BRIEF REPORT

# HOSPITAL EFFLUENTS AS A RESERVOIR OF BETA-LACTAMASE- AND CARBAPENEMASE-PRODUCING *ENTEROBACTERIACEAE*

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## ABSTRACT

The aim of this study was to determine the presence of beta-lactamase- (*bla*) producing *Enterobacteriaceae* in hospital effluent samples from two level II and III hospitals in Lima, Peru. The resistance profile of the isolated bacteria was identified and characterized using the MicroScan system for 18 antimicrobials, and the presence of extended spectrum beta-lactamases (ESBL) (bla$_{CTX-M}$, *bla*$_{SHV}$ *bla*$_{TEM}$, *bla*$_{PER}$) and carbapenemases (*bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$) resistance genes was determined by conventional PCR. Thirty-two isolates were identified (20 *Enterobacteriaceae* and 12 gram-negative bacteria). All the isolated bacteria showed multidrug resistance. ESBL (*bla*$_{TEM}$) and carbapenemase (*bla*KPC, *bla*IMP) genes were found in samples from the hospitals that we evaluated. The release of these microorganisms to public areas and the lack of treatment of the hospital effluents could be an important public health problem.

**Keywords:** Antibacterial Drug Resistance; Multiple Antibacterial Drug Resistance; Antibacterial Agents; Waste Water; Sewerage; Public Health; Public Hospitals; Peru (Source: MeSH NLM).

## INTRODUCTION

Multi-drug resistance in bacteria is considered a health problem, due to the high mortality and the lack of therapeutic options [1]. Previous studies have shown that hospital effluents contain antibiotics and multi-drug resistant bacteria [2,3], especially enterobacteria that produce betalactamases with the capacity to hydrolyze penicillins, cephalosporins, monobactams, carbapenems and beta-lactamase inhibitors [4]. In this sense, hospital effluents are a major reservoir of bacteria with antimicrobial resistance.

Unlike other countries, where hospital effluents are treated in a specialized process [5], only five hospitals in Peru treat their solid waste, but not their liquid waste [6]. In most cases, hospital effluents flow directly into nearby rivers where people are contaminated by using the water to wash their clothes or for personal hygiene [7].

Therefore, the aim of this research was to determine the presence of extended-spectrum betalactamase (ESBL)- and carbapenemase-producing bacteria in the hospital effluents of two hospitals in Lima and to characterize their resistance profile, to provide evidence that can be used to promote control and biosafety measures.

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## THE STUDY

This was a descriptive cross-sectional study conducted at the Laboratory for Research in Molecular Biology of the Universidad Peruana Unión (LIBM-UPeU). Hospital effluent samples were obtained from two hospitals in Lima. The level II Huaycán Hospital, located in the district of Ate has emergency and hospitalization services and receives about 647,000 people [8]. The level III Hipólito Unanue Hospital located in the district of El Agustino has outpatient, emergency, hospitalization, and surgery services; and during 2018, about 200,000 medical attentions were carried out in this hospital [9].

During June and July 2019, Wastewater effluent samples were collected prior to disposal into the sewerage system. Two samples were obtained in 100 mL sterile bottles for each hospital with a difference of 30 minutes between both collections at the same location. Subsequently, they were transported in cold chain (2-8 °C) to the laboratory. The collected samples were diluted with distilled water (1:50 dilution) and 1 mL was inoculated in triplicate in selective media for bacteria with phenotype resistant to penicillins, third-generation broad-spectrum cephalosporins and monobactams (medium, CHROMagar ESBL) and in selective media for bacteria with phenotype resistant to carbapenemases (medium, CHROMagar mSupercarba), according to the manufacturer's instructions [10]. Then, the colonies were reseeded on MacConkey agar, four strains per plate, and incubated at 37 °C for 24 hours. This was done to isolate the colonies and identify them.

Identification and susceptibility profiling was carried out using the MicroScan® automated system (AutoScan-4) and the use of panels for gram-negative bacteria (Dade MicroScan®), following the manufacturer's instructions [10]. Eighteen antimicrobials were used for each strain. The minimum inhibitory concentration (MIC) values were used to interpret the antibiotic resistance profile according to the cut-off points recommended by the Clinical and Laboratory Standards Institute 2020 [11]. Multidrug resistance was defined as the detection of a phenotype resistant to at least one antimicrobial from three or more families [12].

DNA extraction was performed by the bacteria-specific silica gel column-based method with the innuPREP Bacteria DNA kit (Analytikjena, Germany), following the manufacturer's protocol [13]. The isolated DNA was stored at –20 °C until amplification of each gene by conventional PCR. For the detection of ESBL genes ($bla_{CTX-M}$, $bla_{SHV}$, $bla_{TEM}$, $bla_{PER}$) and carbapenemases ($bla_{KPC}$, $bla_{NDM}$, $bla_{VIM}$, $bla_{IMP}$), we used conventional PCR in an endpoint thermal cycler (Bio-Rad, USA) [14,15]. The primers used for this procedure are detailed in Table 1. The amplification products were visualized by electrophoresis in 2% agarose gels at 120v for 55 minutes, according to the protocol standardized at LIBM-UPeU.

The results were processed with Labpro software and exported to Microsoft Excel where they were subsequently analyzed. We obtained the number of isolates identified by hospital. A heat map was prepared with the antibiotic resistance profile of the isolated bacteria for each hospital and for the *bla* gene profile [11].

The study was classified as risk-free research and did not require informed consent. Data management was carried out under strict confidentiality parameters. This research was evaluated and approved by the Ethics Committee of the Universidad Peruana Unión.

### KEY MESSAGES

**Motivation for the study:** Hospital effluents containing antibiotics and multi-drug resistant bacteria are not treated, and it could result into a public health problem.

**Main findings:** The isolated enterobacteria and gram-negative bacteria showed multidrug resistance. ESBL genes ($bla_{TEM}$) and carbapenemases ($bla_{KPC}$ and $bla_{IMP}$) were found in effluents from level II and III hospitals in Lima.

**Implications:** The high presence of enterobacteria and multidrug-resistant gram-negative bacteria that produce $bla_{TEM}$, $bla_{KPC}$ and $bla_{IMP}$ genes in hospital effluents is alarming and should lead to the implementation of wastewater treatment systems before discharge into the sewer system.

## FINDINGS

Thirty-two strains of the *Proteobacteria* phylum were isolated; 14 (43.8%) and 18 (56.2%) were isolated in level II and III hospitals, respectively. In level II hospital, 8 (57.1%) enterobacteria and 6 (42.9%) gram-negative bacteria were

**Table 1.** Primers used for detecting ESBL resistance genes and carbapenemases.

| Genes | Amplicon (bp) | Primer | Sequence |
|---|---|---|---|
| $bla_{CTX-M}$ [14] | 544 | CTX/F<br>CTX/R | TTTGCGATGTGCAGTACCAGTAA<br>CGATATCGTTGGTGGTGCCAT |
| $bla_{TEM}$ [14] | 931 | TEM/F<br>TEM/R | TCCGCTCATGAGACAATAACC<br>TTGGTCTGACAGTTACCAATGC |
| $bla_{SHV}$ [14] | 868 | SHV/F<br>SHV/R | TGGTTATGCGTTATATTCGCC<br>GGTTAGCGTTGCCAGTGCT |
| $bla_{PER}$ [14] | 927 | PER/F<br>PER/R | ATGAATGTCATCACAAAATG<br>TCAATCCGGACTCACT |
| $bla_{KPC}$ [15] | 916 | KPC/F<br>KPC/R | AACAAGGAATATCGTTGATG<br>AGATGATTTTCAGAGCCTTA |
| $bla_{NDM}$ [15] | 512 | NDM/F<br>NDM/R | AGCACACTTCCTATCTCGAC<br>GGCGTAGTGCTCAGTGTC |
| $bla_{VIM}$ [15] | 261 | VIM/F<br>VIM/R | AGTGGTGAGTATCCGACAG<br>ATGAAAGTGCGTGGAGAC |
| $bla_{IMP}$ [15] | 404 | IMP/F<br>IMP/R | GGYGTTTWTGTTCATACWTCKTTYGA<br>GGYARCCAAACCACTASGTTATCT |

F: forward, R: reverse; CTX-M: cefotaxime; TEM: temoniera; SHV: sulfhydryl variable; KPC: Klebsiella pneumoniae carbapenemase; NDM: New Delhi metallo β -lactamase; IMP: Imipenemase metallo β-lactamase; VIM: Verona encoded-integron metallo βlactamase; bp: base pairs; bla: beta-lactamase genes.

identified; and in level III hospital, 12 (66.7%) enterobacteria and 6 (33.3%) gram-negative bacteria were identified (Table 2). A total of 20 (62.5%) enterobacteria were identified; the most frequent were *Enterobacter cloacae* (6/20; 30%), *Escherichia coli* (5/20; 25%) and *Citrobacter freundii complex* (4/20; 20%).

Regarding the resistance phenotype, 100% of isolates from both hospitals showed multidrug resistance, including resistance to beta-lactams, ciprofloxacin, trimethoprim/sulfamethoxazole, erythromycin, tigecycline, and an amikacin-resistant Pseudomonas aeruginosa (Figure 1). The antibiotic resistance profile of enterobacteria (8/20) isolated from level II hospital was amikacin (0.0%), clavulanic acid (37.5%), ampicillin/sulbactam (62.5%), ampicillin (100.0%), aztreonam (100.0%), cefepime (87.5%), cefotaxime (100.0%), ceftazidime (87.5%), cefuroxime (100.0%), ciprofloxacin (87.5%), erythromycin (50.0%), gentamicin (37.5%), imipenem (12, 5%), meropenem (3.1%), piperacillin/tazobactam (9.4%), tigecycline (0.0%), tobramycin (15.6%), trimethoprim/sulfamethoxazole (9.4%).

The antibiotic resistance profile of enterobacteria (12/20) from level III hospital was amikacin (0.0%), clavulanic acid (91.7%), ampicillin/sulbactam (91.7%), ampicillin (100.0%), aztreonam (100.0%), cefepime (100.0%), cefotaxime (100.0%), ceftazidime (100.0%), cefuroxime (100.0%), ciprofloxacin (41, 7%), erythromycin (66.7%), gentamicin (41.7%), imipenem (41.7%), meropenem (21.9%), piperacillin/tazobactam (15.6%), tigecycline (0.0%), tobramycin (6.3%), trimethoprim/sulfamethoxazole (9.4%) (Figure 2).

**Table 2.** Number and frequency of species isolated from effluents of two hospitals in Lima, according to level of healthcare.

| Bacterial species (n=32) | Level II Hospital | | Level III Hospital | |
|---|---|---|---|---|
| | n=14 | % | n=18 | % |
| Enterobacteria | 8 | 57.1 | 12 | 66.7 |
| *Citrobacter freundii complex* | 3 | 21.4 | 1 | 5.6 |
| *Enterobacter cloacae* | WI | 0.0 | 6 | 33.3 |
| *Enterobacter asburiae* | 1 | 7.1 | WI | 0.0 |
| *Escherichia coli* | 3 | 21.4 | 2 | 11.1 |
| *Escherichia vulneris* | WI | 0.0 | 1 | 5.6 |
| *Klebsiella oxytoca* | 1 | 7.1 | 2 | 11.1 |
| Gram-negative | 6 | 42.9 | 6 | 33.3 |
| *Achromobacter xylosoxidans* | WI | 0.0 | 1 | 5.6 |
| *Aeromonas hydrophila* | 1 | 7.1 | WI | 0.0 |
| *Pseudomonas aeruginosa* | 1 | 7.1 | 1 | 5.6 |
| *Pseudomonas* spp. | 1 | 7.1 | WI | 0.0 |
| *Vibrio fluvialis* | 2 | 14.3 | 3 | 16.7 |
| *Yersinia enterocolitica* | 1 | 7.1 | 1 | 5.6 |

WI: without bacterial isolate.

The distribution of the genes detected in level II hospital was 21.4% (3/14, isolates) for the $bla_{TEM}$ gene: *Escherichia coli* (n=2) and *Klebsiella oxytoca* (n=1); 28.6% for the $bla_{KPC}$ gene (4/14, isolates): *Citrobacter freundii complex* (n=2), *Pseudomonas aeruginosa* (n=1) and *Vibrio fluvialis* (n=1); and 7.1% (1/14, isolates) for the $bla_{IMP}$ gene: *Pseudomonas* spp. The distribution of genes identified in the level III hospital was 38.9% (7/18, isolates) for $bla_{TEM}$: *Enterobacter cloacae* (n=2), *Pseudomonas aeruginosa* (n=1), *Yersinia enterocolitica* (n=1), *Klebsiella oxytoca* (n=1), *Escherichia coli* (n=1) and *Escherichia vulneris* (n=1); and 44.4% for $bla_{KPC}$ gene (8/18, isolates): *Enterobacter cloacae* (n=4), *Vibrio fluvialis* (n=3) and *Citrobacter freundii complex* (n=1). We did not find $bla_{CTX-M}$, $bla_{SHV}$ and $bla_{PER}$ genes in any of the ESBL-producing isolates, and we did not find $bla_{NDM}$ and $bla_{VIM}$ genes in any carbapenemase-producing isolates either (Figure 1).

## DISCUSSION

In this study, all the isolated enterobacteria and gram-negative bacteria were multidrug resistant. Amikacin and tigecycline were the most sensitive antibiotics, whereas 70%100% of the isolates were resistant to ampicillins, cephalosporins, monobactams and carbapenemics. ESBL ($bla_{TEM}$) and


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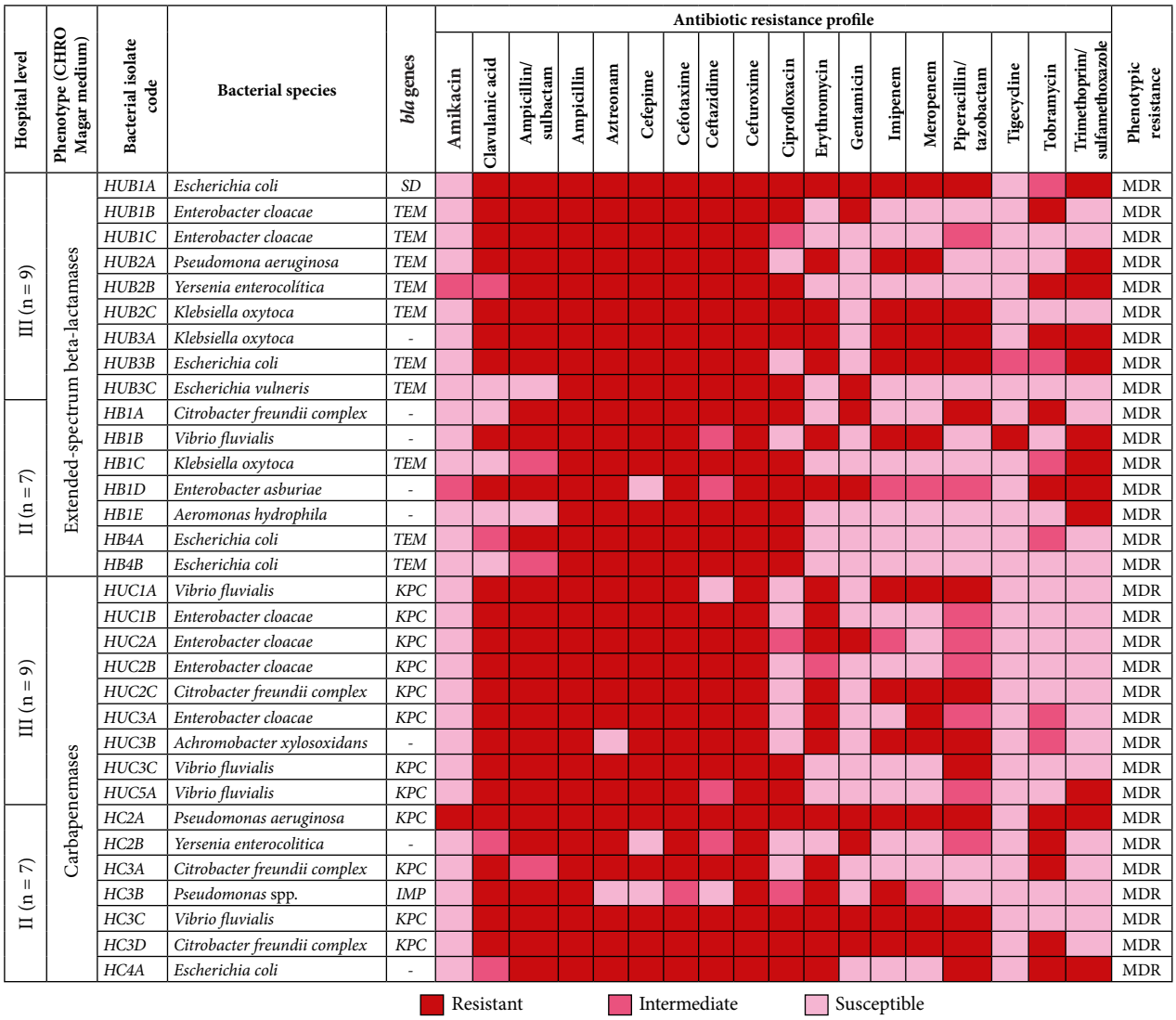


*bla:* beta-lactamase genes; TEM: temoniera; KPC: *Klebsiella pneumoniae* carbapenemase; IMP: Imipenemase metallo β-lactamase; MDR: multi-drug resistance (resistant to at least one antibiotic from 2 or 3 different classes); ND: no detection of ESBL or carbapenemase genes.

**Figure 1.** Antibiotic resistance profile according to phenotype, phenotypic resistance, and betalactamase genes (ESBL and carbapenemases) in enterobacteria and gram-negative bacteria from two hospitals in Lima.

carbapenemase genes ($bla_{KPC}$ and $bla_{IMP}$) were found in level II and level III hospitals, with a higher frequency in the latter level.

Regarding the resistance profile, all enterobacteria and gram-negative bacteria showed resistance to beta-lactam antibiotics, with multidrug resistance in 100% of the isolates. This differs from the description in a hospital in Brazil, where multidrug resistance was found to be 33.3%, with 38.0% resistance to cefoxitin; 27.0% to ceftazidime; 22.0% to cefepime, 13.0% to imipenem, 11.0% to meropenem and 44.0% to aztreonam [16]. It also differs from another study in China, where 85.5% multidrug resistance was found, with 77.4% resistance to trimethoprim/sulfamethoxazole; 66.1% to amoxicillin/clavulanic acid; 61.3% to cefoxitin; and 61.3% to ciprofloxacin [17]. The results of this study show higher resistance rates, however only 3.1% resistance to meropenem was found in level II hospital. One possible explanation could be the lack of a liquid waste treatment system in the evaluated hospitals. This problem could be found in other hospitals in Peru, where liquid waste is not treated, which could be harmful to the population that uses river water for hygiene and agriculture.

On the other hand, ESBL resistant genes were detected in 21.4% and 38.9% of the isolates from level II and III hospitals, respectively, with the $bla_{TEM}$ gene predominating in bacteria, such as *Escherichia coli* and *Klebsiella oxytoca*. Likewise, in a study carried out in Brazil, ESBL genes were detected in 35% of the cases, however,

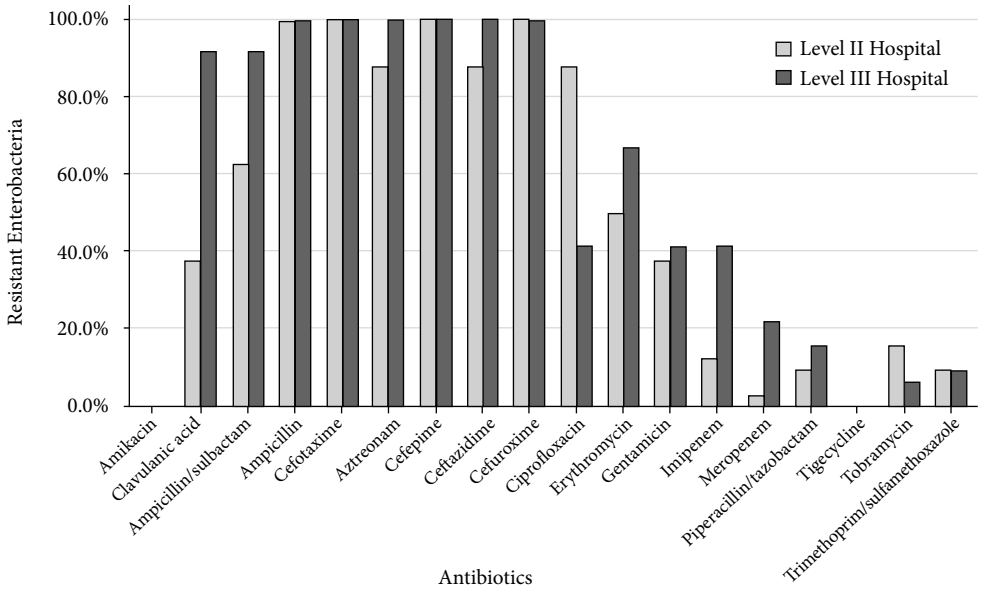


**Figure 2.** Antibiotic resistance profile of enterobacteria from two hospitals in Lima, Peru.

the $bla_{CTX}$ gene predominated in bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Hafnia alvei*. This difference could be due to the susceptibility profiles found are different when the $bla_{CTX}$ gene predominates, since there is high resistance to gentamicin, trimethoprim/sulfamethoxazole, and ciprofloxacin, which occurs mainly because of the plasmid transmission mechanism in these bacteria [16]. Carbapenemics-associated genes were detected in almost 28.6% and 44.4% of the isolates from level II and III hospitals, respectively, with the $bla_{KPC}$ gene predominating in both. In the study from Brazil, the presence of the $bla_{KPC}$ gene associated with ESBL was also detected [16]. On the other hand, in a university hospital in Switzerland, the researchers found the presence of the *OXA-48* gene associated with *Escherichia coli* and *Citrobacter freundii* [18]. However, our study highlights the presence of the $bla_{IMP}$ gene associated with *Pseudomonas* spp.

A higher number of multidrug resistant enterobacteria was found in level III hospital, which could be due to the high demand of a population of more than two million inhabitants in the central and eastern districts of Lima [19]. In contrast, level II hospital, which cares for a population of approximately 200,000 inhabitants [20] and has fewer specialties, had a lower incidence of enterobacteria.

The limitation found in this study was only one sampling point of hospital effluents was accessible; two more sampling points should have been considered, which correspond to points where urban wastewater is mixed with hospital effluent and urban wastewater already mixed with hospital effluents from the public sewer system [17]. Likewise, the results could not be extrapolated to hospitals of other levels of care and in other provinces of Peru. However, according to the review carried out, this is the first study that identifies the phenotype and resistance genes of enterobacteria from effluents from two Peruvian hospitals.

In conclusion, phenotypic multidrug resistance was found in all isolates. Likewise, we found genotypic resistance to ESBL genes ($bla_{TEM}$) and carbapenemases ($bla_{KPC}$ and $bla_{IMP}$), with a greater presence in level III hospital. We recommend to carry on further studies on multidrug resistance of bacteria present in hospital effluent wastewater from hospitals of different levels (I, II, III and IV) in Peru to determine the microbiological quality of hospital effluents, and to implement hospital wastewater treatment systems.

**Authorship contributions:** PMC conceived the original idea, formulated and reviewed the project. JYY participated in project formulation and review. RSM, ARC, ACQ, MLB, MHS participated in project writing, sample collection and experimental development. AFL and DLL participated in experimental development, results analysis, discussion, and critical review. RSM, JYY, ARC, ACQ, MLB, MHS, MGP and PMC participated in the conception of the article, analysis of results, writing of the results, discussion, critical revision, and evaluation of the final version of the manuscript. All authors approved the final version of the manuscript.

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