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Epidemiology and Molecular Characterization of *Staphylococcus aureus* Enterotoxins A and B Isolated from *Clarias gariepinus* from Wild and Pond in the FCT Abuja, Nigeria

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Abstract

Staphylococcus aureus is an important foodborne pathogen associated with food poisoning and other multiple infections in human being. Its presence in fish is of public health concern. *Clarias gariepinus* organs collected from the pond/wild include serious issue due to the fact that they were collected from organs from the pond/wild organs namely intestine, *Staphylococcus aureus* is not a normal flora of fish. The risk of zoonotic transmission to humans highlights the need to evaluate *Staphylococcus aureus* enterotoxin A and B from African catfish (*Clarias gariepinus*) in Federal Capital Territory, Abuja. Four hundred samples of *Clarias gariepinus* organs were collected with two hundred (200) samples each from the ponds/wild and 66 samples from both Gwagwalada and Abuja Municipal Area council and 68 from Bwari respectively and examined for the presences of *Staphylococcus aureus* from the intestines, gills and skin using standard procedures on Baird Parker (BPA) agar plates supplemented with egg yolk emulsion and tellurite solution. Isolates were further identified using conventional biochemical tests: Gram's staining, catalase, production of coagulase, Dnase, Oxidase test, hemolytic zone on 5% sheep blood agar and fermentation of mannose, mannitol, xlylose, maltose, trehalose, lactose, fructose, sucrose and glucose. Isolates were further characterized using Microgen TM STAPH-12S KIT. Antimicrobial sensitivity test was carried out by disc diffusion method on Mueller Hinton agar and was interpreted in line with the Clinical and Laboratory Standard Institute. An overall isolation rate of from the ponds was 5.0% while from the wild was 6.0%

recorded for *Staphylococcus aureus*. Antibiogram of *Staphylococcus aureus* isolates revealed high sensitivity to some of the antibiotics, doxycycline 10 (71.4%), gentamycin 9 (64.2%), clindamycin 7 (50.0%) and ampicillin 6 (42.8%). Similarly, *Staphylococcus aureus* isolates also showed resistance to vancomycin 11 (78.5%), oxacillin, oxtetracycline and trimethoprim-sulfamethoxazole 10 (71.4%) while erythromycin, tetracycline and streptomycin showed 7 (50.0%) respectively. *Staphylococcus aureus* isolates indicated a high percentage of isolates having MARI characteristics of 0.92%. A total 2/6 (33.3%) of the samples were found positive for the presence SEA and 1/6 (16.7%) for SEB. It was concluded that the study confirmed the presence of enterotoxins in African catfish which is a major concern to public health. Preventive and control measures are necessary to tackle this serious food safety concern.

Keywords

Staphylococcus aureus, Antimicrobials, Foodborne Diseases, Public Health Pathogens

1. Introduction

Seafood including fish meat is the chief source of animal protein in the diet. Consumption of seafoods specifically fish is increasing due to its health advantages over red meat and other food products [1]. Food-borne infections are common and constitute an important health and economic burden globally [2].

Staphylococcus aureus is an opportunistic pathogen that causes several diseases such as skin and soft tissue infections [3], food poisoning, and life-threatening complications, such as pneumonia, endocarditis osteomyelitis, and toxic shock syndrome, due to its large arsenal of exotoxins, including enterotoxins, as well as invasion, immune evasion, and antibiotic resistance mechanisms [4]. Several foodborne pathogens have been reportedly associated with fish contamination [5]. *Staphylococcus aureus* is amongst one of the leading causes of food contamination, which can spoil the food by producing lethal enterotoxin [6]. Food borne diseases are identified by presenting various symptoms in the gastrointestinal system, such as: nausea, vomiting, diarrhoea, abdominal pain and fever [7].

Staphylococcal enterotoxin (SEs) are exoproteins which when ingested by humans give rise to symptoms of acute gastroenteritis. SEA is frequently associated with food poisoning outbreaks and accounts for about 75% of the outbreaks in developed countries [8]. SED is the second most prevalent toxin causing food poisoning outbreaks followed by the SECs and SEB [8]. The best measures to prevent enterotoxin associated food poisoning in *S. aureus* rely on preventing the bacteria from contaminating food and discourage continue replication. This is because Staphylococcal enterotoxins are stable to heat treatment and stomach acid [9]. Fish have a microbiota that is dependent on what it exists in the waters where they live, meaning that polluted waters can provide a variety of pathogens; the micro-

organisms in the skin and gastrointestinal content of the fish in life do not invade the sterile muscle package due to the protection of its natural defences. When the animal dies, these microorganisms penetrate the fish, causing deterioration and loss of safety. The presence of *Staphylococcus aureus* in fish and fish products can mainly indicate contamination from the skin, mouth and nostrils of infected handlers or healthy carriers, during stages of capture, transport, storage, processing and preparation processes [10] [11]. Contamination of seafood with drug resistance and virulent species of *S. aureus* can create serious food safety and public health issues. Enterotoxin producing MRSA can be more fatal and problematic compared to its drug sensitive counterpart [12]. *Staphylococcus aureus* has been isolated from fish by some previous researchers like Arslan and Ozdemir, [13] who reported 24% of *Staphylococcus aureus* from fish. Similarly, Haifaa *et al.*, [14] recorded the percentage of 35.5% *Staphylococcus aureus* from the skin, 21% from intestine and 25.8% from the liver respectively. *Staphylococcus aureus* detection from fish, *Oreochromis niloticus*, and wastewater samples from Qarun Lake with a prevalence of 81.5%, suggests that this pathogen may be abundant in lakes [15]. Many species of staphylococci are capable of releasing enterotoxin that can cause gastroenteritis in humans when foods contaminated by these species are consumed [16]. Obaidat *et al.* [17] reported 88.5 prevalence of *Staphylococcus aureus* in imported fish and correlations between antibiotic resistance and enterotoxigenicity. Preventive measures such as practicing hand hygiene measures are important to avoid or reduce contamination of food by *S. aureus*. These procedures must include control of raw materials, proper handling, cleaning and disinfection of equipment from farm to fork. The aim of the present study was to detect Staphylococcal enterotoxins A and B from the organs of African catfish from the wild and ponds from selected area councils in the FCT, Abuja.

2. Material and Method

2.1. Study Area and Design

A cross-sectional study was employed and primary sources (fish ponds/wild) included three randomly selected area councils of Gwagwalada, Kubwa and Amac in the FCT Abuja, which was formed in 1976, from parts of Nasarawa, Niger and Kogi States. The territory is located just north of the confluence of Niger and Benue rivers. It is bordered by Niger State to the West and North, Kaduna to the Northwest, Nassarawa to the East and South and Kogi to the Southwest. Abuja has an estimated human population of 2,690,000 according to Federal Capital Territory Administration [18]. It lies between latitude 8.25 and 9.20 North of the equator, and longitude 6.45 and 7.39 East of Greenwich Meridian. Abuja is geographically located in the centre of the country. The Federal Capital Territory has a land mass of approximately 7315 km^2 of which the actual city occupies 275.3 km^2. It is situated within the Savannah region with moderate climatic conditions.

2.2. Ethical Approval

Ethical approval was sought from Committee on animal use and care ABU Zaria.

2.3. Study Design/Sample Collection

A cross sectional epidemiological study was conducted and freshly caught African catfish (*Clarias gariepinus*) were purchased from the wild/ponds in the three selected Area Councils namely, Gwagwalada 66 each from the wild/ponds, Abuja Municipal Area council 66 each from the wild/ponds and Bwari 68 each from the wild/ponds by using purposive sampling method in the FCT, Abuja totalling 400 samples and transported to bacterial Zoonoses Laboratory Department of Veterinary Public Health and Preventive Medicine Ahmadu Bello University Zaria for analyses.

2.4. Enumeration and Isolation of *Staphylococcus aureus*

This was conducted according to the procedure described by [19]. Ten grams portion each of the intestine and gills were aseptically weighed using a weighing balance and placed in a sterile polythene bag and homogenized in 90 ml of sterile peptone water in a stomacher for two minutes. Using a sterile pipette, one ml from the homogenized sample was inoculated onto Tryptic soy broth containing 6.5% NaCl and incubated at 37˚C for 24 h. The skin swab stick was dropped into 90 ml of peptone water and thereafter one ml was inoculated onto 9 ml of tryptic soy broth (TSB) containing 6.5% NaCl and incubated at 37˚C for 24 h. A loopful of the homogenate was picked using a sterile inoculating wire loop and streaked on Baird-Parker agar supplemented with egg yolk tellurite and incubated at 37˚C for 24 h. Tiny shiny black colonies on BPA are suggestive of *Staphylococcus aureus*, were picked and streaked on nutrient agar slant and incubated for 24 h and subsequently stored at 4˚C in the refrigerator for further identification by standard methods.

2.5. Biochemical Characterization of Isolates

Presumptive *Staphylococcus* species that were gram positive cocci in clusters were subjected to biochemical tests as described by [20] which were; coagulase test, catalase test, Oxidase test, Haemolysin test, DNase test and fermentation of maltose, mannose, lactose, fructose, sucrose trehalose, xylose and D-glulose. These isolates were further confirmed using MicrogenTM STAPH-identification system (Microgen Bioproducts, United Kingdom).

2.6. Antibiotic Sensitivity

Antibiotic susceptibility tests for isolates of *Staphylococcus aureus* were performed according to the Kirby-Bauer method as described by [21] and the evaluation methods of the Clinical and Laboratory standards Institutes [22]. Isolates grown on nutrient agar overnight were suspended in 2 ml sterile normal saline (0.9% sodium chloride solution). A turbidity equivalent was prepared by compar-

ing with a 0.5 MacFarland standards. Bacterial suspensions of 0.1 ml were spread on plates of sterile Mueller-Hinton agar with the help of a sterile cotton swab to form a smooth bacterial lawn. Antimicrobial discs were placed on the plates with disc dispenser and gently pressed down to ensure contact. The plates were allowed to stand for 30 minutes for diffusion of active substances of the agents. Plates were carefully inverted and incubated at 35˚C - 37˚C for 24 hr. The inhibition zone diameter around the disc were measured and compared with the interpretation standards provided by [22]. Multiple antibiotic resistance (MARI) for this study is defined as resistance of isolate to three or more antibiotics [23]. Multiple antibiotic resistance index (MARI) was calculated according to Furtula *et al.* [24] as the ratio of number of antibiotics to which an organism is resistant, to the total number of antibiotics to which an organism is exposed to.

2.7. PCR Detection of Staphylococcal Enterotoxin A and B Genes

2.7.1. DNA Extraction

DNA extraction was performed using suspension of isolated colonies prepared in sterile distilled water following protocol of bacterial DNA extraction kit (DNAzol® BD, USA). Two to three well isolated colonies of suspected *Staphlococcus aureus* isolates were suspended in 0.5 ml autoclaved distilled water. One ml of DNAzol was added in bacterial suspension. The mixture was vortexed vigorously for 15 - 20 seconds and stored at room temperature for 5 minutes then centrifuged for 1 minute at 8000 g. A volume of 0.4 ml of isopropanol was added to the lysate for precipitation of DNA. The precipitated DNA was sedimented by centrifugation at 6000 g for 5 minutes, supernatant was removed and 0.5 ml of DNAzol was added to the DNA pellet. The DNA pellet was vortexed until it was completely dispersed. Then it was centrifuged at 6000 g for 5 minutes. The supernatant was removed and washed, the DNA pellet was mixed with 1 ml of 75% chilled ethanol. Again, it was centrifuged at 7000 g for 5 minutes. The ethanol wash was decanted and stored in the eppendrof tube vertically for 15 minutes to evaporate any residual ethanol. The DNA pellet was then dissolved in 20 ul molecular grade water.

2.7.2. Amplification of Staphylococcal Enterotoxin A and B

A multiplex PCR assay was performed to detect Staphylococcal enterotoxin A and B. The multiplex PCR assay was carried out with the CLASSIC K960 (UK) thermocycler. The PCR amplification was performed using PCR PreMix kit (iNtRON, Korea) and specific primers listed in **Table 1**, in a total volume of 20 µL of mixture containing 0.5 µL of each of the toxin gene-specific primers, 2 µL of template DNA, and 15 µL of double distilled water. The amplification conditions included three steps: initial denaturation at 94˚C for 5 min, 35 cycles of denaturation at 94˚C for 45 sec, primer annealing at 50˚C for 45 seconds, and extension at 72˚C for 45 seconds followed by the final extension at 72˚C for 7 min. The amplified products were then resolved by electrophoresis in 2% agarose gel at 100 V for 60 minutes. Gels were stained with ethidium bromide solution and documentation was done using the Gel Doc system.

Table 1. Primer sequences multiplex PCR and predicted product size for Staphylococcal Enterotoxins.

Gene	Primer Sequence		Reference
SEA	F-5ATGGTTATCAATGTGCGGGTG3 R-3TGAATACTGTCCTTGAGCACCA5	344	Peacock *et al.* [37]
SEB	F-5TGGTATGACATGATGCCTGCAC3 R-3AGGTACTCTATAAGTGCCTGCCT5	196	

3. Data Analysis

Statistical Packages for Social Sciences (SPSS Version 23.0) data obtained was presented using descriptive statistics in percentages, tables, figures and charts. Chi-square was used to test for association and 95% confidence interval was used where appropriate to test for association between levels of contamination in different markets. P-value ≤ 0.05 was considered as significant.

4. Results

In this study, a total of 400 samples were collected from the three area councils of the FCT, Abuja, namely Gwagwalada, Bwari and Abuja municipal area council and subjected to analysis for the presence of *S. aureus* revealed 10 (5.0%) of the samples collected from the ponds and 12 (6.0%) from the wild were positive for *S. aureus* (Table 2).

Table 2. Prevalence of *Staphylococcus aureus* from African catfish (*Clarias gariepinus*) from ponds and Wild from selected area councils in the FCT Abuja.

Type	No. of Samples pond	No. Positive (%)	No. of Samples Wild	No. Positive (%)
		Skin	Gills	Intestine
AMAC	66	3 (4.5)	66	5 (7.5)
Gwagwalada	66	5 (7.5)	66	6 (9.0)
Bwari	68	2 (2.9)	68	1 (1.4)
Total	200	10 (5.0)	200	12 (6.0)

S. aureus isolates subjected to antibiogram revealed 11/14 78.5% resistance to vancomycin, whereas oxytretacycline, Trimethoprim-Sulfamethaxazole and Oxacillin revealed 10/14 (71.4). Cefazolin 9/14 (64.2) with Doxycline and clindamycin showing the least resistance 2/14 (14.2%). *S. aureus* isolates showed susceptibility to doxycycline 10/14 (71.4%) and gentamycin 9/14 (64.2%) and clindamycin 7/14 (50.0%) respectively (Table 3).

Two isolates showed resistance to thirteen antibiotics and one to eleven antibiotics used in the study whereas four isolates showed resistance to nine and one to six to four isolates respectively (Table 4).

Pathogenicity of *S. aureus* isolates was molecularly determined by using two different genes SEA and SEB. The enterotoxins A is more frequently associated to

this organism responsible for food poisoning. It was found in this study that 2/6 (33%) isolates are positive for SEA and 1 (16.7%) are positive for SEB gene (Figure 1). It was also confirmed that none of the isolates are having both SEA and SEB genes.

Table 3. Antibiotic susceptibility of *S. aureus* from African catfish.

Antibacterial Agent	Disk potency (µg)	No susceptible (%)	No intermediate (%)	No resistant (%)
Doxycycline (Dxy)	30	10 (71.4)	00 (0%)	2 (14.2)
Cloxacillin	30	6 (22.2)	00 (0%)	6 (22.2%)
Gentamicin (GN)	10	9 (64.2)	00 (0%)	3 (21.4)
Clindamycin (CLI)	2	7 (50.0)	3 (21.4)	2 (14.2)
Cefazolin	30	3 (21.4)	00 (0%)	9 (64.2)
Oxacillin (OXA)	30	2 (14.2)	00 (0%)	10 (71.4)
Erythromycin (E)	15	3 (21.4)	2 (14.2)	7 (50.0)
Streptomycin (S)	25	4(28.5)	1 (7.1)	7 (50.0)
Ciprofloxacin (CIP)	5	3 (21.4)	1 (7.1)	8 (57.1)
Ampicilin	10	6 (42.8)	2 (14.2)	4 (28.5)
Vancomycin (VA)	30	1 (7.1)	00 (0%)	11 (78.5)
Tetracycline (TE)	30	5 (35.7)	00 (0%)	7 (50.0)
Oxytetracycline (OXY)	30	2 (14.2)	00 (0%)	10 (71.4)
Trimethoprim-Sulfamethaxazole (SXT)	1.23/23.75	2 (7.4)	00 (0%)	10 (71.4)

Table 4. Antibiotic resistance pattern of *S. aureus* from African catfish.

No.	Isolates	Resistance pattern	MARI
1.	A G2	OXA, OXY, SXT, CIP, DOX, CLI, CFZ, CX, VA, TE, GN, S, E	0.92
2.	G S6	OXA, OXY, SXT, CIP, DOX, CLI, CFZ, CX, VA, TE, GN, S, E,	0.92
3.	A G3	OXA, OXY, SXT, CIP, CFZ, CX, VA, TE, GN, S, E	0.78
4.	B I3	OXA, OXY, SXT, CIP, CFZ, CX, VA, TE, S	0.64
5.	B G5	OXA, OXY, SXT, CIP, CFZ, CX, VA, TE, S	0.64
6.	A S4	OXA, OXY, SXT, CIP, CFZ, CX, VA, TE, S	0.64
7.	G S2	OXA, OXY, SXT, CIP, CFZ, VA, TE, S, E,	0.64
8.	B I7	OXA, OXY, SXT, CIP, CFZ, VA	0.42
9.	B S2	OXA, OXY, SXT, CFZ, VA	0.35
10.	A G6	OXA, OXY, SXT, VA	0.28
11.	A I5	VA	0.07

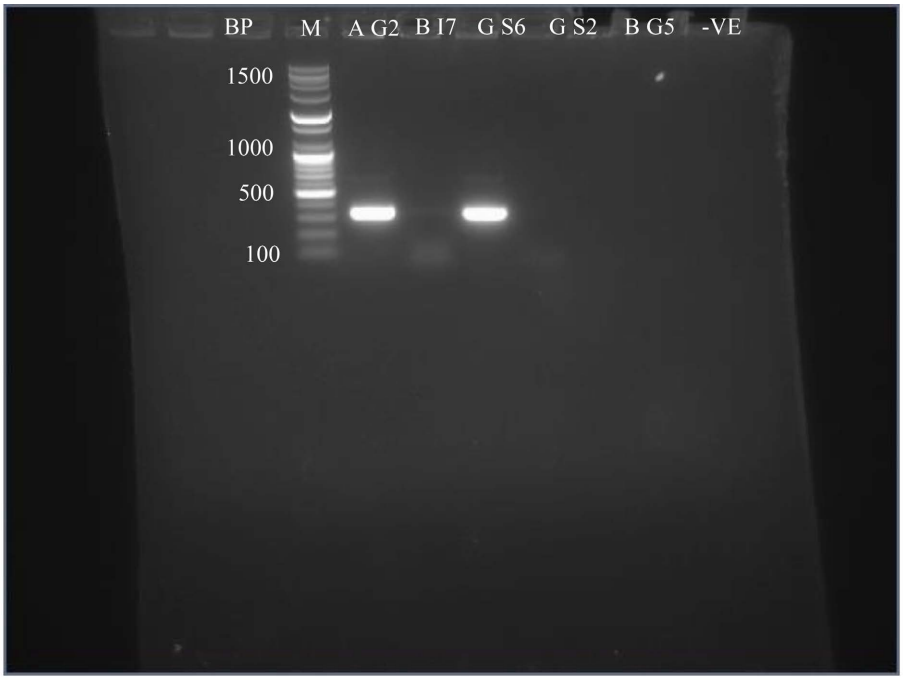

Figure 1. Agarose gel picture of the product of electrophoresis of multiplex PCR amplification of *Sea* (344 bp) and *Seb* (196 bp) genes. Legend Lane M is the molecular weight marker and lanes 1 and 3 positive *S. aureus* isolates for *Sea* and 2 positive *S. aureus* isolates for *Seb* lane 6-ve control.

5. Discussion

Staphylococcus aureus is an important foodborne pathogen, most frequently associated with enterotoxins associated intoxication [25]. Food-borne infections are common and constitute an important health and economic burden globally [2]. Food handlers contribute to food safety, being potential sources of bacteria that cause foodborne diseases due to the introduction of pathogens during its processing, distribution and manipulation [26]. *S. aureus* is not the normal flora of fish species and its presence in seafood and fish cannot be ignored [27]. In this study we found 5.0% prevalence of *S. aureus* from the pond and 6.0% from the wild which are in line with the report of 26% by [13]. Similarly, Bujjamma and Padmavathi [28] reported 24.5% of *Staphylococcus auerus* from fish. Mohammed *et al.* [29] reported 31% *S. aureus* contamination in fish from Nigeria. Furthermore, Rashid *et al.* [30] reported enterotoxigenic methicillin resistant *Staphylococcus aureus* contamination in salted fish from Gwadar Balochistan. Also, Matouke and Nour [31] reported 53% from gills and 57% from the intestines of *Clarias gariepinus* from the characterization of antibiotic-resistant *Staphylococcus aureus* from gills and gastro-intestinal tracts of catfish (*Clarias gariepinus*), and water samples from Jabi Lake, Abuja, Nigeria. The variation may be due to the method of isolation and the sample size as well as the locations and the deeper organs sampled in the present study. Isolation of *Staphylococcus aureus* and its toxins calls for concern. Because the most common way of contamination of fish

is by contact with fish handlers hands, especially in the cases where the fish is handled before or after cooking. Prolonged storage without refrigeration allows the bacteria to grow and form toxins. Since some toxins are heat-stable, the incriminated fish may cause food poisoning even when subjected to further heat treatment [32].

Antibiotic resistance patterns of *S. aureus* isolated from this study showed resistance to multiple antibiotics. The pattern of resistance of *Staphylococcus aureus* isolates showed that vancomycin, oxacillin, oxytretecycline, and Trimethoprim-Sulfamethaxazole heard the highest resistant which agrees with the report of [33] who reported resistance to Tetracycline 18 (64.3%). Vancomycin 17 (60.7%), Erythromycin 17 (60.7), Trimethoprim-Sulfamethaxazole 7 (25) from the isolates of *Staphylococcus aureus* from milk in Zaria Kaduna state Nigeria. However, resistance to vancomycin from this study is not in conformity with the report of Godwin *et al.* [34] who reported 100% susceptibility to vancomycin. Impact of antibiotic resistance may result in severe illness, disability, long hospital stays, increase healthcare cost, treatment failure and even death, Susceptibility of *Staphylococcus aureus* from this study to doxycycline, gentamycin, clindamycin and ciprofloxacin agrees with the reports of Grema *et al.*, [35] who reported considerably level of susceptibility to gentamicin, clindamycin, doxycycline and ciprofloxacin respectively.

MARI of 0.92 recorded from this study may be an indication of high and indiscriminate usage of antibiotics [24]. The high percentage occurrence of multiple antibiotics resistance (MAR) index among the bacterial isolates in this study might have arisen due to common practices such as self-medication and over the counter usage of antibiotics.

Pathogenicity of *S. aureus* isolates was molecularly determined by using two different genes primer representing SEA and SEB. The enterotoxins A is more frequently associated to this organism responsible for food poisoning. It was found in this study that 2/6 (33.3%) isolates are positive for SEA gene while 1/6 (16.7%) of the isolates are positive for SEB genes. The enterotoxin A and B found in this study have also been reported in other works by previous researchers Mohammed *et al.* [29] reported high level of SEA (38%) compared to SEB (35.7%) genes from salted fish in Khartoum states Sudan. Study by Rashid *et al.* [36] revealed 38% SEA and 23% SEB gene presence in the isolates, which shows higher frequency of SEA compared to SEB which agrees with this present study. The detection of staphylococcal enterotoxins in fish is of public health concern as it can lead to food poisoning through the consumption of contaminated fish particularly in retailed outlets. However, further research is needed to check for the distribution of these toxins in fish globally.

6. Conclusion/Recommendations

A significant number of *S. aureus* were isolated from the gills, skin and intestine of fish, which showed resistance to thirteen different antibiotics and were detected

carrying enterotoxins genes SEA and SEB. Proper hygiene practices such as the washing of hands, utensils, and surface with hot, soapy water before and after handling raw fish and also avoid touching the face or other food items while handling raw fish to prevent the transfer of *Staphylococcus aureus* as well as advise to fish farmers on indiscriminate use of antibiotics and discourage the use of antibiotics as growth promoters during feed formulation.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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