Original Article

Detection of Aminoglycoside-Modifying Enzymes Genes in *Staphylococcus aureus* Resistant to Aminoglycoside Antibiotics in Burn and Wound Patients

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Abstract

Background: One of the main sources of infection in hospitals and the general population is *Staphylococcus aureus.* For the treatment of severe staphylococcal infections, aminoglycosides are frequently utilized. The primary mode of this antibiotic resistance is the production of aminoglycoside-modifying enzymes (AMEs). **Objectives:** This study's objectives were to identify the aminoglycoside-resistant *S. aureus* strains from burns samples and molecular detection of AMEs genes among these stains. **Materials and Methods:** In this investigation, 230 burn wounds yielded 63 different *S. aureus* strains. Gentamicin (10 mg), amikacin (10 mg), kanamycin (30 mg), tobramycin (10 mg), streptomycin (25 mg), and neomycin (30 mg) are aminoglycoside antibiotics used in this study to investigate their activity against bacterial isolates, the *aac (6´) Ie/aph (2´), aph (3´)-IIIa1, ant (4´)-Ia1, and mecA* genes were identified using the PCR technique. **Results:** Of 63 clinical *S. aureus* isolates verified by phenotypic testing, 50 isolates resistant to aminoglycosides in primary screening showed that the highest levels of antibiotic resistance were seen in Kanamycin, which involved 40 isolates (63,4%), while the lowest resistance was found in amikacin and gentamicin, also 12 isolates (24%) confined mecA gene. Furthermore, 32 isolates (64 %) had gene *aph*(3)-IIIa and six isolates (12 %) had *aac (6´)Ie/aph (2˝)* gene. While the presence of the *ant (4´)-Ia1* gene wasn't observed in any of the samples. **Conclusion:** The gene *aph(3´)-IIIa1* was the highest and responsible for the mechanism resistance to aminoglycoside in *S. aureus.* Multiple drug-resistant *S. aureus* strains were found to arise in settings where aminoglycoside antibiotics were used frequently.

Keywords: Aminoglycosides, drug resistance, *mecA*, *Staphylococcus aureus*

INTRODUCTION

The body's initial and biggest barrier is the skin. It protects the human body and maintains a constant conversation with the exogenous environment, which is full of pathogens from outside the body, ultraviolet (UV) radiation, allergens, and chemical irritants. Therefore, in order to defend the host against unfavorable assaults and aggressions, a dynamic cutaneous ecosystem has evolved. This ecosystem consists of two main components: a sophisticated immune system and a typical flora with numerous commensal microorganisms like bacteria, fungus, and viruses that make up the skin microbiota, or cutaneous microbiome. To create a biological and immunological barrier, both are interrelated and mutually regulated.[1] When a human organism is suddenly exposed to energy levels that are more than what its physiologic tolerance can handle, injury occurs physically.[2] Since the skin serves as the first line of defense against invading organisms, severe burns frequently result in wound infections because necrosis creates an ideal habitat for microbial invasion and growth. One of the leading factors in burn patient mortality is wound infection.[3]

Iraq is one of many developing countries with a high rate of burn injuries, yet there are few reliable sources of information about the epidemiology of burns in these nations. After falls, traffic accidents, and interpersonal

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confrontations, burns are the fourth most prevalent type of injury.[4] Gram-positive organisms located in hair follicles and sweat glands eventually colonize the originally sterile burn wound surfaces.[5] Women and children, especially younger children, are at greater risk of burns, and most of these injuries occur in the home. This may be partly explained by women spending more time at home, participating in activities such as preparing and serving hot drinks, cooking and heating water, and being exposed to equipment and devices such as space heaters and stoves. In addition to these exposures, the design and layout of the house, availability of safe places to play, developmental stage, and supervision practices are likely to impact on burn risk for children.[6] The risk of invasive sepsis from several procedures has been demonstrated to be reduced by early excision.[7] As a result of early grafting, topical and systemic antimicrobials, and strict infection control measures, the field of burn wound microbiology has also seen significant change. These interventions have gradually come to be regarded as necessities for burn care nearly everywhere. Local nosocomial microorganisms and hospital stay length are two additional crucial variables that affect burn wound infection.[8] *Staphylococcus aureus* is a notable example of prevalent infections in injury abrasions. Both a common cause of infections ranging from minor illnesses to fatal ones, *S. aureus* is commensal to humans. *S. aureus* colonization has been linked to slower wound healing, a greater need for surgical treatments, and longer stays at burn facilities.[9] By medical practitioners' hands, by fomites, the patient's nose and skin surface, as well as during intervention, the burn wound surfaces of hospitalized patients receive microbial delivery.[10] Aminoglycosides are one of the antibiotic classes that are crucial in. the management of staphylococcal infections. These are frequently combined in a synergistic manner with either beta-lactam or glycopeptides, particularly for the treatment of difficult staphylococcal infections.[11] The inactivation of antibiotics by genetically encoded aminoglycoside-modifying enzymes (AMEs) is the primary mechanism of aminoglycoside resistance.[12] In general, five groups of AMEs, which include the following, mediate resistance to aminoglycosides: aminoglycoside-60-*N*-acetyltransferase/2″-*O*-phosphoryltransferase. Acetyltransferase (AAC) (6′)/aminoglycoside phosphotransferase (APH)(2″) encoded by the *aac*(*6′*)/*aph*(*2″*) gene; aminoglycoside-30-*O*-phosphoryltransferase III [APH(3′)-III] encoded by *aph*(*3′*)-*IIIa* gene; aminoglycoside-40-*O*-phosphoryltransferase.I.[aminoglycoside nucleotidyltransferase (ANT)(4′)-I] encoded by *ant(4′)-Ia* gene; aminoglycoside-9-*O*-nucleotidyltransferase I.[ANT(9)-I] encoded by the "*ant* (6)-*I*," and aminoglycoside-6-O-nucleotidyltransferase I [.ANT (6)-I] encoded by the ant (6)-I g*ene*.[12,13]

Clinically, the staphylococci with the most common AMEs include ANT (4′)-I, AAC (6′)/APH(2′″), and APH (3″)-III, which change therapeutically important aminoglycosides such as tobramycin, gentamicin, and kanamycin.[13] Resistance to aminoglycosides in staphylococci is mostly brought on by AMEs. AAC, APH, and ANT are the three kinds of AMEs that have so far been found. Medication inactivation by AMEs such as APHs is the most significant mechanism of aminoglycoside resistance in staphylococci. AMEs can be chromosomally or plasmidically encoded. The most prevalent AME among staphylococcal strains is *aac (6′)/aph (2′″)*. The *aac (6″)/aph(2′″)* gene encodes the bi-functional enzyme *aac (6″)/aph (2′″)*.[14,15] Additionally, staphylococcal isolates also contain the genes for ANT(4″)-I and APH(3′)-III, which are both encoded by the *ant (4′)*-*Ia* and *aph (3′)-IIIa* genes, respectively.[16-18] This study aimed to investigate the relationship between the antibiotic resistance patterns and aminoglycosides resistance gene in *S. aureus* isolated from wound and burn infections.

MATERIAL AND METHODS

Sample collection and bacterial isolates

The study population included 230 participants with burn wound infection admitted to the burn unit in Imam Sadiq Hospital, Hilla Teaching Hospital, and Specialized Burn Center in the Medical City of Baghdad and Kufa from February to September 2022. Swabs from burn wounds were obtained from all patients registered in the study and sunk into Stuart's mode of transportation. Of these 230, only 63 burn wound swabs were *S. aureus*. Swabs were taken from the infected wound after all traces of medicament were removed. After collection, all swabs were cultured on nutrient agar, blood agar, and mannitol salt agar and incubated at 37ºC for 24 h. Morphological examination of these swaps and colonial morphology, production of β hemolysis on blood agar, and production of pigmentation on mannitol salt agar revealed 63 isolates of *Staphylococcus* that were confirmed to be *S. aureus* by coagulase mannitol fermentation [Figure 1] and ultimately confirmed by the Vitek 2 Advanced Expert System (bioMerieux, Marcy l'Etoile, France).

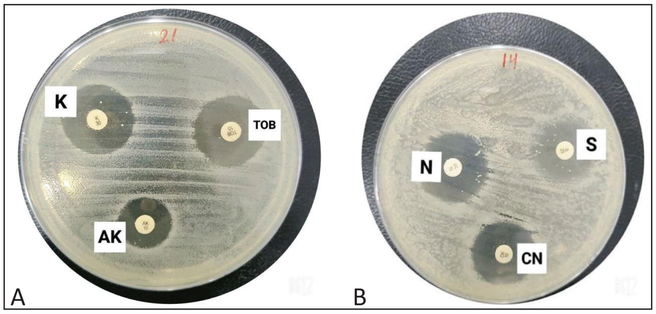

Figure 1: Susceptibility test of *Staphylococcus aureus* isolates to aminoglycoside antibiotics. (A) For amikacin (AK), tobramycin (TOB) and kanamycin (K). (B) For streptomycin (S), neomycin (N), and gentamycin (CN)

Antibiotics susceptibility test

The Kirby–Bauer method was used for the establisher test[19] and using the following aminoglycoside antibiotics for *S. aureus* isolates, gentamicin (10 mg), amikacin (10 mg), kanamycin (30 mg), tobramycin (10 mg), streptomycin (25 mg), and neomycin (30 mg) (Bioanalyase/Turkey) Preparation of Mueller Hinton plates It following the manufacturer's directions and inoculum preparation (turbidity standard). Colonies from overnight cultures of the tested isolates were transferred to a tube containing 5 mL of normal saline to create the inoculums, resulting in a culture with 1.5×10^8 CFU/mL when adjusted to McFarland standard tube No. 0.5. Following incubation, the inhibitory zone's diameters were measured in millimeters (mm). The diameter of the inhibition zone for individual antimicrobial agents was translated in terms of sensitive (S), intermediate (I), and resistant (R) categories by comparison with the manufacturing-provided standard (CLSI 2020)[20] [Figure 2].

Yazi Abdullah Jassim DNA extraction and polymerase chain reaction (PCR)

Using a commercially available DNA extraction kit(G-spinTM Genomic DNA Extraction Kit for Bacteria), genomic DNA was extracted. Using the exact primers specified in the manufacturer's instructions, PCR was used to find the mecA gene. For detection of the *aac (6′)-Ielaph (2″)*, *aph (3′)-IIIa*, and *ant (4′)* genes, which are the most common AMEs among the staphylococci, the PCR method was performed with specific primers [Table 1]. All PCR mixtures were prepared in a 25 µL volume containing 12.5 µL of PCR master mix (Promega), 1 mmol of each primer, 3 µL of DNA template, and 7.5 µL of deionized water. The mixtures were placed in a PCR machine (Cleaver, USA), and the PCR products were then visualized by electrophoresis in agarose gel, stained with ethidium bromide, and examined under UV illumination. The programming of the thermocycler is described in Tables 2 and 3.

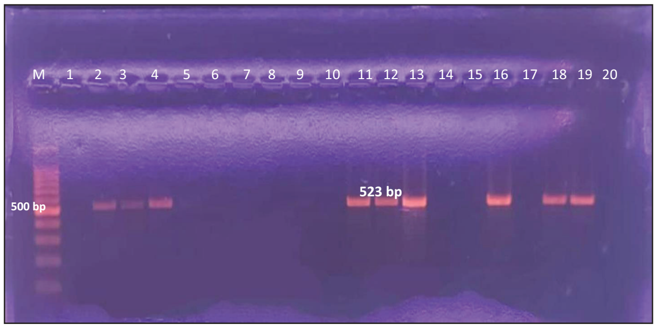

Figure 2: Gel electrophoresis of aph(3)-IIIa gene for *Staphylococcus aureus* bacteria in voltage (85 V) time (90 min) and 5 µL of PCR product loaded in well.[1-20] Lane M: DNA ladder (100bp). wells[2-4,11-13,16,18,19] are positive to presence this gene while[1,5-10,14,15,17,20] are negative to this gene

Ethical approval

The study was carried out in conformity with the ethical standards set forth in the Helsinki Declaration. Before a sample was taken, it was done with the patient's verbal and analytical consent. According to the document, a local ethics commission evaluated and approved the study protocol, as well as the subject information and permission form number M220109, on January 17, 2022, to get this approval.

RESULTS

During the Period from February to September 2022, 230 burn wound sample swaps were collected, and only 63 were *S. aureus* from a variety of clinical samples, including burn 50 and wound 13. The distribution of isolates is detailed in Table 4. This study included six aminoglycosides antibiotics determined by the disc-diffusion method, which are amikacin, gentamycin, tobramycin, kanamycin, streptomycin, and neomycin. The results analyzed the sensitivity pattern of antibiotics against *S. aureus,* as in Figure 1.

The phenotypic prevalence of *S. aureus* drug-resistant to aminoglycoside antibiotics isolates to amikacin were 30, gentamycin 30, tobramycin 36, kanamycin 40, streptomycin 34 and neomycin 34 isolates. Many of the isolates were multidrug-resistant *S. aureus* (MDR); the percentage of resistance to these antibiotics is shown in Table 5.

Based on PCR analysis, only 50 *S. aureus* burn wound isolates were resistant to aminoglycosides in primary

Table 1: Primer sequences

Target gene	Primer sequence	Size product (bp)	Reference
aac(6′)/aph(2″)	5′CAGAGCCTTGGGAAGATGAAG-3′ 5′-CCTCGTGTAATTCATGTTCTGGC-3′	348	[21]
aph(3)-IIIa	5′-GGCTAAAATGAGAATATCACCGG-3′ 5′-CTTTAAAAAATCATACAGCTCGCG-3′	523	
ant(4)-Ia	3′-5′-CAAACTGCTAAATCGGTAGAAGCC 5′-GGAAAGTTGACCAGACATTACGAA-3′	294	
mecA	5′-CCT AGT AAA GCT CCG GAA-3′ 5′-CTA GTC CAT TCG GTC CA-3′	314	[22]

Table 2: The PCR program for the mecA gene amplification

No.	Step	Temperature (ºC)	Time	No. of cycle
1.	Initial denaturation	95	3 min	1
2.	Denaturation	95	30 s	35
3.	Annealing	54	30 s	
4.	Extension	72	30 s	
5.	Final extension	72	5 min	1

Table 3: The PCR program for the aac(6′)/aph(2″), aph(3)-IIIa, and ant(4)-Ia gene amplification

No.	Step	Temperature (ºC)	Time	No. of cycle
1.	Initial denaturation	95	3 min	1
2.	Denaturation	95	30 s	35
3.	Annealing	55	40 s	
4.	Extension	72	40 s	
5.	Final extension	72	5 min	1

Table 4: Distribution of *Staphylococcus aureus* isolation

Sample sources	Sample collection locations			
	Hilla Teaching Hospital	Imam Sadiq Hospital	Specialized Burn Center/Najaf	Specialized Burn Center/Medical City, Baghdad
Burns no. = 50 (79.4%)	–	10 (15.9%)	26 (41.3%)	14 (22.2%)
Wounds no. = 13 (20.6%)	13 (20.6%)	–	–	–
Total no. = 63 (100%)				

Table 5: Percentage antibiotic susceptibility test for pathogenic bacteria

Antibiotics	Antibiotic susceptibility of *S. aureus* of 63 (%)		
	R	I	S
AK	47.6	17.4	34.9
CN	47.6	4.7	47.6
TOB	57.1	–	42.8
K	63.4	–	36.5
S	53.9	11.1	43.9
N	53.9	3.1	42.8

AK: amikacin, CN: gentamycin, TOB: tobramycin, K: kanamycin, S: streptomycin and N: neomycin

detection, 12 isolates (24%) contained *mecA* gene. Also, 32 isolates (64 %) had *aph(3′)-IIIa1* gene, and six isolates (12 %) had *aac(6′)Ie/aph (2″)* gene. While the presence of the *ant (4′)-Ia1* gene was not observed in any of the samples [Figures 2–4]. Of these 50 *S. aureus* isolates that contain resistance genes, only 15 of them contained more than one resistance gene. Their distribution was 10 samples containing *aph (3)-IIIa* with *mecA* genes and only 5 containing *aph (3)-IIIa* with *aac (6′)/aph(2″)* genes, as in Tables 6 and 7].

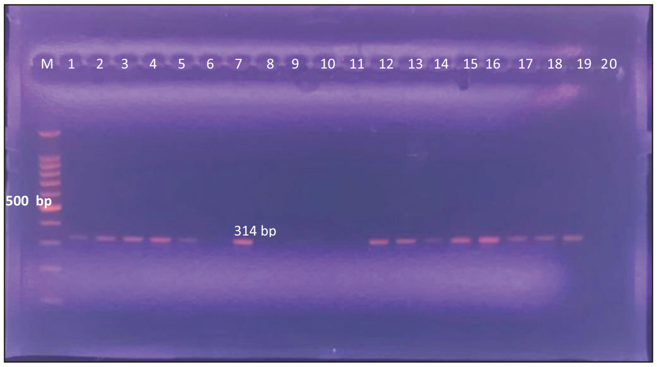

Figure 3: Gel electrophoresis of mecA gene for *Staphylococcus aureus* bacteria in voltage (85 V) time (90 min) and 5 µL of PCR product loaded in well.[1-20] Lane M: DNA ladder (100bp). All these samples are positive for the presence of this gene except well[6,8-11,20] are negative

DISCUSSION

Burning injury is the localized reaction of tissue to the transfer of energy from a source that is physical (mechanical, thermal, electrical, electromagnetic, or chemical), with or without a systemic reaction.[23] Infections are a major contributor to illness and humanity in hospitalized burn patients; 75% of all fatalities from thermal injuries are caused by burns covering more than 40% of their total body's exterior. The rate of nosocomial infections is higher in burn patients due to a number of factors, including the nature of the burn injury, the patient's immunocompromised status, age, the extent of the injury, and the depth of the burn, furthermore variables related to microbes, including kind and number of organisms, production of enzymes and toxins,

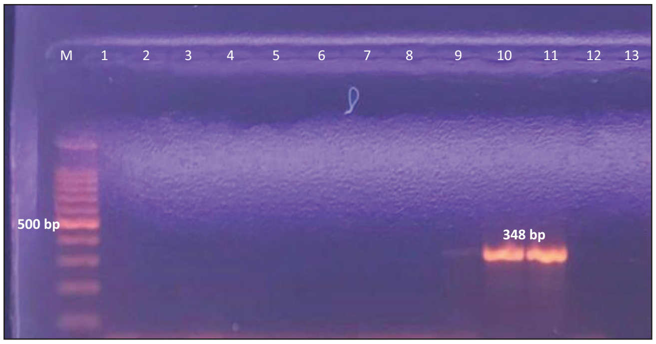

Figure 4: Gel electrophoresis of aac(6′)/aph(2″) gene for *Staphylococcus aureus* bacteria in voltage (85 V) time (90 min) and 5 µL of PCR product loaded in well.[1-20] Lane M: DNA ladder (100 bp). All these samples are negative for the presence of this gene except well[10,11] are positive

Table 6: Genotyping distribution of aminoglycoside resistance genes for *Staphylococcus aureus* in the study

Gene	No.	Percentage
aph(3)-IIIa	32	64
aac(6′)/aph(2″)	8	16
mecA	10	20
Total	50	100

Table 7: Genotyping distribution of aminoglycoside resistance genes with *mecA* gene for *Staphylococcus aureus* in the study

Genes	No. (100%)
aph(3)-IIIa + mecA	10 (20%)
aph(3)-IIIa + aac(6′)/aph(2″)	5 (10%)

colonization of the burn wound site, and systemic spread of the colonizing organisms.[24] Taneja *et al.*,[25] study and other earlier research have demonstrated that a variety of bacterial species are capable of easily infecting burn wounds. The majority of species were discovered to be *S. aureus* and *P. aeruginosa.* Interestingly, the variation in bacterial flora and the colonization rate changes over time after the initial infection.

According to the findings of our study, analysis of the resistance patterns to aminoglycoside antibiotics revealed that Tobramycin, which had 36 isolates (57.1%), and Kanamycin, which had 40 isolates, had the highest rates of antibiotic resistance. This was according to the studies done by Rahimi *et al.*,[26] in Isfahan, where gentamicin and kanamycin resistance levels were higher than in the current study. Nevertheless, they agreed with Sattari *et al.*[27]'s findings, which reported 30% tobramycin resistance, which is higher than the findings of our study.

Additionally, amikacin and gentamycin, which included 30 isolates (47.6%), showed the lowest levels of antibiotic resistance. According to the studies done by Schmitz *et al.,*[28] in Europe, where it was reported that more than 30% of cases had phenotypic resistance to gentamicin and amikacin. The findings of this study precisely match those of that study, while it was not consistent with the study of Abdal *et al.*[29] in Qom and Ghotaslou *et al.*[30] in Tabriz, who showed that 59 isolates with the highest level of antibiotic resistance were found to be resistant to gentamicin (52.21%).

The reasons for these variations in some reports from various places can be traced to strain variations according to location. Aminoglycoside antibiotics are occasionally used in combination with beta-lactam and aminoglycoside antibiotics to increase the efficiency of these medications. The 30S subunit of ribosomes is where aminoglycosides connect in order to stop bacterial translation and exert their antibiotic actions.[31]

Resistance to aminoglycosides is because of the presence of enzymes that cause changes in aminoglycoside antibiotics.[32] A number of genes, including *aac (6′) Ie/ aph (2′)*, *aph (3′)-IIIa1*, and *ant (4′)-Ia1*, code for these enzymes. Transposons, for example, are movable genetic components that can move around a strain.[33] The three enzymes AAC (6′)/APH(2′), APH (3′)-III, and ANT (4′), which are encoded by the genes *aac (6′) Ie/aph(2′), aph (3′)-IIIa1*, and *ant (4′)-Ia1,* respectively, being among the most common modifying enzymes in several species of *S. aureus* and have significant medical and clinical significance.[34] The activity of the *aac (6′) Ie/aph (2″)* enzyme causes resistance to gentamicin, kanamycin, and tobramycin; the presence of the *ant (4′)-Ia1* gene causes resistance to neomycin, tobramycin, and amikacin; and the *aph (3′)-IIIa1* enzyme causes of kanamycin and tobramycin resistance.[32,34] We need to utilize a suitable selection of aminoglycoside antibiotics, such as CN, K, AK, and TOB, for the phenotypic identification of bacteria that are resistant to these drugs.[32] Additionally, efflux pumps may be to blame for the resistance to many antibiotics.[35] Failure to use appropriate initial strain identification of bacteria resistant to aminoglycosides using antibiotic discs, limited sensitivity of phenotypic approaches, and lack of precise and quick identification are among the causes of failure to treat resistant infectious strains.[36]

The PCR approach can be used to find genes that spread antibiotic resistance-causing enzymes, which is a highly effective and delicate technique for identifying resistant bacteria. The results of the molecular studies also showed that the *aph (3)-IIIa* gene was more frequently present in isolates that were resistant to aminoglycosides, while the *aac(6′)/aph (2′″)* gene was not found in the isolates. This indicates that the *aph (3)-IIIa* gene is present and is the most important method of resistance to aminoglycosides in *S. aureus*. These results were inconsistent with those of studies by Liakopoulos *et al.*[37] and Choi *et al.*,[38] which concluded that the gene *aac(6″)/aph(2″)* was the most significant and was responsible for the mechanism of resistance to aminoglycoside in *S. aureus*.[37,38]

According to investigations on the identification of the genes causing resistance to aminoglycoside antibiotics, the *aac* (6′) *Ie/aph*(2″) gene and the *aph*(3′)-*IIIa1* gene have the highest prevalence rates. Shokravi *et al.*[39] and Malek Hosseini *et al.*[40] have reported these findings. The *aac(6′) Ie/aph(2″)* gene has the highest frequency in this investigation. However, *aph(3′)-IIIa1* is used instead of *ant(4′)-Ia1* with a prevalence of 11.50%, which is inconsistent with the findings of the current study. These variations in outcomes could be brought about by various geographical factors, distinctive genotypic traits of bacteria, the source of the sample, and the type of samples. The technique, quantity, and duration of utilizing aminoglycoside antibiotics were some of the most significant variables that set this study's prevalence of antibiotic resistance, specifically to aminoglycoside antibiotics, apart from other research. By altering the pattern of antibiotic delivery and refraining from administering different antibiotics during therapy, we can stop genetic transfers from occurring. Furthermore, we can infer that the coadministration of different antibiotics from different classes may lead to the emergence of MDR bacteria.

CONCLUSION

MDR strains were found to arise in settings where aminoglycoside antibiotics were used frequently.

The gene *aph(3′)-IIIa1* was the highest and responsible for the mechanism of resistance to aminoglycoside in *S. aureus.*

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Nil.

Conflicts of interest

There are no conflicts of interest.

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