by the phosphatase activity of bacterial isolates, it becomes para-nitrophenol (*p*-NP), turning the solution yellow (Behera *et al*., 2017).

Assessing the effect of salinity on alkaline phosphatase production

Five isolates of spore forming beneficial *Bacillus* sp (*B. aryabhattai* -E/S2/19/03; *B. pumilus* - E/S3/19/03; *B. subtilis* - E/S4/19/02; *B. altitudinis* - E/S1/20/04; *B. paramycoides*- E/S2/19/05) were selected for the study Bacterial cultures grown in marine broth at a concentrated of 10^8CFU.ml^{-1} were inoculated and maintained under different salinity conditions 0,15,25,35 ppt. The nutrient broth was supplemented with marine salt to produce required salinity. ALP assay was performed after 48 h of incubation.

Microcosm study to analyse the potential of isolates to mobilize phosphorus from aquaculture sediment to water

The study was carried out in a set up with fourteen glass tanks (2×1×1 ft). In each tank, the aquaculture pond sediment was placed to 5cm thickness and 25L of seawater. The physicochemical parameters of sediment such as soil texture, pH, organic carbon, available-P and total P were studied. The five selected *Bacillus* strains isolated in this study and a commercial PPB (FOZBACT, comprised of *B. megaterium* from Ashwathy Green Enterprises Pvt. Ltd) were cultured in 25 ml of marine broth for 3 days with final concentration of 10^8CFU.ml^{-1}. After centrifugation and washing, the pellet of 5 ml of the bacterial cultures were inoculated into the microcosm set up. The initial water quality parameters in the microcosm study were pH-8.2; Alkalinity-160 mg.L^{-1}; salinity-35 ppt; ammonia-0.0365 mg.L^{-1} and phosphorus-0.31 mg.L^{-1}. The initial sediment contains 33.16-36.08 ppm of available-P, 581.32-596.32 ppm of total phosphorus and 0.4% of organic carbon; 69%, 12%, 19% of sand, silt and clay respectively. The study was conducted in duplicates. The tanks without bacterial inoculation were maintained as controls. The available phosphorous, organic carbon and soil texture were analysed before the start of the experiment. Both water and soil samples were collected from the microcosm set up on day 0,2,4,6,8,10,12 and 14 and analysed the phosphorus concentration in water, available phosphorus content and ALP activity of sediment samples. The PPB counts were also recorded on initial and

Design of the ALP gene primers

Genes	Primers name	Sequence (5′-3′)	Size (bp)	Reference
phoD	ALPS-F730	CAGTGGGACGACCACGAGGT	370	Fraser *et al*., 2017
	ALPS-R1101	GAGGCCGATCGGCATGTCG		

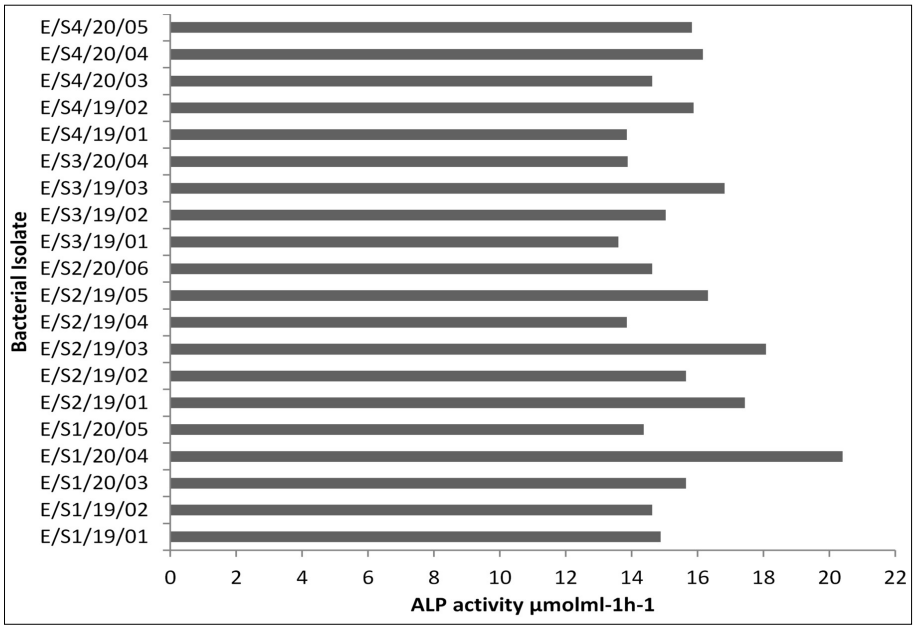

Fig 1: Alkaline phosphatase activity of 20 nos. of isolated bacterial strains.

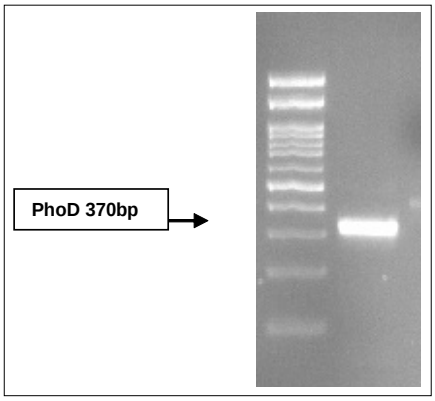

Fig 2: PCR amplification of ALP gene bacterial isolates.

Table 1: Partial 16S rRNA gene sequences of *Bacillus* isolates and their accession number.

Bacterial species	Isolate code	Accession no.
Bacillus subtilis	E/S4/19/02	OP296256
Bacillus altitudinis	E/S1/20/04	OP296257
Bacillus paramycoides	E/S2/19/05	ON567106
Bacillus pumilus	E/S3/19/03	ON567107
Bacillus aryabhattai	E/S2/19/03	ON567093

final days in order to ascertain the survival of inoculated isolates in the set up.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, Ilinois, USA). The one way analysis of variance test was used to evaluate significant differences all mean separations were carried out by Duncan multiple range tests to a significance level of 0.05.

RESULTS AND DISCUSSION

Isolation and identification of phosphatase producing bacteria

PPB isolated in this study was identified as *Pseudomonas* sp. (8 nos.), *Bacillus* sp.(6 nos.), *Brevundimonas* sp.(3 nos.), *Achromobacter* sp.(2 nos.) and *Arthrobacter* sp. (1 no.). The ALP activity observed in the PPB isolates obtained in this study is shown in Fig 1. The ALP activities of PPB isolates were in the range of 13.6±0.021 -20.4±0.015 µmolml^{-1}h-^{1}. The bacterial isolate E/S1/20/04 (*B. altitudinis)* exhibited the maximum activity of 20.4±0.015 µmolml^{-1}h-^{1}. The isolates that showed ALP activity more than 16 µmolml^{-1}h^{-1} were E/S2/19/03 (*B. aryabattai*); E/S2/19/01 (*B. anthracis*); E/S3/19/03 (*B. pumilus*); E/S2/19/05 (*B. paramycoides*); E/S4/20/04 (*Arthrobacter* sp.) and E/S4/19/02 (*B. subtilis*).

Ravikumar *et al*. (2007) reported that *Pseudomonas*, *Vibrio* and *Bacillus* are the three major groups of bacteria present in the mangrove sediments are involved in the production of extra-cellular phosphatase enzymes. Barik *et al*. (2001) reported that the ALP activity assay of *Bacillus* sp and *Pseudomonas* sp isolated from freshwater aquaculture pond were 12.013 mg of p-NPmL^{-1}1h^{-1} and 11.388 mg of p-NPmL^{-1}1h^{-1} respectively. Abhijith *et al*. (2017) reported that *B. cereus* strain APT23 and *B. thuringiensis* strain isolated from mangrove region exhibited 10.04±4.04 µg p-NP released g^{-1} h^{-1} per 1×10^{8} cfu in 72h.

ALP activity at varying salinity

Fig 2 shows the presence of target PhoD gene in PCR amplification. ALP activity observed in the isolates at varying salinity (0, 15, 25 and 35 ppt) is shown in Fig 3. All the isolates had the ability to grow and exhibited ALP activity in salinity ranges from 0 to 35 ppt. At 0ppt salinity, the isolates exhibited poor growth rate and low ALP

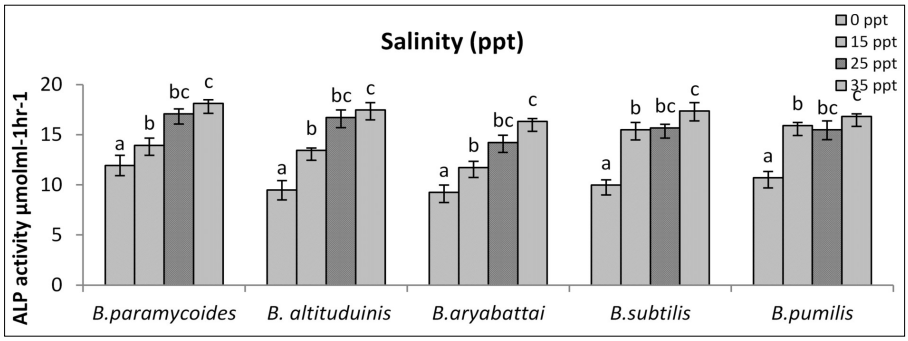

Fig 3: ALP activity of *Bacillus* isolates at varying salinity.

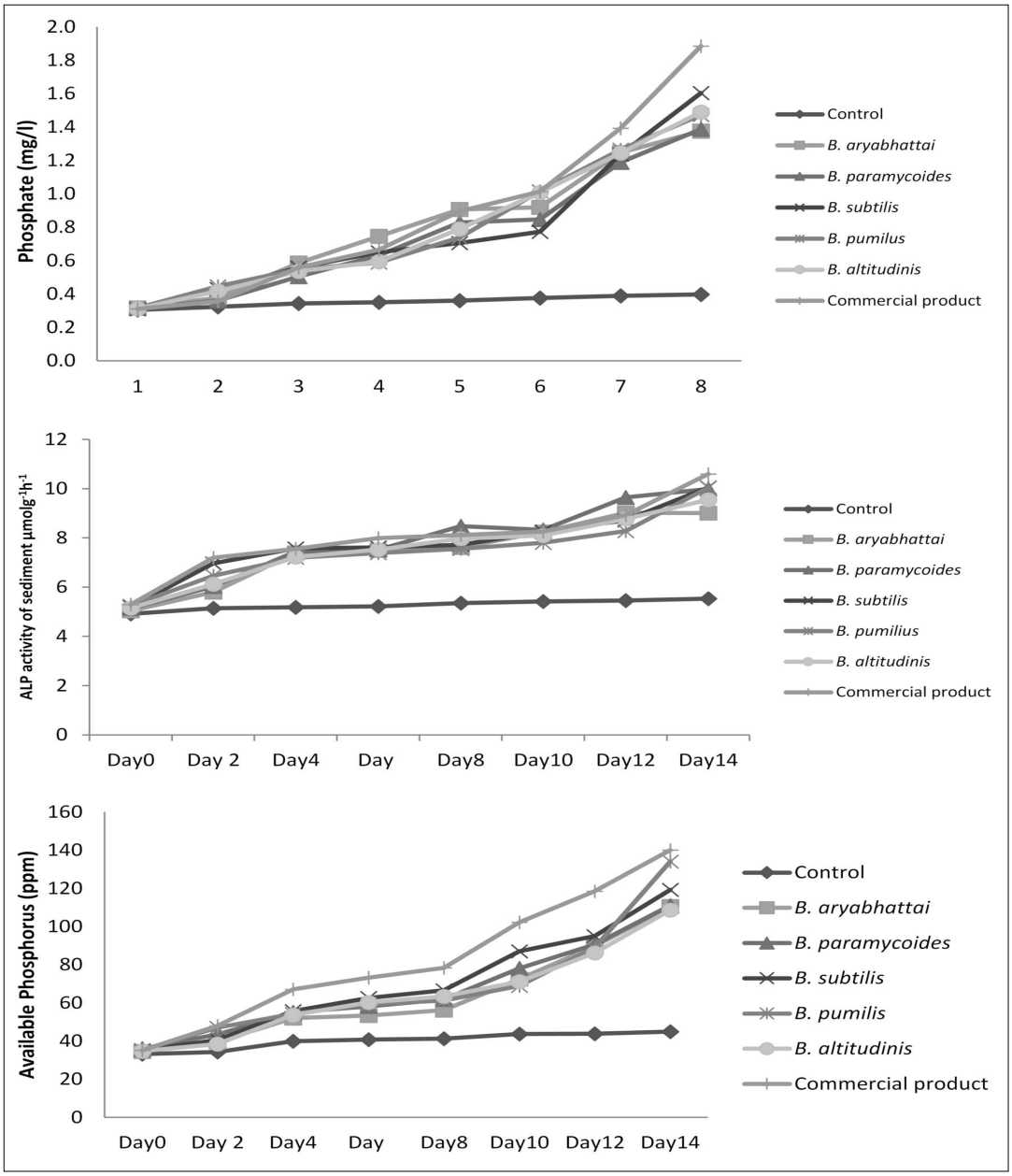

Fig 4: Microcosm study-Phosphate in Water, ALP activity and Available-P in Sediment.

activity. All the five isolates showed significantly higher ALP activity at 35 ppt. The present study clearly revealed that salinity has a great impact on the growth as well as ALP activity of the isolates. Though these isolates showed maximum activity at 35 ppt, they showed activity at zero saline condition too. Thus, these isolates could also be used in brackish water aquaculture and inland saline aquaculture.

Microcosm study to analyse potential of isolates to mobilize phosphorus from aquaculture sediment to water

Phosphate level in water and ALP activity and available-P in the sediment during the microcosm experiment is shown in Fig 4. The control tank showed stable available P, ALP activity in sediment and orthophosphate in water from 0 to 14. All the tanks inoculated with five isolates collected in the study and commercial PPB product. A gradual increase in ALP activity and available-P in sediment and orthophosphate in water were observed up to 10 days and the variation in the activity could be observed from day 10 -14. During the study period, there is a significant increase in phosphorus in water as well as ALP activity and available-P concentration in sediments was observed between control and treatment tanks. In control tank, the phosphorus content in water was maintained in the range of 3.05-3.97mg/l whereas, in commercial P bacterial product inoculated tanks, P concentration was steadily increased and observed as 1.88 mg/l at 14th day. Among the treatment groups, *B. subtilis* treatment tanks showed maximum P in water ie. 1.60 mg.L^{-1} followed by *B. altitudinis* -1.49 mg.L^{-1} and *B. pumilus*-1.47 mg.L^{-1}. The soil ALP activity (µmol g^{-1}h^{-1}) was in the order of commercial P products (10.59) > *B. pumilus* (10.05) e" *B. subtilis* (10.04) > *B. paramycoides* (9.99) > *B. altitudinis* (9.59) > *B. aryabhattai* (9.01). In terms of available-P content in sediments on 14th day, maximum was recorded in tanks with commercial P products (140.06 ppm) followed by *B. pumilus* (134.0 ppm). With regard to available-P, there is no significant ($p<0.05$) difference observed between *B. pumilus* and commercial product. *B. pumilus* had significantly ($p<0.05$) increased available-P content from 35.44 ppm on 0th day to 134.05 ppm on 14th day. With respect to Soil ALP activity and Water - P, except control, all treatments showed on par with commercial product.

The survival of inoculated bacterial isolates in treatment tanks was assessed by counting PPB in water and sediment samples at the beginning and end of the study. The initial and final PP count during the study period were in the range of 11-21 cfu/ml and 410-483 cfu/ml in water samples and 7-13 cfu.g^{-1} and 2-4×10^{4} cfu.g^{-1} in soil samples.

Soil organic matter (SOM) content and clay content positively influenced the ALP activity. The exchange of P between sediment and water is complex phenomenon influenced by bacterial activity due to the abundance of PPB, out of total microbial mass of soils (Alongi, 1988). The bacterial ALP activity of sediments varied based on the physicochemical properties of soil, organic matter content, C/N ratio and total P content (Djordjevic *et al*., 2003). The increase in ALP activity in sediment samples of all treatment tanks with the PPB inoculum indicated that the inoculated bacterial isolates hydrolyze the organic phosphorus by producing extracellular phosphatases in the sediment which has remitted in the increase in the available P content in sediment and phosphorus in water. Similar experiment with the isolates from various sources has been carried out to demonstrate the mineralising capacity in agriculture soil (Mader *et al*., 2011; Piromyou *et al*., 2011; Nassal *et al*., 2018). Thus, the application of PPB as biofertilizer in aquaculture pond will aid in hydrolysis of organic P as well as drastically reduce the usage of P based chemical fertilizers.

Soluble phosphate of above 0.20mg/l may be indicative of medium to high and highly productive fish ponds (Adhikari *et al*., 2017). Based on available-P, soil could be classified as low (<30ppm), medium (30-60ppm) and high (>60ppm). The phosphate level in water of microcosm study was observed to be above the recommended optimal value. But the increase in available P content of sediment was in the range of high productivity category. In the present study, water-P, ALP activity and available-P of sediment increased up to 2.46-3.75 times, 0.63-0.92 times and 1.41-2.11 times respectively.

CONCLUSION

This study describes the isolation, characterization and identification of PPB bacteria from rhizospheric sediments of *A.marina* from the Ennore region. As the study region belongs brackishwater category, the saline tolerate bacteria were isolated which can able to work at salinity conditions. Further, the spore forming *Bacillus* sp are considered as indicator for successful and wide range of applications in agriculture and aquaculture. Knowledge on mineralizing potential of the native bacterial isolates with respect to various environmental parameters will be helpful in the development of management activities utilizing these isolates as biofertilizer in aquaculture.

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Conflict of interest: None.

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