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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

# Predicting tigecycline susceptibility in multidrug-resistant *Klebsiella* species and *Escherichia coli* strains of environmental origin

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## Abstract

Tigecycline (TGC) is an important antimicrobial agent used as a last resort for difficult-to-treat infections mainly caused by carbapenem-resistant *Enterobacteriaceae*, but TGC-resistant strains are emerging, raising concerns. In this study, 33 whole-genome characterized multidrug-resistant (MDR) strains (*Klebsiella* species and *Escherichia coli*) positive mainly to *mcr-1*, *bla*, and/or *qnr* from the environment were investigated for TGC susceptibility and mutations in TGC resistance determinants, predicting a genotype–phenotype relationship. TGC minimum inhibitory concentrations (MICs) of *Klebsiella* species and *E. coli* ranged from 0.25 to 8 and 0.125 to 0.5 mg/L, respectively. In this context, KPC-2-producing *Klebsiella pneumoniae* ST11 and *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* ST4417 strains were resistant to TGC, while some *E. coli* strains of ST10 clonal complex positive for *mcr-1* and/or $bla_{\text{CTX-M}}$ exhibited reduced susceptibility to this antimicrobial. Overall, neutral and deleterious mutations were shared among TGC-susceptible and TGC-resistant strains. A new frameshift mutation (Q16stop) in RamR was found in a *K. quasipneumoniae* strain and was associated with TGC resistance. Deleterious mutations in OqxR were identified in *Klebsiella* species and appear to be associated with decreased susceptibility to TGC. All *E. coli* strains were determined as susceptible, but multiple point mutations were identified, highlighting deleterious mutations in ErmY, WaaQ, EptB, and RfaE in strains exhibiting decreased susceptibility to TGC. These findings demonstrate that resistance to TGC is not widespread in environmental MDR strains and provide genomic insights about resistance and decreased susceptibility to TGC. From a One Health perspective, the monitoring of TGC susceptibility should be constant, improving the genotype–phenotype relationship and genetic basis.

**Keywords** *Enterobacterales* · Tigecycline · Antimicrobial resistance · Aquatic ecosystems

## Introduction

Bacterial species, highlighting *Klebsiella pneumoniae* and *Escherichia coli*, exhibiting multidrug resistance have been spreading at the human-animal-environment interface worldwide, supporting antimicrobial resistance as a multifactorial and public health problem [1]. The environment is a hotspot for xenogenetic pollutants, spreading antimicrobial resistance to different sectors [2, 3]. In clinical settings, tigecycline (TGC), a bacteriostatic antimicrobial agent belonging to the glycylcycline class, has been used as a last resort for difficult-to-treat infections caused by multidrug-resistant (MDR) and carbapenem-resistant *Enterobacteriaceae* (CRE) [4]. In addition, TGC has good activity against extended-spectrum β-lactamase-producing *Enterobacteriaceae* and antimicrobial-resistant Gram-positive bacteria [5]. In this context, TGC monotherapy is used to treat skin, soft-tissue, and intra-abdominal infections caused by CRE [6]. Worryingly, MDR and CRE clinical strains exhibiting resistance to TGC have been reported, threatening the success of antimicrobial therapy [7, 8].

TGC is similar in structure to tetracyclines, acting as an inhibitor of bacterial protein translation via reversible binding to the 30S ribosomal subunit. This antimicrobial agent has a glycyclamide moiety to the 9-position and was developed to overcome the main molecular tetracycline resistance mechanisms, highlighting Tet proteins. In this

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regard, TGC resistance is mediated by chromosomal or accessory gene-encoded mechanisms [6, 9]. Investigation about TGC susceptibility has been carried out mainly in *Enterobacteriaceae* strains from humans and food-producing animals [10, 11], but not in environmental strains. By studying bacterial resistance in environmental bacteria, it is possible to gain insights into the complex dynamics of TGC resistance transmission among different ecosystems. Therefore, this study aimed to determine TGC susceptibility, provide genomic insights, and understand the genotype–phenotype relationship of resistance or decreased susceptibility to TGC in MDR strains from the Brazilian environment.

## Material and methods

### Bacterial strains

Thirty-three MDR strains (20 *E. coli* and 13 *Klebsiella* species) previously whole-genome characterized by our research group were selected. Strains were isolated from aquatic environments (rivers, streams, and sewage treatment plants) and agricultural soils from the Southeast and Midwest regions of Brazil. These strains belonged to various sequences types, including high-risk clones, were resistant to critically important antimicrobials, highlighting polymyxins, carbapenems, extended-spectrum cephalosporins, and/or fluoroquinolones, and carried a broad resistome, spotlighting *mcr-1* (*mcr-1.1*, *mcr-1.26*), *bla* ($bla_{KPC-2}$, $bla_{NDM-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, $bla_{CMY-2}$, $bla_{OXA-2}$, $bla_{OXA-9}$, $bla_{TEM}$), and/or *qnr* (*qnrS1*, *qnrB1*, *qnrB19*, *qnrVC1*) (Supplementary Table S1).

### TGC susceptibility testing

Susceptibility assays were performed by disk diffusion and/or broth microdilution methods. For *E. coli* strains, the tigecycline susceptibility was first tested by disk diffusion method using disks of tigecycline (15 µg) (Liofilchem, Italy). Afterward, the minimum inhibitory concentration (MIC) for tigecycline (Sigma-Aldrich, USA) was determined for *E. coli* and *Klebsiella* sp. strains by broth microdilution method using freshly prepared (< 12 h) BBL™ Mueller Hinton II Broth (BD, USA). *E. coli* ATCC® 25922™ was used as quality control. The cell viability was assessed using resazurin (0.02%). The results were interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for *E. coli* (Zone diameter: susceptible ≥ 18 mm, and resistant < 18 mm; MIC: susceptible ≤ 0.5 mg/L, and resistant > 0.5 mg/L) [12] and *Klebsiella* species (MIC: susceptible ≤ 2 mg/L, and resistant > 2 mg/L) [13].

### Whole-genome sequence-based analysis

TGC resistance determinants [*E. coli*: AcrB, EmrY, MarR, Lon, WaaQ, EptB, RfaC, RfaE, LpcA, RpsI, and RpoB; *Klebsiella* species: RamR, RpsJ, and OqxR; Both: AcrR, Tet(A), Tet(X), and TMexCD1-TOprJ1)] were mapped and extracted from genomes using Geneious Prime® v.2022.2.2 (Biomatters Ltd., New Zealand). Subsequently, the sequences were aligned using Clustal Omega v.1.2.3 [14]. For mutation analysis, wild-type strains *E. coli* K-12 substr. MG1655 (GenBank accession number U00096.3), *K pneumoniae* MGH 78578 (GenBank accession number CP000647), *K. quasipneumoniae* subsp. *similipneumoniae* ATCC® 700603™ (GenBank accession number CP014696), and *K. quasipneumoniae* subsp. *quasipneumoniae* (GenBank accession number NZ_CCDF00000000) were used to compare specie-specific sequences. Specifically, *E. coli* EC168wt was used as wild-type strain for WaaQ and *E coli* plasmid RP1 (GenBank accession number X00006) for Tet(A). Sequences of *Bacteroides thetaiotaomicron* transposon CTnDOT (GenBank accession number AJ311171) and *Klebsiella pneumoniae* plasmid pHNAH8I-1 (GenBank accession number MK347425) were used to identify Tet(X) and TMexCD1-TOprJ1, respectively [7, 15–18]. Mutations were predicted as neutral or deleterious using Protein Variation Effect Analyzer software [19]. The frequency of mutated sequences was analyzed using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

### Genotype–phenotype relationship

Discrepant results between genotype (neutral or deleterious) and phenotype (susceptible or resistant) were confirmed by repeated disk diffusion and/or MIC testing.

### Data availability

Genomic sequences have been deposited in the National Center for Biotechnology Information under accession numbers available in Supplementary Table S1.

## Results

### TGC susceptibility and genotype–phenotype relationship in *Klebsiella* species

TGC MICs ranged from 0.25 to 8 mg/L in *Klebsiella* species. KPC-2-producing *K. pneumoniae* ST11 strain (EW666), and *K. quasipneumoniae* subsp. *quasipneumoniae* ST4417 strain (SWT10) exhibited resistance to

TGC. A stop codon was found in RamR (Q16stop) of SWT10 and point mutations were identified in OqxR and Tet(A). No amino acid substitutions were found in AcrR and RpsJ, and the Tet(X) and TMexCD1-TOprJ1 sequences were not detected. In BLASTn analysis, no Q16stop RamR sequence was found, evidencing a new RamR variant related to TGC resistance (MIC 8 mg/L). Deleterious mutations V130A in *K. pneumoniae* strains, and M46I and E117A in *K. quasipneumoniae* subsp. *quasipneumoniae* of OqxR were identified and appears to be associated with a decrease in TGC susceptibility. The single (V130A) and multiple (M46I, E117A) point mutations found in OqxR were assigned as frequent and infrequent, respectively (Table 1; Supplementary Table S2).

Despite the several neutral mutations detected in Tet(A), none of the profiles seem to be exclusively related to TGC resistance. Furthermore, BLASTn analysis revelated that mutated Tet(A)-type sequences (Type 1: I5R, V55M, I75V, T84A, S201A, F202S, V203F; and Type 2: I5R, S12C, V55M, I75V, T84A, H132Y, G155S, G156S, T176M, C178I, S201A, F202S, V203F, A206V, V212I, V228A, A231T, A245T, T247L, A271T, A272T, T289A, F296L, M323V, L389F, A393V) are frequent in *Enterobacteriaceae*, especially in *K. pneumoniae*, *E. coli*, and *Salmonella* Infantis (Table 1; Supplementary Table S2).

## TGC susceptibility and genotype–phenotype relationship in *E. coli* strains

All *E. coli* strains were susceptible to TGC since presented zone diameter sizes > 21 mm and MIC values from 0.125 to 0.5 mg/L. There was no correlation between zone diameters sizes and MIC values. In general, multiple point mutations (neutral or deleterious) were identified in AcrB, EmrY, AcrR, MarR, WaaQ, EptB, LpcA, RfaC, RfaE, and Tet(A). On the other hand, no amino acid substitutions were identified in sequences of Lon, RpsI, and RpoB, and the Tet(X) and TMexCD1-TOprJ1 sequences were not found. Deleterious mutations were found in ErmY (L252V, L370M), AcrR (H115Y), WaaQ (A109T, L153V), EptB (L80Q, R157H, D246N), RfaC (A42T, R130C), and RfaE (T302P), which were distributed among *E. coli* with different TGC MICs. A mutated Tet(A) sequence (I5R, V55M, I75V, T84A, A93T, S201A, F202S, V203F), denominated Type 3, was found in a strain with a MIC of 0.25 for TGC and was determined as infrequent (Table 2).

In this context, the mutations L252V in EmrY, A109T and L153V in WaaQ, R157H, and D246N in EptB, seem to be related to decreased susceptibility to TGC (MIC 0.5 mg/L). Furthermore, unique neutral mutations found in strains with TGC MIC of 0.5 mg/L, including M320I in

**Table 1** TGC susceptibility and amino acid substitutions in TGC resistance determinants of *Klebsiella* species strains

| Strain (ST)[1] | TGC MIC (mg/L)[2] | Amino acid substitutions[3] | | |
|---|---|---|---|---|
| | | RamR | OqxR | Tet(A) |
| SWT10 (ST4417) | **8** | **Q16stop** | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| EW666 (ST11) | **4** | WT | **V130A** | – |
| EW671 (ST307) | 2 | WT | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| EW706 (ST5569) | 2 | WT | WT | – |
| EW606 (ST6325) | 2 | WT | **M46I, E117A** | – |
| EW807 (ST340) | 1 | WT | **V130A** | I5R, S12C, V55M, I75V, T84A, H132Y, G155S, G156S, T176M, C178I, S201A, F202S, V203F, A206V, V212I, V228A, A231T, A245T, T247L, A271T, A272T, T289A, F296L, M323V, L389F, A393V |
| EW608 (ST11) | 1 | WT | **V130A** | – |
| EW775 (ST340) | 1 | WT | **V130A** | – |
| EW158 (ST661) | 1 | WT | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| EW160 (ST4415) | 1 | WT | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| EW704 (ST30) | 1 | WT | WT | – |
| EW714 (ST889) | 1 | WT | WT | – |
| EW185 (ST4416) | 0.25 | WT | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |

[1]Sequence type, ST

[2]Tigecycline, TGC; Minimum inhibitory concentration, MIC. The MIC value of the control strain was 0.25 mg/L; Resistance is shown in bold

[3]Substitutions predicted in silico to be deleterious are highlighted in bold. Wild-type, WT; Gene not found, –

**Table 2** TGC susceptibility and amino acid substitutions in TGC resistance determinants of *E. coli* strains

| Strain (ST)[1] | TGC MIC (mg/L)[2] | Amino acid substitutions[3] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AcrB | EmrY | AcrR | MarR | WaaQ | EptB | LpcA | RfaC | RfaE | Tet(A) |
| EW659 (ST10) | 0.5 | WT | M320I | WT | WT | – | WT | WT | T144I, E224K | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| EW717 (ST349) | 0.5 | H596N | WT | WT | G103S, Y137H | S70A, N127K, E154D | Q305E, A505V | WT | E201D | WT | – |
| EW159 (ST1665) | 0.5 | WT | WT | WT | G103S, Y137H | **L153V**, E154D | I46V, A557T, E559V | WT | M114L, **R130C** | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| EW625 (ST6157) | 0.5 | H596N | **L252V** | WT | G103S, Y137H | V82I | A505V | G44D | E85K | WT | – |
| EW827 (ST1775) | 0.5 | WT | WT | WT | G103S, Y137H | – | **D246N**, A505V, E516A, A557T, A561V | WT | T144I, E224K | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| S526 (ST1146) | 0.5 | WT | WT | WT | G103S, Y137H | N127K, E154D | I46V, **R157H**, A557T, E559V | WT | M114L, **R130C** | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| S662 (ST345) | 0.5 | WT | L339V | WT | G103S, Y137H | N127K, E154D | I46V, A505T, A557T, E559V | WT | G30E | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| S663 (ST448) | 0.5 | WT | V511I | WT | G103S, Y137H | **A109T**, N127K, E154D | I46V | WT | G30E | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| EW232 (ST223) | 0.25 | WT | V511I | WT | G103S, Y137H | N127K, E154D | I46V, **L80Q**, A557T, E559V | WT | M114L | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| S376 (ST906) | 0.25 | WT | V511I | WT | G103S, Y137H | N127K, E154D | I46V | WT | WT | WT | I5R, V55M, I75V, T84A, A93T, S201A, F202S, V203F |
| EW92 (ST131) | 0.125 | H596N, S1043N | I240L | WT | G103S, Y137H | WT | A505V | WT | A101T | Q473L | – |
| EW222 (ST648) | 0.125 | WT | WT | **H115Y** | A53E, G103S, Y137H | V82I | S331T, A505V | A43G | L173K | **T302P**, L330F | – |
| EW239 (ST354) | 0.125 | WT | WT | WT | G103S, Y137H | V82I | S331T, A505V | EW | **A42T**, A81T | WT | – |
| EW655 (ST744) | 0.125 | WT | I184V, L339V, **L370M** | WT | WT | – | WT | WT | T144I, E224K | WT | – |
| EW658 (ST131) | 0.125 | H596N, S1043N | I240L | WT | G103S, Y137H | WT | A505V | WT | A101T | WT | – |

**Table 2** (continued)

| Strain (ST)[1] | TGC MIC (mg/L)[2] | Amino acid substitutions[3] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AcrB | EmrY | AcrR | MarR | WaaQ | EptB | LpcA | RfaC | RfaE | Tet(A) |
| EW697 (ST12841) | 0.125 | WT | WT | WT | WT | – | WT | WT | T144I, E224K | WT | – |
| EW698 (ST1720) | 0.125 | WT | WT | WT | G103S, Y137H | N127K, E154D | I46V | WT | G30E | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| EW715 (ST744) | 0.125 | WT | I184V, L339V, **L370M** | WT | WT | – | WT | WT | T144I, E224K | WT | – |
| S366 (ST189) | 0.125 | WT | I184V, L339V, **L370M** | WT | K99E, G103S, Y137H | N127K, T149I, E154D | WT | WT | M114L, **R130C** | WT | I5R, V55M, I75V, T84A, S201A, F202S, V203F |
| S802 (ST131) | 0.125 | H596N, S1043N | I240L | WT | G103S, Y137H | WT | A505V | WT | A101T | Q473L | I5R, V55M, I75V, T84A, S201A, F202S, V203F |

[1]Sequence type, ST

[2]Tigecycline, TGC; Minimum inhibitory concentration, MIC; The MIC value of the control strain was 0.25 mg/L

[3]Substitutions predicted in silico to be deleterious are highlighted in bold. Wild-type, WT; Gene not found, –

EmrY, G44D in LpcA, E85K in RfaC, and A135E in RfaE, may also be related to decreased susceptibility to TGC (Table 2). Therefore, the genotype–phenotype relationship should be further investigated. Worryingly, *E. coli* strains of ST10 clonal complex with decreased susceptibility to TGC were positive for *mcr-1* and/or $bla_{\text{CTX-M}}$ genes (Supplementary Table S1).

In general, frequent mutations were identified among the TGC resistance targets studied. On the other hand, mutations L252V and M320I in EmrY, E85K and G44D in RfaC, have not yet been identified. In addition, some single point mutation or mutation profiles in AcrB, EmrY, MarR, WaaQ, EptB, and RfaC were infrequent, highlighting the identification of a single sequence identical to profiles I46V, R157H, A557T, E559V and I46V, A505T, A557T, E559V of EptB (Supplementary Table S2).

## Discussion

The acquisition of resistance or decreased susceptibility to TGC in *Enterobacteriaceae* is complex and multifactorial. Among the known mechanisms associated with these phenomena in *K. pneumoniae* and *E. coli*, overexpression of RND-type efflux pumps caused by mutations in their transcriptional activators and/or repressors is the most common. Additionally, mutated sequences of the lipopolysaccharide biosynthesis pathway, ribosomal S10 protein (RpsJ), and RNA polymerase β subunit (RpoB) were also reported [6, 15, 16, 20]. Moreover, plasmid-encoded Tet(A), Tet(X) and TMexCD1-TOprJ1 have been associated with resistance or reduced susceptibility to TGC, drawing attention to rapid spread mediated by horizontal gene transfer [17, 18, 21]. In the latter, TMexCD1-TOprJ1 confers multidrug resistance and was already identified in *Enterobacteriaceae* from human- and animal-associated samples, and even in a *mcr-8.1*-bearing plasmid of a *Klebsiella pneumoniae* strain from chicken [18].

In *Klebsiella* species, efflux-mediated resistance mechanisms are commonly associated with TGC resistance. In this regard, RamR was involved in the overexpression of RamA, which upregulated the expression of the AcrAB efflux pump. A frameshift mutation was found in a TGC-resistant *K. quasipneumoniae* subsp. *similipneumoniae* strain from a sewage treatment plant, supporting the TGC resistance mediated by this mechanism [8, 10, 22]. Deleterious mutations in OqxR, a local repressor responsible for the downregulation of the OqxAB efflux pump, were found mainly in strains with decreased susceptibility to TGC. The V130A mutation has been identified in TGC-resistant and TGC-susceptible strains, supporting our findings [8, 18, 22].

In *E. coli* strains, TGC resistance may involve alterations in a variety of genetic loci. Mutations in targets encoding

AcrAB and EmrY efflux pumps may mediate TGC resistance, while amino acid substitutions in its regulators (AcrR, MarR, Lon) seem to be more associated with this phenotype [15, 16, 24]. In addition, the overexpression of the AcrAB efflux pump can concomitantly be associated with resistance to fluoroquinolones and TGC [25]. Amino acid substitutions in targets (WaaQ, EptB, RfaC, RfaE, LpcA) that affected the lipopolysaccharide biosynthesis pathway were reported in strains with MICs below of resistance breakpoint. These mutated targets may cause a significant reduction of porins, causing the slow rate of porin-independent diffusion of TGC and leading to reduced susceptibility to TGC. In addition, a relatively low-level resistance associated with mutated sequences of efflux regulatory network and lipopolysaccharide core biosynthesis pathway has been described, endorsing our results [15, 16]. Despite the great diversity of mutations in the beforementioned targets, no data have been found to support their exact role in TGC susceptibility. Therefore, the phenotype-genotype relationship should be further explored and mutations, especially deleterious ones, should be experimentally confirmed.

The Tet(A) mutants have been related to reduced susceptibility to TGC and can act synergistically with RamR mutated sequences to increase the level of TGC resistance [8, 17]. Although Tet(A) sequence Type 1 has already been associated with resistance or reduced susceptibility to TGC since presented mutations in the interdomain region that affect its affinity for TGC, our findings evidence that it is widespread in TGC-susceptible strains, contrasting with its role in TGC resistance [11, 17, 26, 27]. In addition, Tet(A) sequences Type 2 and 3 were identified in strains with low MICs, showing that these variants also have no role in TGC resistance. Consequently, selective pressure by TGC can promote the formation of Tet(A) mutants, leading to TGC resistance [28].

Despite the excellent performance of genomic analyzes to predict susceptibility to fluoroquinolones, β-lactams, and aminoglycosides in *E. coli* and *K. pneumoniae*, this does not seem to happen for TGC, especially for *E. coli* [29]. It is important to emphasize that the genomic results to infer susceptibility depend on the local epidemiology and can be affected by the phylogenetic distance to the reference strains [30, 31]. In clinical settings, this discrepancy can affect the development of clinical metagenomics and, consequently, the use of pathogen-specific antimicrobials coupled with rapid diagnostics [32].

In summary, our findings corroborate that rates of TGC-resistant *E. coli* strains are still low when compared to *Klebsiella* species and demonstrate that resistance to TGC is not widespread in environmental MDR strains. However, some strains presented reduced susceptibility to TGC and may evolve resistance through multifactorial mechanisms, including multisubstrate efflux pumps. Furthermore, it is possible to infer that the selective pressure of environmental contaminants does not strongly interfere with the selection of TGC-resistant strains. Finally, our results have limitations, such as the limited number of strains used and lack of experimental evidence, but provide genomic insights about resistance and decreased susceptibility to TGC. Therefore, constant monitoring of TGC susceptibility should be carried out within a One Health perspective, improving the genotype–phenotype relationship and genetic basis.

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**Author contribution** **J.P.R.F.:** Conceptualization, formal analysis, investigation, data curation, writing—original draft, writing—review & editing. **E.G.S.:** Conceptualization, funding acquisition, supervision, project administration, writing—review & editing.

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**Data availability** All data analyzed during this study are included in this article.

## Declarations

**Ethics approval** No ethical approval was necessary for the present study.

**Consent to participate** Not applicable.

**Consent for publication** All authors agreed with the final version of the manuscript.

**Conflict of interest** The authors declare no competing interests.

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