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Co-resistance of Antibiotics and Heavy metals in Bacterial Strains Isolated from Agriculture Farm and Soap Industry

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ABSTRACT: *In this study, a relationship between antibiotic and heavy metal resistance was estimated among culturable bacterial strains of agriculture farm and soap industry soil. A total of 27 bacterial strains were isolated and screened for their antibiotic and heavy metal resistance by supplementing LB agar medium with variable concentrations of respective stress. On LB-agar medium, agriculture farm soil harboured more cultivable bacterial strains (17 bacterial strains) as compared to the soap industry soil (10 bacterial strains). Whereas, minimum inhibitory concentration of antibiotics for bacterial strains ranged from 20µg/ml to 5000µg/ml, while MIC of heavy metals had a range of 20µg/ml-2000µg/ml for Nickel, Copper, and Mercury whereas the minimum inhibitory concentration of lead and chromium was up to 10,000µg/ml and 250,000µg/ml respectively. A high rate of co-resistance for Streptomycin with Lead and copper and Ampicillin with lead was observed in 90% of industrial soil bacterial strains. In conclusion, multiple antibiotic resistance and antibiotic-heavy metal co-resistance in bacterial strains could be due to contamination of soil with any sort of heavy metals or the diversity of population inhabiting that particular site. Antibiotic resistance can also be attributed to the horizontal gene transfer in bacteria.*

Keyword: Antibiotic resistance, Heavy metal tolerance, Co-resistance

INTRODUCTION

The evolution and spread of resistance genes is a major challenge in bacteria, these days (Liguori et al., 2022). Bacteria become resistant to antibiotics or heavy metals by mutations or by acquisition of resistance genes from other micro-organisms (Riaz et al., 2021). There are some other genes in the environmental bacterial strains which may act as resistance genes. Resistant bacteria release their mobile genetic elements having resistance characteristics into their surrounding environment such as the sewage or surface water. These mobile genetic elements are then acquired by the neighbouring bacteria thus, gaining the resistance potential (Haudiquet et al., 2022). It has been reported that contaminated soil has more number of plasmid bearing bacteria than other sites. Some evidences also reveal cross resistance of antibiotics and metals in the isolates of contaminated environments (Sinigani and Younessi, 2017). This contamination of soil usually occurs due to the human activities in any particular area such as the use of manure or pesticide in agriculture field or the use of various chemicals in industries and their release in the industrial effluents. Sometimes, these effluents are used to water the agricultural lands which may cause the transfer of these heavy metals or toxic chemicals into the fields. According to the previous reports, plasmids have also been developed from genes of non-agricultural lands (Rajendran et al., 2022). Soil bacteria are a reservoir of mobile genetic elements which can be

transmitted to other bacteria without any restriction of taxonomic affiliation (Heuer et al., 2008).

Heavy metals are also a major concern in the environment as they are neither destroyed nor degraded. Some heavy metals are required in trace amounts by bacteria because they incorporate in the cofactors or enzymes. But bacteria may become toxic to even very low concentrations of heavy metals as they either bind to the DNA or release oxygen radicals (López-Maury et al., 2002). To survive under heavy metal stress conditions bacteria have acquired several adaptation mechanisms to combat the uptake of heavy metals. These mechanisms include efflux of metals outside the cell, metal accumulation and complexation inside the cell, and reduction of heavy metals to less toxic states. These resistance adaptations can be chromosomal, transposon or plasmid mediated (Sevgi et al., 2010).

In last decades it has been reported that bacteria with antibiotic resistance can arise in environment due to co-resistance or cross-resistance to the heavy metal resistance or the co-regulation of resistance pathways. This co-resistance of antibiotics and heavy metals is usually linked genetically due to mobile genetic elements like plasmids or transposons (Akinbowale et al., 2007). Antibiotic resistance genes are associated with other genetic elements. Whenever the primary selection of these associated genetic elements such as heavy metal resistance genes occurs, it ultimately causes the selection of antibiotic resistance genes. Thus, when

heavy metal resistant bacteria survive in heavy metal polluted environment, it creates a positive selective pressure for antibiotic resistance genes owing to the genetic heritage that they share (Di Cesare et al., 2016). The objectives of this study were the isolation and enumeration of the bacterial strains in the soil of agriculture farm and soap industry. Moreover, identification of the heavy metal and antibiotic resistant bacteria and determination of their minimum inhibitory concentrations was determined and to find a correlation of antibiotic and heavy metal tolerance in bacteria.

MATERIAL and METHODS

Soil sampling Sites

Soil samples were collected from two different sites including Agriculture farms, University of the Punjab, Lahore, Pakistan (31°29'49.46"N, 74°18'23.22"E) and Chemical Contaminated soil from a Soap industry in Sheikhupura, Pakistan (31°37'29.14"N, 73°54'46.23"E). Samples were collected aseptically in zip-lock bags. The physical characteristics of the collected samples, such as, pH and temperature were measured. The collected samples were brought on ice to the laboratory under aseptic conditions and samples were used after serial dilution for the isolation of strains.

Enumeration of total culturable bacteria in soil samples

For the isolation of total culturable bacteria from selected soil samples, one gram of each soil sample was added in 9ml of sterile distilled water and dilutions were prepared by subsequently

mixing 1ml in 9ml of autoclaved distilled water. An aliquot of 50 μ l sample was plated on L-agar. After incubation of 24 hours at 37°C, colony forming unit (CFU/ml) of the bacterial strains was calculated. Morphologically distinct colonies were picked and re-streaked to obtain pure cultures. Presumptive identification of these bacterial strains was done by catalase, oxidase, and starch hydrolysis tests (Cappuccino and Sherman, 2008).

16SrRNA Sequencing

The 16S rRNA sequencing of highly antibiotic and heavy metal resistant strain was done to confirm its taxonomic identity. Purified and isolated bacterial colony of strain GS-17 was sent to Macrogen (Seoul, Korea) for sequencing. Almost complete 16S rRNA sequence was determined with primers 785F (GGATTAGATACCCTGGTA) and 907R (CCGTCAATT CMTTTRAGTTT). The sequence obtained from this technique was analyzed by BLAST. With the help of Clustal W version 1.6, the close neighboring sequences were aligned and downloaded. Phylogenetic tree was constructed in MEGA-X software, using the neighbor joining algorithm with 1,000 replicates in the bootstrap method (Kumar et al., 2018).

Identification of heavy metal tolerant bacterial strains

Heavy metal tolerance profiling of selected strains was done on LB agar medium. To identify the heavy metals resistance potential of selected strains;

K₂CrO₄, CuCl₂, HgCl₂, NiCl₂, and PbCl₂ heavy metals were used. A stock solution (1g/10ml), was prepared and autoclaved. LB agar was supplemented with varied heavy metal concentrations (ranging from 100 to 2500µg/ml) and bacterial strains were streaked on it. The minimum concentration of heavy metal at which bacteria could not grow was considered as its minimum inhibitory concentration (Akinbowale et al., 2007).

Identification of antibiotic resistant bacterial strains

Isolated bacterial strains were also identified for their antibiotic resistance potential and the minimum inhibitory concentration of antibiotics for inhibiting bacterial growth. Ampicillin, Streptomycin and chloramphenicol were supplemented in LB agar medium. The concentration of antibiotics in LB agar medium ranged from 10µg/ml to 1000µg/ml, 5000µg/ml and 15,000µg/ml for chloramphenicol, ampicillin and streptomycin, respectively. The minimum

concentration of antibiotic at which bacterial strains could not survive was considered its minimum inhibitory concentration (Akinbowale et al., 2007).

RESULTS

Physical Parameters of soil samples

Samples for this study were collected from Agriculture farms, University of the Punjab, Lahore, Pakistan and Soap industry soil, Sheikhpura, Pakistan and abbreviated as GS and CS, respectively. The temperature and pH of the samples recorded is given in table 1.

Table 1: Physical parameters of soil samples

Sample	Temperature (°C)	pH
Agriculture Farms (GS)	28±0.3	6.9±0.1
Soap Industry Soil (CS)	30±0.2	7.2±0.05

Morphological and Physiological characterization of strains

A total of 27 strains were obtained from two different soil samples. From agriculture farm soil, 63% of the total strains were retrieved while, from chemical contaminated soap industry soil 37% bacterial strains were isolated.

Morphologically, strains were diverse in their color i.e. white, off-white, pink, orange and yellow. The colony morphology of these strains also varied from each other. Out of the total bacterial strains, 60% were gram positive rods, 33% gram negative rods, 7% gram positive cocci while no isolate was gram negative cocci. A total of 16

bacterial strains (60%) were catalase positive, 7 (26%) were oxidase positive while only 5 (18.5%) were positive for both tests. None of the strains showed any potential of starch hydrolysis.

Enumeration of total bacterial count

The CFU/g of soil sample was higher for agriculture farm soil i.e. between 1×10^6 and 1.2×10^8 cells while the CFU/g of soap industry soil ranged from 1.4×10^6 and 6×10^6 . Higher number of

bacteria in agricultural soil can be due to the nutrient rich environment in soil.

16SrRNA sequencing and Phylogenetic Analysis

The antibiotic and heavy metal resistant strain, GS-17, showed 99% homology to *Bacillus endophyticus*. The 16SrRNA sequence was submitted to GenBank with the accession number OM736154. The phylogenetic tree of this strain with neighboring members of *Bacillus* genera is given in Fig. 1.

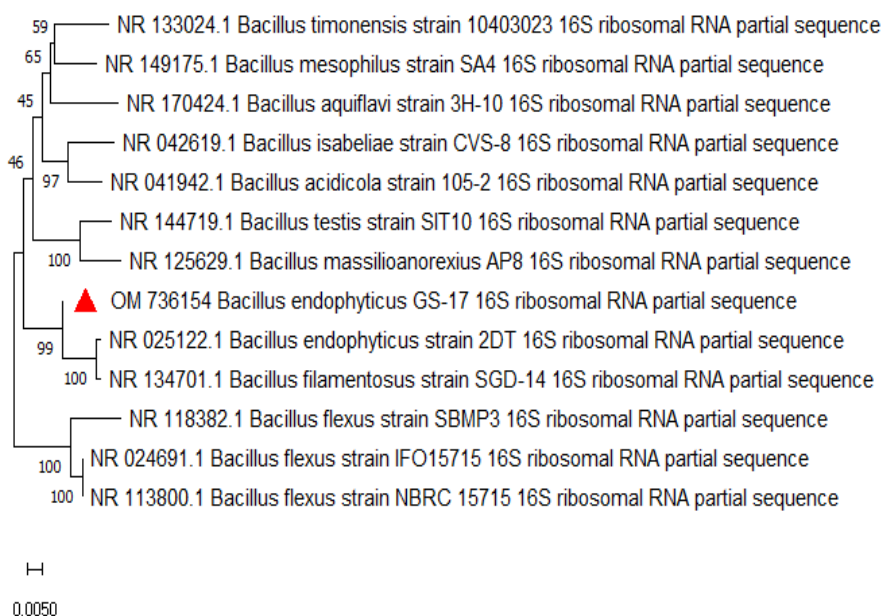


Fig. 1. Phylogenetic tree of antibiotic and heavy metal resistant bacterium GS-17 based on 16S rRNA gene sequencing and compared with other neighboring members of *Bacillus* genera.

Heavy Metal Resistance Profiling

Bacterial strains were screened for their resistance profiling against 5 different heavy metals. Lead was tolerated by maximum number of bacterial strains i.e. 65% and 90% of GS and CS sample bacterial strains, respectively. The percentage resistant strains of agriculture farm soil sample against Copper, Nickel Chromium and Mercury were 10 (59%), 3 (18%), 11 (65%) and 7 (41%) strains, respectively. Whereas the heavy metal tolerant bacterial strains in soap industry soil sample were 10% (Cr), 20% (Ni), 50% (Hg) and 90%

(Cu). Only 12 (44%) bacterial strains could resist all of the heavy metals together. Minimum inhibitory concentration for different bacterial strains ranged from 1000µg/ml to 250,000µg/ml for Chromium, 500µg/ml to 2000µg/ml for Nickel, 1000µg/ml to 10,000µg/ml for Lead, 1000µg/ml to 1500µg/ml for Copper and least resistance was observed against Mercury that ranged from 20µg/ml to 150µg/ml. Site specific comparison of heavy metal resistance in different isolated strains is shown in Fig. 2.

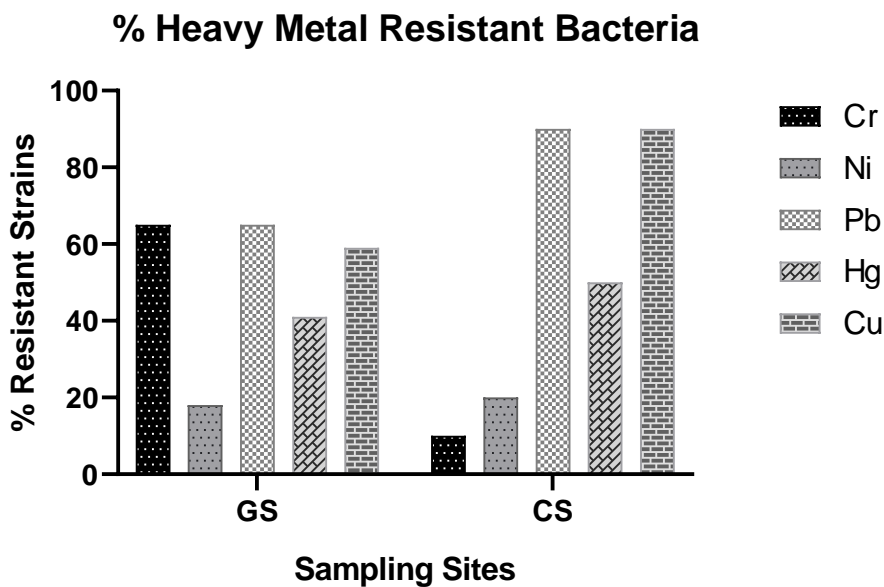


Fig. 2. Box plot of two sampling sites, showing the percentage resistant bacteria against each heavy metal i.e. Cr (Chromium), Ni (Nickel), Pb (Lead), Hg (Mercury), and Cu (Copper).

Antibiotic resistance profiling

Out of total bacterial strains, 81.5% (22 of 27) were resistant to ampicillin, 29% (6/27) were able to resist streptomycin while only 48.1% bacterial strains were resistant to chloramphenicol. However, only 7.4% (2/27) could resist all of the

antibiotics. Minimum inhibitory concentration of streptomycin for these bacterial strains ranged from 70µg/ml to 200µg/ml where maximum concentration could be tolerated by 5 (50%) bacterial strains of soap industry soil bacteria while, only 1 strain (3.7%)

of agriculture farms could tolerate highest concentration of streptomycin. For ampicillin, minimum inhibitory concentration had a range of 150µg/ml to 5000µg/ml for soap industry isolates where 50% bacterial strains (5/10) had the potential to resist 5000µg/ml concentration whereas, the minimum inhibitory concentration of ampicillin for agriculture farm soil is 150µg/ml to 1000µg/ml and the maximum

concentration was tolerable by 7.4% bacteria only. Chloramphenicol at the concentration of 20µg/ml could not be tolerated by most of the bacterial strains but 2 bacterial strains (7.4%) of GS sample and 1 isolate (10%) of CS soil sample were able to resist even 1000µg/ml concentration of chloramphenicol. Comparison of antibiotic resistance by bacteria of different soil samples is given in Fig. 3.

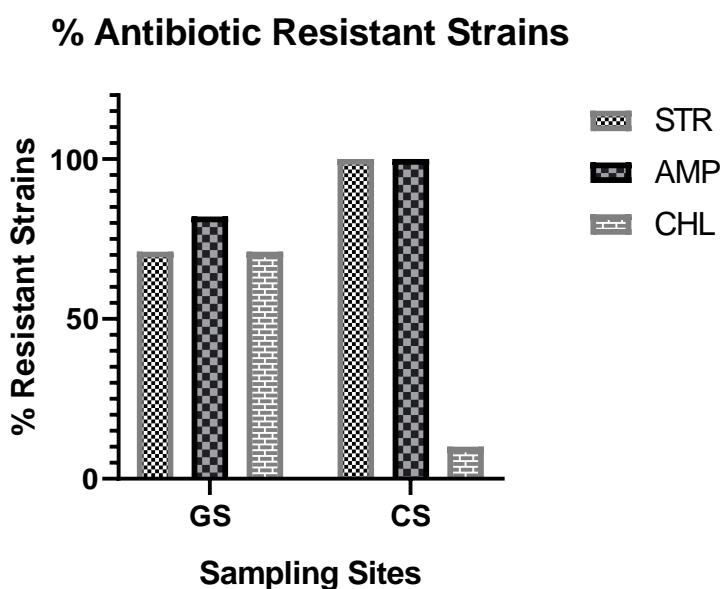


Fig. 3. Box plot of two sampling sites showing the percentage resistant bacteria against each antibiotic i.e. STR (streptomycin), AMP (Ampicillin) and CHL (chloramphenicol)

Co-Resistance of Antibiotics and Heavy Metals

Some of the bacterial strains had co-resistance of antibiotics and heavy metals as presented in figure 3 (heat map of antibiotics and heavy metal co-resistance). In soap industry soil bacterial strains, 90% strains presented

co-resistance of Streptomycin with lead and copper. Moreover, co-resistance of Ampicillin with lead was also observed in 90% bacterial strains of soap industry soil. In agriculture farm soil, maximum co-resistance of Ampicillin with chromium and lead was shown by 65% bacterial strains (Fig. 4).

Co-Resistance of Antibiotics and Heavy Metals in selected sites

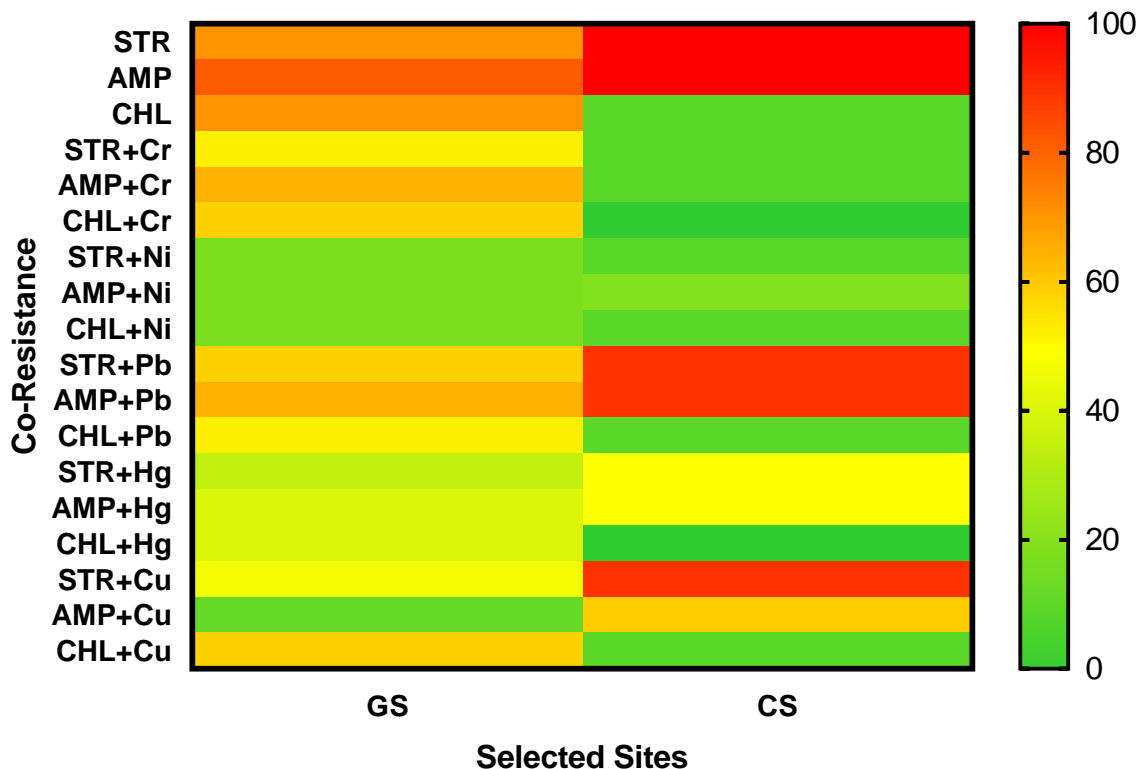


Fig. 4. Heat map of antibiotic-heavy metal Co-resistance in selected sites. Red color indicates the highest percentage of bacterial strains (100%) containing resistance for heavy metals and antibiotics while green color indicates minimum number of bacterial strains (0%). Where, GS= Agriculture farm Soil and CS= Soap industry Soil, STR= Streptomycin, AMP= Ampicillin, CHL= Chloramphenicol, Cr= Chromium, Ni= Nickel, Pb= Lead, Hg= Mercury, and Cu= Copper.

DISCUSSION

The bacterial strains in this study were found to be resistant to antibiotics and heavy metals to a great extent. This resistance pattern of bacteria towards antibiotics and heavy metals is highly influenced by the anthropogenic activities as described previously in many studies (Pei et al., 2006). The overuse of antibiotics in daily routine as

well as untreated dumping of industrial effluent has led to remarkable negative impact on human health. It has also promoted transfer of resistance genes among different bacteria for the purpose of their survival (Najar et al., 2020). The presence of antibiotic resistance in agriculture farm bacterial strains can be attributed to the use of manure in the fields for the purpose of better growth of

vegetables (Li et al., 2022). Use of manure in agricultural lands is a traditional practice. Manure helps to improve the soil fertility but its long term effects on environment are unknown. In animal husbandry, a myriad of antibiotics is utilized which causes the release of antibiotic resistance genes in animal manure. If this manure is not treated properly or directly applied to the agricultural land it consequently allows the horizontal gene transfer of these resistance genes to the indigenous bacteria of that land thus making them antibiotic resistant (Tyrrell et al., 2019). It has been reported that antibiotics degrade in soil but heavy metals are ubiquitous in nature and are increasing due to industrialization and urbanization (Sinigani and Younessi, 2017). In this study, it was observed that antibiotic (streptomycin, chloramphenicol and ampicillin) and heavy metal (Cr, Cu, Ni, Hg and Pb) resistance co-existed in some of the bacterial strains. This resistance of both antibiotics and heavy metals in bacteria occurs due to agricultural or sewage contaminations which may not only increase the antibiotics, but also increases the heavy metal concentration in soil (Quinton and Catt, 2007). Maximum resistance against antibiotics and heavy metals was observed in the bacterial strains of soap industry soil. Heavy metal resistance is prevalent in chemical contaminated soils (Thomas et al., 2020). As discussed in previous studies, heavy metal resistance is genetically linked to antibiotic resistance in bacteria especially on plasmids thus bacteria possessing heavy

metal resistance can easily acquire antibiotic resistance (Das et al., 2013). The high resistance patterns of bacterial strains towards mercury and lead can be particularly due to the resistance genes present on transposable elements. A group of researchers, demonstrated a linkage of mercury with antibiotics on plasmids in *Enterobacteriaceae* (Essa, Julian, Kidd, Brown, & Hobman, 2003). Other researchers have also reported co-occurrence of lead and antibiotics resistance in *Pseudomonas*, *Corynebacterium*, *Bacillus* and *Enterobacter* in the bacterial strains of heavy metal contaminated soil bacterial strains. According to Knapp et al. Nickel is also considered to possess antibiotic resistance genes correlated to heavy metal resistance genes (Knapp et al., 2011).

The variation in resistance profiles of bacterial strains is also dependent on the sampling sites. The metal contaminations in natural soil are different from the soil which is under human influence thus the resistance mechanisms in both sites may vary. Moreover, antibiotic resistance pattern of the bacterial strains is also dependent on the physical and chemical properties of soil. In this study, agricultural soil bacterial isolates showed more resistance towards chloramphenicol as compared to streptomycin and ampicillin. While in soap industry soil, streptomycin and ampicillin resistant bacterial strains were more prevalent. This difference in resistance patterns is attributed to the variation in exposure of the soil to various contaminants from wastewater, manure, effluents or other

sources that contain antibiotic resistance genes (Ashelford et al., 2000).

CONCLUSION

Co-resistance of antibiotics and heavy metals was predominant in soap industry soil samples. Soap industry soil is usually contaminated with the chemicals being used in the processing thus promoting the growth of resistant bacteria in this soil. When these resistant bacteria survive in such polluted environment, it creates a positive selective pressure for antibiotic resistance genes as well, owing to their genetic linkage on mobile genetic elements.

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ETHICAL APPROVAL

The study was approved by the institutional ethical review committee.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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