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Discovery of NDM-1 inhibitors from natural products

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ABSTRACT

Objectives: Human health is seriously threatened by metallo-β-lactamase (MBL)-mediated bacterial antimicrobial resistance, among which New Delhi metallo-β-lactamase 1 (NDM-1) has received great attention due to its extensive substrate profile and high lateral gene transfer. Currently, there is no inhibitor of NDM-1 available in clinical therapy, thus making an urgent need for research and development of novel NDM-1 inhibitors.

Methods: A natural compound library was screened to determine potential inhibitors of NDM-1 based on its crystal structure. Five known NDM-1 inhibitors were used as positive controls for the computer screening protocol. Based on the screening results, the half maximal inhibitory concentration (IC_{50}) of several potential NDM-1 inhibitors was determined using purified NDM-1. The potential interaction between the inhibitor and NDM-1 was analysed using docking.

Results: Five potential NDM-1 inhibitors were discovered with IC_{50} values ranging from 3.348 ± 1.35 μM (hesperidin) to 214.1 ± 13.37 μM (stevioside). The most active inhibitor, hesperidin, acts directly on key residues near the NDM-1 active site.

Conclusion: A series of NDM-1 inhibitors was discovered using virtual screening, which allows for improved screening efficiency and reduced costs. Considering the low toxicity of these compounds, they may be used as potential lead compounds for the development of NDM-1 inhibitors.

1. Introduction

Clinical use of antibiotics plays an important role in the treatment of bacterial infections, reducing mortality and prolonging human life. However, the emergence of bacterial antimicrobial resistance has led to a dramatic decline in the efficacy of antibiotics. The prevalent mechanisms of bacterial resistance include reduced cell wall permeability, antibiotic efflux and enzyme-mediated drug degradation [1–3]. Accompanied by the worldwide use of β-lactams, β-lactamase-mediated antimicrobial resistance has drawn ever more concern as the enzyme-encoding genes are often located on transferable units and are easily spread between pathogens [4]. Based on their molecular properties and/or amino acid sequences, β-lactamases can be divided into classes A, B, C and D, with classes A, C and D exhibiting hydrolytic capability depending on a serine in the active site. Class B requires either one or two zinc ions as the nucleophile and thus are known as metallo-β-lactamases (MBLs) [5]. There are three subclasses of MBLs (B1, B2 and B3), of which the class B1 New Delhi metallo-β-lactamase 1 (NDM-1) has attracted extensive attention in recent years as it confers resistance to almost all classes of β-lactam antibiotics with the exception of the monobactam aztreonam [6]. NDM-1 has been identified mainly in *Escherichia coli*, *Acinetobacter* spp. and *Klebsiella pneumoniae* [7] and is encoded on a readily transferable plasmid, facilitating its transmission [8]. Ongoing research has suggested that the bla_{NDM-1} gene conferring resistance is now widely spread in the world. Currently, 17 NDM variants have been reported [9], whilst there are no effective inhibitors of NDM-1 (or MBLs) in the clinic. Pathogens possessing bla_{NDM-1} may cause a real and formidable threat to human health in the coming future. Discovering new NDM-1 inhibitors as antibiotic adjuvants is important to avert future catastrophic pandemics [10].

The combination of a serine-β-lactamase inhibitor with a β-lactam has been used clinically to treat antimicrobial-resistant infections. However, serine inhibitors such as clavulanic acid, sulbactam, etc., have no effect on MBLs, whilst metal chelators such as ethylene diamine tetra-acetic acid (EDTA), phenanthroline, pyridine dicarboxylic acid, etc. have no clinical significance owing to their side effects [11,12]. In the past few years, many chemical scaffolds showing effective inhibitory activity against NDM-1 have been discovered. For example, in our previous study [13], we designed and synthesised thiophene-carboxylic acid derivatives as

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potential inhibitors of NDM-1. In vitro experiments demonstrated that these inhibitors combined with meropenem can effectively inhibit the growth of *E. coli* producing NDM-1. Other authors explored a series of bisthiazolidines that were designed to mimic the general backbone of β-lactam antibiotics because of their ability to inhibit MBLs [14,15]. Nitrogen-containing heterocyclic compounds have also become attractive candidates as MBL inhibitors. Yang and colleagues reported a series of triazolyl thioacetamides with inhibitory activity against NDM-1 [16,17]. Brem et al. demonstrated that cyclic boronates can inhibit MBLs in vitro as well as having activity in cells producing NDM-1 [18]. However, no MBL inhibitors have been approved as drugs [19]. With the rapid spread of NDM-1 globally, screening and development of new high-efficiency NDM-1 inhibitors is particularly urgent.

With the development of computer technology, virtual screening is increasingly being applied to new drug development. For example, Kang et al. identified a series of compounds that inhibit clinically relevant MBLs by screening 1.5 million compounds [20]. The best compound identified had a half maximal inhibitory concentration (IC_{50}) of 19 ± 2 μM. Brindisi et al. found a 'hit' compound by performing structure-based high-throughput docking that was able to restore β-lactam (cefoxitin) susceptibility in a co-ordinated whole-cell assay of *E. coli* strains producing VIM-2 based on the three-dimensional structure of VIM-2 [21].

The current study used the molecular docking function in MOE2016 software (Chemical Computing Group Inc., Montreal, Québec, Canada) to virtually screen active small-molecule compounds from a natural compound library. Several potential NDM-1 inhibitors were purchased and kinetically screened to validate the experimental conditions.

2. Materials and methods

2.1. Virtual screening

2.1.1. Preparation of protein target for virtual screening

The crystal structure of NDM-1 and ampicillin complex at pH 5.5 (Bis–Tris) was downloaded from the Protein Data Bank (PDB) database with a resolution of 1 Å (PDB ID: 5ZGE). Extracting NDM-1 and ampicillin structures separately and performing pre-treatment (hydrogenation, charge, structure correction, etc.) was done using MOE2016.

2.1.2. Validation of docking method

The root mean square deviation (RMSD) and GBVI/WSA dG scores were used as indicators. RMSD represents the difference between the conformation produced by docking and the experimentally detected conformation (PDB ID: 5ZGE). The GBVI/WSA dG score calculates the binding free energy between the ligand and the receptor protein in a docking conformation. The smaller the score, the more stable the binding between the receptor and ligand. Multiple docking procedures with different scoring functions (or in different forcefield) were performed using the NDM-1 and ampicillin structures extracted in the previous step, and the final docking method was determined as the one that produced the smallest RMSD.

To verify the accuracy of the GBVI/WSA dG scoring function and the selected forcefield conditions, five known NDM-1 inhibitors [22] were selected and their structures were built and minimised under $MMFF_{94}$ forcefield by MOE2016. The correlation between the efficacy of the inhibitor and the scoring results was determined by docking with the receptor protein. If the absolute value of the scoring result is better, the corresponding IC_{50} is smaller, indicating that the scoring function and the selected forcefield conditions meet the requirements, thereby the scoring range when screening the desired small molecule ligand was established.

2.1.3. GBVI/WSA dG scoring

The GBVI/WSA dG is a forcefield-based scoring function that estimates the free energy of binding of the ligand from a given pose. The calculation formula is as follows [23]:

$$\Delta G \approx c + \partial\left[\frac{2}{3}(\Delta Ecoul + \Delta Esol) + \Delta Evdw + \beta\Delta SAweighted\right]$$

where c represents the average gain/loss of rotational and translational entropy, and α,β are constants that were determined during training (along with c) and are forcefield-dependent. If not using an AMBER forcefield, the parameters will be set by default to the MMFF-trained parameters. *Ecoul* is the coulombic electrostatic term that is calculated using currently loaded charges, using a constant dielectric of 1; *Esol* is the solvation electrostatic term that is calculated using the GB/VI solvation model; *Evdw* is the van der Waals contribution to binding; and *SAweighted* is the surface area weighted by exposure. This weighting scheme penalises the exposed surface area.

2.1.4. Preparation of a natural compound library

A natural compound library containing 2177 compounds was used to identify potential inhibitors. Before virtual screening, MOE2016 Wash application was conducted to remove possible system structure errors (alkali–metal–oxygen single bonds, protonated strong acids, deprotonated strong bases) in the database. After structural proof-reading, the Database Partial Charges panel was used to calculate and set the partial charge of all molecules in the MOE database. The Database Minimization panel was used to minimise the structure, add hydrogen, and set the current forcefield partial charge.

2.1.5. Natural compound database screening

The NDM-1 crystal structure (PDB ID: 5ZGE) with the ampicillin removed was used as a receptor, and the small molecule ligands in the natural compound library were sequentially docked. The small molecule ligands with the highest GBVI/WSA dG scores were screened.

2.2. Construction of the NDM-1 strain

The wild-type bla_{NDM-1} gene lacking the signal peptide was synthesised [Bio-Bioengineering (Shanghai) Co., Ltd., Shanghai, China] and was then cloned between *Eco*RI and *Hind*III restriction sites into a pET-28a plasmid harbouring a kanamycin resistance gene [24]. The constructed plasmid encoding NDM-1 was transferred into *E. coli* BL21(DE3). The NDM-1 enzyme was expressed and purified as described by Liu et al. [25]. *Escherichia coli* BL21(DE3) carrying pET28a-NDM-1 was placed in LB medium containing kanamycin (50 mg/mL) and was cultured to an optical density at 600 nm of 0.6–0.8. Protein production was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The culture was further incubated at 18 °C for 14 h. Cells were suspended in 30 mM phosphate-buffered saline (pH 7.3). Following sonication, the supernatant was removed and was added to a Ni-NTA column (QIAGEN, San Diego, CA). The target protein was eluted with a gradient of 30 mM Tris–HCl buffer (pH 7.3, containing 0.5 M NaCl) with different concentrations of imidazole and was then desalted to a reaction buffer [20 mM HEPES (Sangon) using a HiTrap™ Desalting column (GE Healthcare, Shanghai, China)], freeze-dried and stored at −80 °C.

2.3. Determination of IC_{50} values

Natural products (purity ≥98%) were purchased from Shanghai Li Ding Biological Technology Co., Ltd. (Shanghai, China) and cefuroxime sodium was from Shenzhen Li Jian Pharmaceutical Co., Ltd.

(Shenzhen, China). A UV-1800 spectrophotometer (Shanghai MAPADA Instrument Co., Ltd., Shanghai, China) was used to determine the IC_{50} kinetic parameter. The total reading time was 100 s and the interval was 1 s. NDM-1 (final concentration 10 nM) supplemented with 50 mM HEPES buffer containing 100 μM Zn^{2+} was pre-incubated in a 30 °C dry bath for 5 min, allowing Zn^{2+} to occupy its active site sufficiently. Enzyme and inhibitors at different concentrations were then incubated for another 5 min and were transferred into the quartz cuvette. Cefuroxime sodium (final concentration 60 μM) was added and mixed immediately and the initial rate of substrate hydrolysis was recorded. All experiments were repeated three times. The inhibition rate was then calculated using the following equation, and IC_{50} values were calculated by plotting the regression equation for the inhibitor concentration against the percentage of the average inhibition rate [26]:

$$I = \left(1 - \frac{V_i}{V_0}\right) \times 100\%$$

where V_0 is the initial rate of reaction without inhibitor and V_i is the initial rate of reaction at different inhibitor concentrations.

Additional information regarding the methods used is given in the Supplementary material.

3. Results

3.1. Verification of model accuracy

Multiple docking procedures with different scoring function (or in different forcefields) were performed using the NDM-1 structure with ampicillin removed. The final docking method was determined as the one that produced the smallest RMSD. In the best procedure, GBVI/WSA dG scoring function and Amber14: EHT forcefield were chosen. To verify the accuracy of the model, five known NDM-1 inhibitors [22] were selected for docking with the receptor protein based on the established docking parameters. Docking results and IC_{50} values are shown in Table 1. The docking scores and IC_{50} values of different inhibitors were linearly fitted. The results are shown in Fig. 1. The GBVI/WSA dG value is linear with IC_{50}, and the R^2 value is 0.989. This verifies the accuracy of the GBVI/WSA dG scoring function and the selected forcefield conditions, which lays the foundation for virtual screening of the natural compound library.

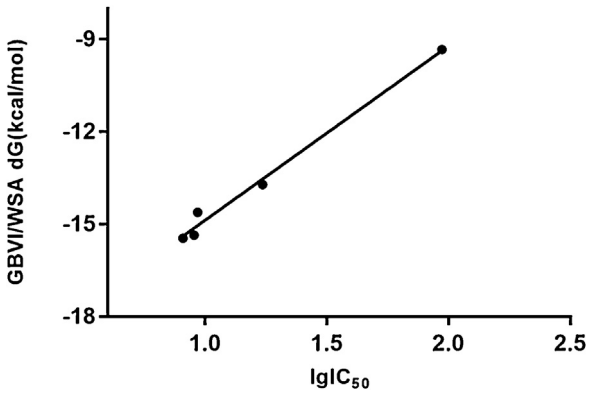
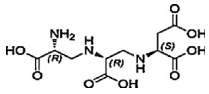
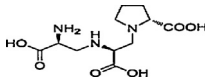
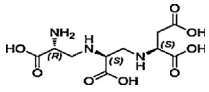
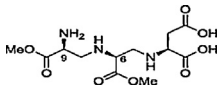
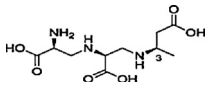


Fig. 1. Plots of $\log_{10}$ IC_{50} values of inhibitors and GBVI/WSA dG values calculated by the molecular docking function in MOE2016 software. IC_{50}, half maximal inhibitory concentration.

3.2. Screening results

The known inhibitors in Table 1 were added to the natural product library using MOE2016 to virtually screen the natural compound library under the selected parameters. The scores of known inhibitors are located in the top 3% of all results. Taking into account possible errors during the calculation of binding energy, the compounds that scored in the top 6% based on the screening criteria resulted in 140 natural compounds for further evaluation. Molecular docking refinement and minimisation of these 140

Table 1
Docking scores, IC_{50} and $\log_{10}$ IC_{50} values for different known NDM-1 inhibitors.

Compound number[a]	Structure	GBVI/WSA dG value	IC_{50} (μM)[a]	Log_{10} IC_{50}
(R,R,S)-AMA		−15.4573	8.1 ± 0.61	0.908
7		−15.3548	9.0 ± 1.3	0.954
(R,S, S)-AMA		−14.6164	9.3 ± 0.45	0.968
5		−13.7085	17.2 ± 1.0	1.235
2		−9.3288	94.0 ± 0.37	1.973

IC_{50}, half maximal inhibitory concentration.
[a] The selected inhibitors were all synthesised in the same laboratory; the compound number and IC_{50} value are from Zhang et al. [22].

Table 2
Structure of hit compounds.

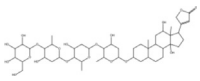
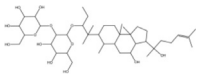
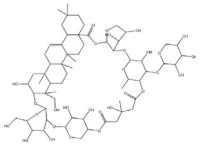
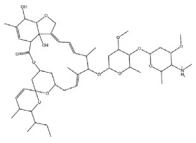
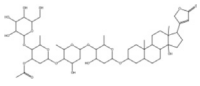
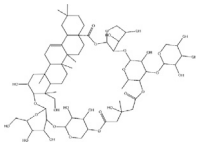
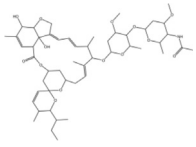
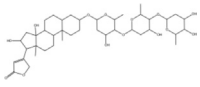
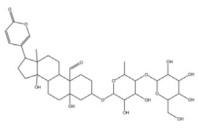
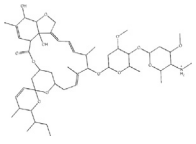
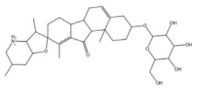
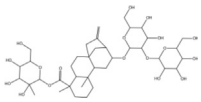
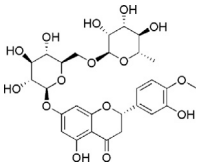
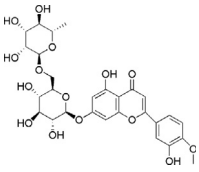
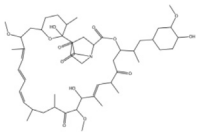
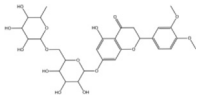
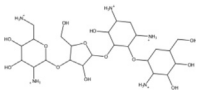
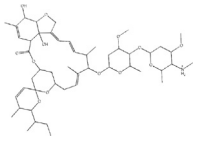
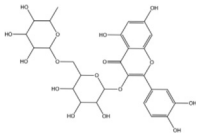
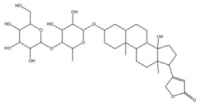
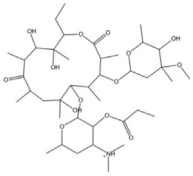
Compound	Structure	GBVI/WSA dG score	Affinity (kcal/mol)
1		−13.09	−12.62
2		−12.96	−15.01
3		−11.91	−12.47
4		−11.91	−15.07
5		−11.71	−11.96
6		−11.66	−15.02
7		−11.25	−14.33
8		−11.24	12.43
9		−11.20	12.77
10		−10.49	−13.44
11		−10.48	−16.36

Table 2 (*Continued*)

Compound	Structure	GBVI/WSA dG score	Affinity (kcal/mol)
12		−10.29	−9.81
13		−10.25	−12.61
14		−10.08	−10.19
15		−9.96	−9.84
16		−9.86	−9.29
17		−9.27	−12.13
18		−9.26	−9.23
19		−9.05	−11.41
20		−8.87	−10.84
21		−8.79	−11.60

C–
O–

Table 2 (*Continued*)

Compound	Structure	GBVI/WSA dG score	Affinity (kcal/mol)
22		−8.29	−10.90

mpounds were carried under $MMFF_{94}$ forcefield; each restored 30 constellations and rescored by GBVI/WSA dG value. Based on the interaction and affinity of ligands and receptors, 22 potential candidate compounds were chosen, and the results are shown in Table 2.

3.3. Inhibition analysis

The enzyme inhibitory activities of 6 easy-to-purchase compounds from the top 22 compounds were validated by in vitro experiments (Table 3). Apart from compound **3** that had no inhibitory effect, the other compounds showed an inhibitory effect on NDM-1. The most active compound was hesperidin, with an IC_{50} of 3.348 ± 1.35 μM.

3.4. Analysis of the interaction between hesperidin and NDM-1

Based on the docking results, the interaction between hesperidin and NDM-1 was analysed and the results are shown in Fig. 2. The interaction diagram shows that the phenyl ring and hydroxyl groups of hesperidin interacts with His122, Asn220 and other amino acids near the binding site, respectively (amino acid residue numbering is consistent with PDB ID: 5ZGE). At the same time, the hydroxyl group and the oxygen atom on the oxygen-containing six-membered ring directly interacts with the Zn^{2+} ion

Table 3
IC_{50} values of validated candidate compounds against NDM-1.

Compound	Name	IC_{50} (μM)[a]
3	Ginsenoside (Rg3)	No inhibition
13	Stevioside	214.1 ± 13.37
14	Hesperidin	3.348 ± 1.35
15	Diosmin	20.740 ± 1.92
18	Paromomycin	20.764 ± 2.21
20	Rutin	15.53 ± 1.34

IC_{50}, half maximal inhibitory concentration.
[a] Assays were performed in triplicate; IC_{50} values are shown as the mean ± standard deviation.

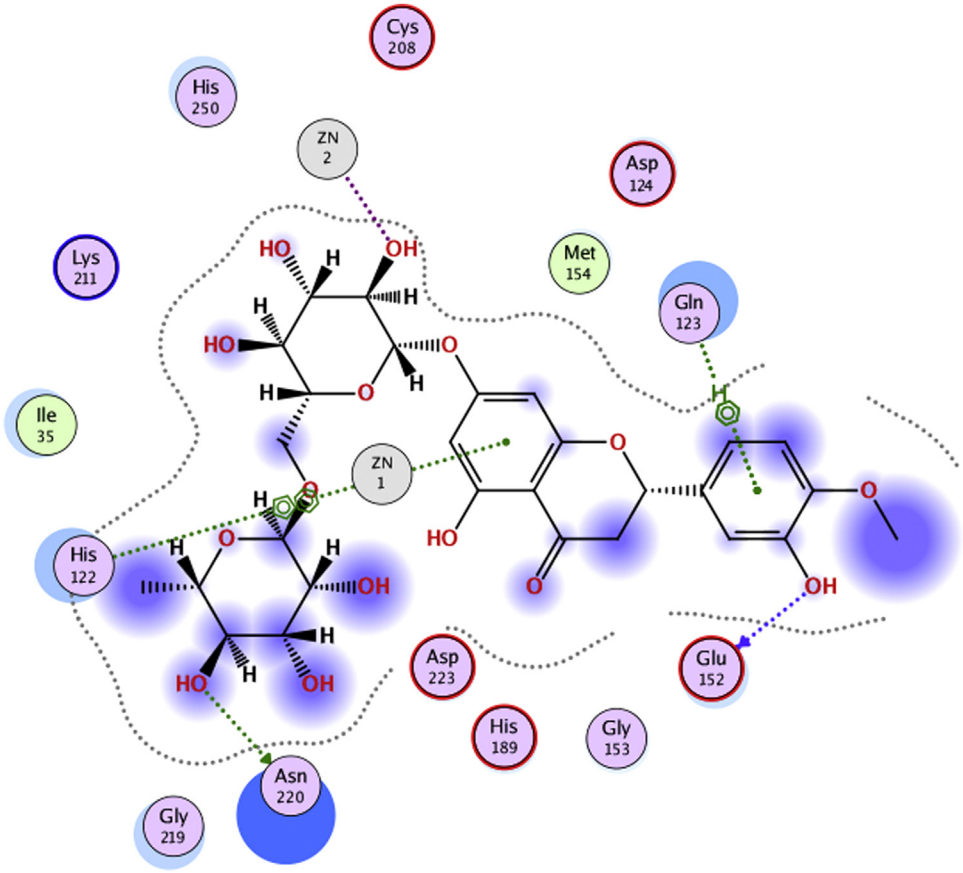


Fig. 2. Interaction between NDM-1 and hesperidin.

of the active centre to form a hydrogen bond. These interactions modes may be used for further optimisation of the lead compound. On the other hand, hesperidin is widely used as a major component of vitamin P to enhance capillary resistance. It has low toxicity, biocompatibility and may be used as a potential lead compound for the development of NDM-1 inhibitors.

4. Discussion

NDM has attracted much attention owing to its extensive hydrolytic profile against β-lactams as well as the current lack of effective MBL inhibitors against NDM-1-producing superbugs. In this work, we have discovered a series of NDM-1 inhibitors using a virtual screen from a natural product library based on the crystal structure of NDM-1. Interestingly, although a relatively small compound database was used in this study, positive compounds of different scaffolds and higher activities were discovered compared with the report of Kang et al. [20]. The most active compound, hesperidin, has an IC_{50} of 3.348 ± 1.35 μM.

In addition, it is worth noting that the ginsenoside (Rg3) has a higher docking score than known inhibitors; however, experiments have shown that it has no inhibitory effect on NDM-1. These results indicate that virtual screening relying on molecular modelling techniques can select potentially effective candidate compounds from a large number of organic compounds and avoid blind screening, thereby greatly reducing the cost of discovering active lead compounds. However, errors in the calculation process cannot be ignored. In practice, the virtual screening results should be comprehensively analysed in combination with experience and experimental validation.

5. Conclusion

Pathogenic bacteria producing NDM-1 pose a real and escalating threat to human health. Although a number of compounds with great inhibitory activity have been reported, they still need to meet several requirements, including good synergistic effect with antibiotics, broad-spectrum inhibition and reasonable pharmaceutical properties. There is no doubt that natural products perform an important function for drug development, whilst virtual screening is more efficient than traditional screening methods. In this paper, a variety of NDM-1 inhibitors were discovered from a natural product repository through virtual screening. Among these inhibitors, hesperidin showed the highest activity against NDM-1, with an IC_{50} value of 3.348 ± 1.35 μM. Considering the low toxicity of hesperidin, it may serve as a potential lead compound for NDM-1 inhibitor development.

At present, virtual screening plays an increasingly important role in the discovery of MBL inhibitors. More and more potential inhibitors have been discovered by this method. At the same time, with the development of computer technology, novel screening techniques have also been developed, such as screening based on nuclear magnetic resonance (NMR) technology and fragment-based drug discovery (FBDD). These developments have also achieved good results [27–29] and have brought hope for the development of MBL inhibitors.

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Competing interests

None declared.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.02.003.

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