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# Intrahospital spread of carbapenem-resistant *Pseudomonas aeruginosa* in a University Hospital in Florianópolis, Santa Catarina, Brazil

## Disseminação Intrahospitalar de *Pseudomonas aeruginosa* em Hospital Universitário de Florianópolis, Santa Catarina, Brasil

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**ABSTRACT**

**Introduction:** Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) has been isolated with increasing frequency in Brazilian hospitals. Since June 2003, its detection in a teaching hospital in the city of Florianópolis, Brazil, has increased. This study aimed to investigate the minimal inhibitory concentration (MIC), presence of Metallo-β-lactamase (MβL) and a possible clonal relationship among the isolates. **Methods:** The study included 29 CRPA and seven isolates with reduced susceptibility. The MIC was determined by agar-dilution. Detection of MβL was performed by Double Disk Sinergism (DDS) and Combined Disk (CD). The MβL gene was verified by PCR and nucleotide sequence analysis. Epidemiological typing was performed by pulsed-field gel electrophoresis. **Results:** Among the 29 carbapenem-resistant isolates, polymyxin B presented 100% susceptibility and piperacillin/tazobactam 96.7%. Seventeen (62%) strains were verified as clonal (A clone) and among these, six isolates indicated phenotypically positive tests for MβL and harbored the $bla_{SPM-1}$ gene. The first CRPA isolates were unrelated to clone A, harbored $bla_{IMP-16}$ and were phenotypically positive only by CD. **Conclusions:** The spread of a high-level of resistance clone suggests cross transmission as an important dissemination mechanism and has contributed to the increased rate of resistance to carbapenems. This study emphasizes the need for continuous surveillance and improved strategies for infection control in this institution.

**Key-words**: *Pseudomonas aeruginosa.* Carbapenem resistance. Nosocomial infections.

**RESUMO**

**Introdução:** O isolamento de *Pseudomonas aeruginosa* resistente aos carbapenêmicos (PARC) tem sido cada vez mais frequente nos hospitais brasileiros. O presente estudo investigou a concentração inibitória mínina (CIM), a presença de metalo-β-lactamases (MβL), e uma possível relação clonal entre PARC isoladas entre junho de 2003 a junho de 2005, em um hospital escola na cidade de Florianópolis, Brasil. **Métodos:** O estudo incluiu 29 PARC e sete isolados com suscetibilidade reduzida. A CIM foi determinada por diluição em ágar. A detecção de MβL foi realizada por sinergismo de duplo disco (SDD) e disco combinado (DC). Genes para MβL foram pesquisados por PCR e confirmados pela análise da sequência de nucleotídeos. A tipagem epidemiológica foi realizada por gel de eletroforese em campo pulsátil. **Resultados:** Entre os 29 isolados resistentes aos carbapenêmicos, 100% apresentaram suscetibilidade a polimixina B, e 96,7% a piperacilina/tazobactam. Dezessete (62%) destes isolados pertenciam a um mesmo clone (clone A); entre estes, seis isolados apresentaram testes fenotípicos positivos para MβL e carreavam o gene $bla_{SPM-1.}$ O primeiro isolado PARC não foi relacionado ao clone A, carreava o gene $bla_{IMP-16}$ e foi fenotipicamente positivo somente por DC. **Conclusões:** A propagação de um clone com alto nível de resistência sugere a transmissão cruzada como um importante mecanismo de disseminação e tem contribuído para o aumento nos níveis de resistência aos carbapenêmicos. Este estudo enfatiza a necessidade de vigilância contínua e melhoramento nas estratégias de controle de infecção nesta instituição.

**Palavras-chaves:** *Pseudomonas aeruginosa.* Resistência aos carbapenêmicos. Infecção nosocomial.

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## INTRODUCTION

*Pseudomonas aeruginosa,* one of the main microorganisms that cause nosocomial infections[1,2], is known for its intrinsic resistance to a range of antimicrobial agents[3]. It can also become resistant to all commercially available antimicrobial agents by developing numerous resistance mechanisms[2,3]. For two decades, carbapenems were considered an excellent therapeutic choice for such infections. However, *Pseudomonas aeruginosa* resistant to this class of antimicrobial agent has been isolated with increasing frequency in Brazilian hospitals, mainly in Intensive Care Units[4,5]. Studies suggest that infection caused by carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) significantly increases mortality in critically ill patients[6,7]. The most common form of resistance is through either lack of drug penetration (i.e. porin mutations and efflux pumps) and/or carbapenem-hydrolyzing β-lactamases, such as metallo-β-lactamases (MβL)[3]. It has been suggested that in the absence of MβL, high imipenem resistance rates in isolates can show great genomic variability, which can be associated with continuous selection of resistant mutants[8]. However, in many geographic regions of Brazil, this has been associated with the dissemination of an epidemic clone that produces SPM MβL[9]. Clinical isolates of CRPA have been increasingly detected since June 2003 in a teaching hospital in the City of Florianópolis, Santa Catarina, Brazil. The aim of this study was to investigate the minimum inhibitory concentration (MIC) for antipseudomonas antimicrobials, the presence of MβL and a possible clonal relationship among isolates.

## METHODS

### Bacterial isolates

This study included clinical isolates of CRPA from patients at the University Hospital, Federal University of Santa Catarina (HU/UFSC), between June 2003 and June 2005. Twenty-nine isolates

showed high-level resistance to carbapenems and seven isolates presented reduced susceptibility. The samples came mainly from patients in the Intensive Care Unit (32.8%) and Internal Medicine Unit III (19.8%), but isolates from other inpatient units were also collected. The bacteria were identified by conventional biochemical tests in accordance with the published recommendations[10].

**Antimicrobial susceptibility test**

The MIC of bacterial isolates was determined for each of nine antimicrobial agents (amikacin, ceftazidime, aztreonam, cefepime, ciprofloxacin, imipenem, meropenem, piperacillin/tazobactam and polymyxin B), performed by the agar dilution method and interpreted in accordance with CLSI[11]. *Pseudomonas aeruginosa* ATCC 27853 was used for quality control.

**Phenotypic detection of metallo-β-lactamase**

Two methods were used to screen the isolates for MβL detection: the double-disk synergy test[12] (DDS), using 2-mercaptopropionic acid (MPA) (Sigma, Steinheim, Germany), and the ceftazidime disk (CAZ - 30μg), placed 20 mm away; and the combined disk[13] (CD) test, using disk of imipenem (IMI - 10μg) with and without ethylenediaminetetraacetic acid (EDTA) (930μg) (Invitrogen, SanDiego, USA). DDS test results were considered positive if the growth inhibition zone increased or if a ghost zone appeared, while the CD test was considered positive if the increase in zone diameter was ≥ 7mm. SPM-1-producing *Pseudomonas aeruginosa* and IMP-producing *Acinetobacter baumannii* were used as positive controls.

**Molecular detection of metallo-β-lactamase genes**

All isolates were tested for the presence of $bla_{SPM-1}$, $bla_{IMP}$, $bla_{VIM}$ genes by polymerase chain reaction (PCR) using primers, as previously described[14]. Total DNA was obtained by boiling bacterial cells. PCR conditions used for $bla_{SPM}$ and $bla_{VIM}$ were performed according to Toleman et al[15], while the conditions used to detect the $bla_{IMP}$ gene were previously described by Gales et al[16]. Positive controls for $bla_{SPM-1}$, $bla_{VIM-1}$ and $bla_{IMP}$ genes were run simultaneously.

**Sequencing**

Amplicons obtained from an SPM-producing isolate and from the IMP-producing isolate were sequenced, using the set of primers previously described. The amplification products for the $bla_{IMP-1}$ and $bla_{SPM-1}$ genes were purified using a GFX-TM PCR purification kit (Amersham Bioscience, Piscataway, USA). The sequences were identified with MegaBACE™ (Amersham Bioscience, Piscataway, USA), analyzed with ChromasPro version 1.33 (Technelysium Pty LTDA), and compared with GenBank database sequences using BLAST tool (http://www.ncbi.nih.gov/BLAST).

**Pulsed-field gel electrophoresis (PFGE)**

DNA of all isolates was prepared as described previously[17] and cleaved with *Spe*I (10U) (Fermentas, Glen Burnie, USA) at 37°C. Electrophoresis was performed on a CHEF - DRIII (Bio-rad Laboratories, Hercules, USA) for 23h at 6V/cm at 12ºC and pulse times from 5 to 60 s. The gels were analyzed with Gel-Pro Analyzer 4.0 and NTSYS 2.02 software. Clusters of possibly related isolates were identified using the Dice similarity coefficient and unweighted pair-group method with arithmetic averages (UPGMA). Identical isolates were assigned the same capital letter. Isolates with more than 90% similarity were assigned as a subtype of the major type, which was designated with the same capital letter followed by an Arabic number (e.g. A1, A2, A3, A4).

## RESULTS

*Pseudomonas aeruginosa* resistant to carbapenems (imipenem and meropenem), were isolated from the urinary tract (37.9%), bloodstream (31%), respiratory tract (13.8%) and from other anatomical sites (17.3%). All of them were susceptible to polymyxin B, and 96.7% to piperacillin/tazobactam. Susceptibility to the other antimicrobial agents tested was infrequent, 6% to aztreonam and cefepime; 10% to ciprofloxacin; 16.7% to amikacin and 43% to ceftazidime. Six out of the seven isolates that showed reduced susceptibility to carbapenems presented reduced susceptibility only to meropenem (MIC 8μg/mL), while the remaining isolate showed reduced susceptibility to both carbapenems tested (meropenem and imipenem) (MIC 8μg/mL).

Isolates that showed intermediate susceptibility to carbapenems presented negative results in the phenotypic test for MβL. When the 29 carbapenem-resistant isolates were tested, seven MβL-producing isolates were detected by the CD test and six isolates by DDS. When PCR was used to detect MβL genes, six of the seven isolates phenotypically positive for MβL yielded a 650 bp product compatible with a fragment amplified from $bla_{SPM-1}$, while one isolate yielded a 590 bp product compatible with a fragment amplified from $bla_{IMP}$. The remaining isolates did not generate PCR products. The results of PCR for $bla_{SPM-1}$ confirmed the findings of both phenotypic methods

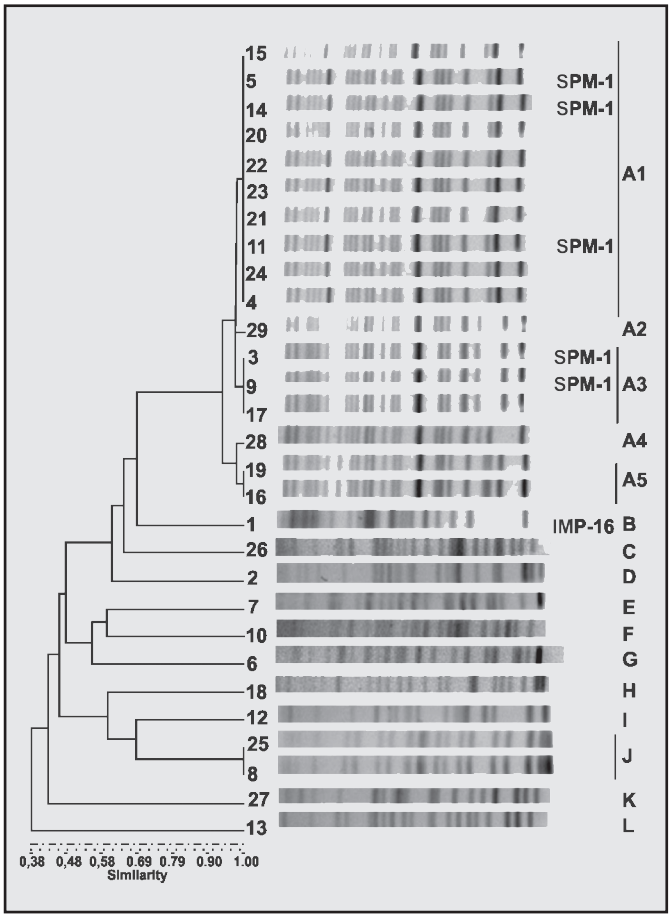


**FIGURE 1 - Dendrogram and electrophoresis showing profiles of PFGE restriction fragments cleaved with *Spe*I enzyme from 29 isolates of carbapenem-resistant *Pseudomonas aeruginosa* isolated in a teaching hospital in Florianópolis, Santa Catarina, Brazil, during the period from July 2003 to July 2005.**

**TABLE 1 - Genotypic and phenotypic data for the characterisation of 29 isolates of carbapenem-resistant *Pseudomonas aeruginosa* used in this study.**

| Isolates | | | MIC(μg/mL) Antimicrobials** | | | | | | | | | MβL detection* phenotypic | | MβL detection* genotypic | PFGE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | Data of isolation | Unit*** | AMI | CAZ | AZT | CPM | CIP | IMI | MER | PTZ | POL | DDS | CD | MβL | profile |
| 1 | 06/2003 | ICU | >512 | >256 | 16 | 256 | 32 | 32 | 128 | 16/4 | 0.25 | - | + | IMP-16 | B |
| 2 | 09/2003 | IM III | 32 | 64 | 32 | 16 | >32 | 8 | 128 | 16/4 | 0.50 | - | - | - | D |
| 3 | 03/2004 | IM II | >512 | >256 | 16 | >256 | 32 | >128 | >128 | 16/4 | 0.25 | + | + | SPM-1 | A3 |
| 4 | 03/2004 | SUI | >512 | 8 | 128 | 32 | 32 | 32 | 128 | 16/4 | 0.50 | - | - | - | A1 |
| 5 | 04/2004 | ICU | >512 | >256 | 16 | >256 | 32 | >128 | >128 | 8/4 | 0.50 | + | + | SPM-1 | A1 |
| 6 | 05/2004 | IM II | 64 | 4 | 64 | 16 | 16 | 16 | 64 | 8/4 | 0.50 | - | - | - | G |
| 7 | 09/2004 | ICU | 128 | 8 | 64 | 16 | 16 | 16 | 64 | 16/4 | 0.50 | - | - | - | E |
| 8 | 10/2004 | ICU | 2 | 1 | 4 | 2 | <1 | 32 | 128 | <1/4 | 0.25 | - | - | - | J |
| 9 | 10/2004 | IM III | >512 | >256 | 16 | >256 | 32 | >128 | >128 | 32/4 | 0.25 | + | + | SPM-1 | A3 |
| 10 | 11/2004 | SU I | 16 | 2 | 16 | 16 | 8 | 32 | 64 | 8/4 | 0.25 | - | - | - | F |
| 11 | 11/2004 | SU I | >512 | >256 | 16 | >256 | 32 | >128 | >128 | 8/4 | 0.50 | + | + | SPM-1 | A1 |
| 12 | 11/2004 | ICU | 2 | 1 | 2 | 2 | <1 | 16 | 64 | <1/4 | 0.50 | - | - | - | I |
| 13 | 01/2005 | SU II | 4 | 8 | 8 | 8 | <1 | 16 | 16 | 16/4 | 0.50 | - | - | - | L |
| 14 | 01/2005 | IMI | 8 | >256 | 16 | >256 | 32 | >128 | >128 | 8/4 | 0.25 | + | + | SPM-1 | A1 |
| 15 | 01/2005 | IM III | >512 | >256 | >256 | >256 | 32 | 32 | 128 | 128/4 | 0.25 | - | - | - | A1 |
| 16 | 01/2005 | IM III | >512 | 32 | 32 | 16 | 32 | 32 | 64 | 32/4 | 0.25 | - | - | - | A5 |
| 17 | 02/2005 | ICU | >512 | 8 | 128 | 128 | 32 | 32 | 128 | 16/4 | 0.12 | - | - | - | A3 |
| 18 | 03/2005 | ICU | 8 | 16 | 8 | 8 | <1 | 16 | 16 | 16/4 | 0.25 | - | - | - | H |
| 19 | 03/2005 | IM III | >512 | 32 | 32 | 16 | 32 | 32 | 64 | 32/4 | 0.25 | - | - | - | A5 |
| 20 | 03/2005 | ICU | >512 | 8 | 64 | 32 | 32 | 32 | 128 | 8/4 | 0.25 | - | - | - | A1 |
| 21 | 03/2005 | IM III | >512 | >256 | 16 | 256 | 32 | 32 | 128 | 16/4 | 0.25 | - | - | - | A1 |
| 22 | 04/2005 | ICU | >512 | 8 | 128 | 64 | 32 | 32 | 128 | 16/4 | 0.12 | - | - | - | A1 |
| 23 | 04/2005 | ICU | >512 | 64 | 64 | 16 | 16 | 32 | 128 | 16/4 | 0.25 | - | - | - | A1 |
| 24 | 05/2005 | IM III | >512 | 16 | 64 | 32 | 32 | 32 | 128 | 8/4 | 0.25 | - | - | - | A1 |
| 25 | 05/2005 | IM III | >512 | 8 | 64 | 32 | 32 | 32 | 128 | 8/4 | 0.25 | - | - | - | J |
| 26 | 05/2005 | E | 128 | 8 | 32 | 16 | 16 | 16 | 64 | 16/4 | 0.25 | - | - | - | C |
| 27 | 05/2005 | E | 8 | 16 | 64 | 16 | <1 | 32 | >128 | 16/4 | 0.25 | - | - | - | K |
| 28 | 06/2005 | E | >512 | 32 | 32 | 32 | 32 | 32 | 64 | 16/4 | 0.50 | - | - | - | A4 |
| 29 | 06/2005 | IM II | >512 | >256 | 16 | >256 | 32 | >128 | >128 | 8/4 | 0.50 | + | + | SPM-1 | A2 |

*(-) not found.

**AMI: amikacin, CAZ: ceftazidime, AZT: aztreonam, CPM: cefepime, CIP: ciprofloxacin, IMI: imipenem, MER: meropenem, PTZ: piperacillin/tazobactam, POL: polymyxin.

***ICU: Intensive Care Unit, General medicine, IM III: Internal Medicine Unit III, IM II: Internal Medicine Unit II, IM I: Internal Medicine Unit I, Surgical, SUII: Surgical Unit II, SUI: Surgical Unit I, E: Emergency.

used (DDS and CD). The positive isolate for $bla_{IMP}$ by PCR was also positive in the CD test, but produced a false negative result in DDS. Sequencing of the respective PCR products confirmed that the genes implicated were $bla_{SPM-1}$ and $bla_{IMP-16}$ (**Table 1**).

PFGE genotyping of the samples, isolated at the HU/UFSC during the period studied (**Figure 1**), revealed the presence of a clone (clone A) that included 17 (62%) of the 29 samples of CRPA and comprised all SPM-1 positive strains. The remaining eight were unrelated, except for two strains that were clonal (clone J).

IMP-16 producing *Pseudomonas aeruginosa* was the first strain resistant to carbapenems at the HU/UFSC and was not related to any other isolates.

The first *Pseudomonas aeruginosa* representatives of A clone (subtype A3), isolated in March 2004, harbored $bla_{SPM-1}$ gene. In the following months, five additional positive $bla_{SPM-1}$ isolates belonging to A clone subtypes were isolated in different units of the hospital.

## DISCUSSION

*Pseudomonas aeruginosa* was one of the main pathogens involved in nosocomial infection during the study period. Carbapenems were considered an excellent therapeutic choice for treatment of these infections; however, the increasing resistance to these agents verified in the institution (13% in 2003, 32% in 2004 and 44% in 2005) was concerning.

All isolates were susceptible to polymyxin B, currently used for empirical treatment of *Pseudomonas aeruginosa* infections in severely ill patients, particularly those in Intensive Care Units in Brazilian hospitals. Piperacillin-tazobactam was also a viable alternative treatment (96.7% susceptibility) and all isolates producing MβL were susceptible to this drug. According to previous studies, piperacillin-tazobactam could be a reliable treatment option for MβL-producing *Pseudomonas aeruginosa* when used appropriately[7,18,19]. Expression of high-level resistance to various antimicrobial agents may involve

different mechanisms, such as the production of enzymes, a reduction in the permeability of the external membrane and overexpression of efflux systems[2,20,21].

MβL-producing isolates presented high MICs for both carbapenems (>128μg/mL) and other β-lactams (≥256μg/mL), except aztreonam (16μg/mL). Aztreonam is not a good substrate for MβL, including SPM-1[15]; however, its reduced susceptibility could be explained by the possible additional mechanisms of resistance to β-lactams carried by this strain[9]. The isolates belonging to clone A, which do not carry MβLs, showed lower MICs, especially to Imipenem (32 to 64μg/mL), and somewhat higher for meropenem (64 to 128μg/mL).The overexpression of the MexAB-OprM efflux system could explain this finding, since the hydrophobic chains of meropenem seem to be a better substrate than imipenem in this system[22]. Four isolates sensitive to ceftazidime (MIC 8μg/mL), were resistant to cefepime and carbapenems. According to Hocquet et al[21], this phenotype could be due to overexpression of *amp*C and the MexXY-OprM[21] efflux system. This clone probably has multiple resistance mechanisms, which, according to Maniati et al[23], would explain the high MIC for carbapenems[23].

Unrelated isolates presented variable susceptibility profiles, some of which showed resistance only to carbapenems. A possible resistance mechanism of these isolates could be due to the loss of porin (OprD)[24]. The expression of different resistance mechanisms to *Pseudomonas aeruginosa* reveals the diverse sensitivity of phenotypic profiles in the susceptibility test of this microorganism.

Attempts to standardize phenotyping techniques to detect MβL have encountered various obstacles, such as the differences observed among this class of enzymes and the variation in test results according to the species of bacteria studied. Picão et al[25] suggested that DDS is the best method for testing different species of bacteria with diverse MβLs[25]. The phenotypic methods used in this study proved to be satisfactory in identifying SPM-1-producing isolates, the main MβL detected in Brazilian hospitals. Nevertheless, DDS failed to identify the isolate carrying $bla_{IMP-16}$. The IMP-16 enzyme was characterized in a *Pseudomonas aeruginosa* isolate in a hospital in the City of Brasília in 2001[26]. *Pseudomonas aeruginosa* which produces this enzyme was also isolated in Santa Catarina in 2003, suggesting that the $bla_{IMP-16}$ variant is circulating in Brazil. These findings justify the use of both methods, DDS and CD, to improve the sensitivity of MβL phenotyping.

One clonal type (clone A) was verified as predominant among the carbapenem-resistant isolates and was identified in all units at the HU/UFSC. This finding suggests that this clone is better adapted to the hospital environment. Alternatively, a continuous source of new acquisition of this microorganism may have occurred, since the other CRPA isolates did not disseminate clonally. The presence of a predominant clone among the resistant strains also indicates that cross-transmission between patients is an important mechanism for the dissemination of CRPA in this hospital. The main isolation site for this microorganism was the urinary tract, followed by the catheter (**Table 1**). These two sites are frequently identified in infection associated with inappropriate manipulation of invasive devices. Other Brazilian authors also reported the urinary tract as the main isolation of clonal strains CRPA[6,9]. Another interesting finding was the isolation of clone A predominantly in general medicine clinics (52.9%), mainly in the Internal Medicine Unit III (35%) (**Table 1**), in contrast to that described by other reports[4,5]. These results highlight the need for improved measures to control nosocomial infection and show that the manipulation of invasive devices is one of the main procedures that require intervention measures.

Clonal dissemination of SPM-producing *Pseudomonas aeruginosa* has been described in several Brazilian States: São Paulo, Ceará, Bahia, Paraná and the Federal District[9]. The same clone was also described in Rio de Janeiro in a subsequent study[27]. Reports of isolates from Pernambuco, Amazonas and Minas Gerais states described clonal dissemination of SPM-1-producing *Pseudomonas aeruginosa*[4,28,29]. The isolates from Santa Catarina were compared with the clone described by Gales et al[9] and it proved to be the same clone, known as the Brazilian epidemic clone. Identification of a single clone in a country of continental dimensions like Brazil, could be explained by environmental dissemination[9], community dissemination[30] or even dissemination by some common source distributed nationally.

The production of MβL did not represent a frequent mechanism of carbapenem resistance in HU/UFSC. However, 62% clonality in CRPA suggests cross transmission as an important mechanism of dissemination, especially outside the Intensive Care Unit. The spread of this clone, with a high-level of resistance and the challenge to eliminate it from the hospital environment, has contributed to the increased rate of resistance to carbapenems. The results of this study emphasize the need for continuous surveillance and improved strategies for infection control in this institution.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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