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Short Communication

Genome-based insights into the resistomes and mobilomes of two *Providencia rettgeri* strains isolated from wound infections in Madagascar

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ABSTRACT

Objectives: A molecular analysis was performed of two *Providencia rettgeri* (*P. rettgeri*) strains (Pr 297 and Pr 269) collected in 2007 and 2009 from wound swabs of patients admitted to the intensive care units at Joseph Ravoangy Andrianavalona hospital and the Military Hospital in Antananarivo, Madagascar.
Methods: The two *P. rettgeri* isolates were subjected to susceptibility testing. Whole genome sequencing was performed to characterise the antibiotic resistance genes, genomic islands and mobilomes (integrons, plasmids and insertion sequences).
Results: All isolates were found to be multidrug-resistant. Antibiotic-resistant genes described were amongst eight different classes of antimicrobial agents. Thirty insertion sequences and twelve genomic islands were predicted in each genome. Class 1 and class 2 integrons were found in both genomes, with gene cassette regions encompassing *arr-2 - cmlA5 - bla*$_{OXA-10}$ *- ant (3")-Ia* and *dfrA1 - sat2 - ant (3")-Ia – orfX*, respectively. IncA/C2, ColM and ColE1-like plasmids were described harbouring *bla*$_{CMY-30}$,*qnrD* and *aac(6')-Ib-cr4* genes, respectively. Phylogenetic analysis showed that Pr 297 and Pr 269 isolates were genetically identical and clustered with *P. rettgeri* strains described in the USA and Spain.
Conclusions: It is believed that this is the first molecular characterisation of wound infection pathogens from Madagascan patients and the first description of *P. rettgeri* co-producing CMY-30, OXA-10 and AAC (6')-Ib-cr4 enzymes. The diversity of the resistance determinants and mobile genetic elements was probably due to extensive horizontal gene transfer events, highlighting the need to conduct further molecular monitoring studies to understand the genomic plasticity of resistant bacteria in Madagascan hospitals.

1. Introduction

Providencia belongs to the Enterobacteriaceae family, which together with *Proteus* and *Morganella* genera form the tribe *Proteeae*. This species is an opportunistic pathogen associated with diverse human infections, especially catheter-related urinary tract infections. It is sometimes associated with wounds infections, which are part of common post-surgical medical challenges [1]. Due to their increased toll on morbidity and financial loss, wound infections are becoming a major concern among patients and healthcare practitioners [2]. In developing countries these infections are generally neglected and the few studies conducted so far have focused on the antimicrobial susceptibility pattern of these pathogens [3]. Other frequent causative microorganisms related to wound infections include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococci*, *Escherichia coli, Klebsiella pneumoniae*, *Proteus* spp. and *Pseudomonas aeruginosa* [4].

Like several developing countries, the data on antibiotic resistance is scarce in Madagascar [5]. Furthermore, the molecular mechanisms underlying this resistance remain unclear; notably those related to pathogenic bacterial isolates infecting wounds. Recently, Lai et al. showed that methicillin-resistant *Staphylococcus aureus* (MRSA) and Enterobacteriaceae resistant to third-generation cephalosporin bacteria were the key pathogens responsible

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for wound infections in six countries in sub-Saharan Africa, including Madagascar, during 2004–2016. The wound pathogenic bacteria that were described showed high levels of resistance to antibiotics with clinical relevance [6]. The limited collection of bacteria from wound infections at the Institute Pasteur of Madagascar in Antananarivo has shown emergence of multi-drug-resistance. More alarmingly, analysis of this database revealed a high level of resistance to β-lactams, aminoglycosides and quinolones in bacteria from the *Proteeae* group. There was particular interest in two pathogens, which, in contrast to others, showed full resistance to third-generation cephalosporins and fluoroquinolones, which are commonly used antibiotics to threat infections in Madagascar [7].

The current study aimed to characterise at a molecular level the genetic factors involved in the multidrug resistance from two clinical strains of *Providencia rettgeri* (*P. rettgeri*) isolated from hospitalised patients in intensive care units (ICUs). An in-depth molecular analysis of resistomes (antibiotic resistance genes) and mobilomes (insertion sequences, integrons, genomic islands and plasmids) of these two pathogens was conducted.

2. Material and methods

2.1. Bacteria isolates

Providencia rettgeri 297 (Pr 297) was isolated from a 26-year-old man in November 2007 and *P. rettgeri* 269 (Pr 269) from a 63-year-old woman in December 2009. Pr 297 and Pr 269 were isolated from two wound swabs of the patients who were admitted to the ICUs at Joseph Ravoangy Andrianavalona hospital (for Pr 297) and the Military Hospital (for Pr 269) in Antananarivo, Madagascar. Species identification was first carried out using the API 20NE system (bioMérieux, Marcy-l'Etoile, France) and then confirmed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS, Bruker Daltomics, Bremen, Germany).

2.2. Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed using the agar diffusion method as recommended by the Antibiogram Committee of the French Microbiology Society (ACFMS 2015). The following antimicrobial agents were tested: piperacillin, piperacillin/tazobactam, ceftazidime, cefotaxime, cefoxitin, cefepime, trimethoprim/sulfamethoxazole, gentamycin, amikacin, tobramycin, ertapenem, imipenem, ciprofloxacin and nalidixic acid.

2.3. DNA extraction and whole genome sequencing

DNA was extracted with the cador Pathogen 96 QIAcube HT Extraction Kit (Qiagen, Paris, France) on a Qiacube HT from 5 mL of liquid cultures grown overnight at 37 °C in Luria-Bertani infusion medium following the manufacturer's protocol. Purity and DNA quantity were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Whole genome sequencing (WGS) using the Illumina technology was performed as previously described [5].

2.4. Genome analysis

Antimicrobial resistant genes were screened using KmerResistance 2.2 software from the Centre for Genomic Epidemiology [8]. The genomes of Pr 297 and Pr 269 were also screened to identify the mobile genetic elements (MGE) associated with antimicrobial resistance, including insertions sequences (ISs), integrons and genomic islands (GIs), as previously described [5]. In silico detection and typing of plasmids was performed using PlasmidFinder [9]. The WGS of all identified MGEs were manually inspected and annotated using Geneious software. All open reading frames (ORF) were predicted and the related genes were BLAST searched against the National Center for Biotechnology Information (NCBI) nucleotides database.

2.5. Phylogenetic tree construction

The phylogenetic tree was generated by core-genome alignment using the Harvest suite version v1.1.2 as described by Simo Tchuinte et al. [5]. Genomic data used in the phylogenetic tree were downloaded from the NCBI database, including WGS of *P. rettgeri* isolates PrDmel1 (CM001774.1), Pr 729/12 (LYBX01000001.1), Pr TUM9994 (BGM101000001.1), PR002 (NXKD010000100.1), Pret_2032 (QKNQ01000001.1), PR1 (NOWC01000095.1), CCBH11880 (JSEQ01000001.1), FDAARGOS_330 (CP27418.1) and H1736 (CVLT01000001.1).

3. Results and discussion

Providencia rettgeri is an environmental bacterium that is considered to be an emerging human pathogen and has been associated with several nosocomial infections [2]. The current study described two clinical *P. rettgeri* isolates (Pr 297 and Pr 269) associated with wound infections of two patients hospitalised in ICUs.

Antibiotic susceptibility testing showed that Pr 297 and Pr 269 were resistant to piperacillin, cefoxitin, ceftazidime, cefotaxime, gentamycin, tobramycin, trimethoprim/ sulfamethoxazole, nalidixic acid and ciprofloxacin, but remained susceptible to piperacillin/ tazobactam, cefepime ertapenem, imipenem and amikacin. To further characterise these strains at a molecular level, WGS of these two isolates was performed. In silico analysis revealed the presence of acquired antibiotic resistance genes (ARGs) to β-lactams (bla_{OXA-10} and bla_{CMY-30}), sulphonamides (*sul 1* and *sul 2*), aminoglycosides (*aac(3)-Iva*, *ant(3'')-Ia*, *aph(3'') Ib*, *aph(4)-Ia*, *aph(6)-Id*), phenicols (*cmlA5* and *floR*), tetracycline (*tet*(A), *tet*(B)), and rifampicin (*aar2*) in both isolates. Additionally, ARGs to quinolones (*qnrD*, in genomes of both bacteria) and fluoroquinolone (*aac(6')-Ib-cr4*, only in *P. rettgeri* 269) were identified. This study represents the first report of *P. rettgeri* producing CMY-30, OXA-10 and AAC(6')-Ib-cr4 enzymes. Furthermore, genes encoding resistance to trimethoprim *dfrA1* and *dfrA23* were also detected in Pr 269 and Pr 297, respectively. Based on phenotypic and genotypic results, Pr 297 and Pr 269 were defined as multidrug-resistant strains (MDR).

Mobile genetic elements play a pivotal role in the adaptation of bacterial populations, allowing them to rapidly cope with hostile conditions, including the presence of antimicrobial compounds [10]. Several types of mobile elements associated with antimicrobial and heavy metal resistance were identified in the two Pr 297 and Pr 269 genomes. Amongst 30 insertion sequences (IS) predicted in the two genomes with ISL3, the most represented were ISPlul 5 and Tn*3* transposons. Additionally, 12 genomic islands were predicted in both strains and further analysis revealed the presence of seven ARGs, transposable elements in the GIs, and mercuric resistance determinants present only in *P. rettgeri* 269 (Fig. S1). Genomic islands are described as clusters of genes of probable horizontal origin driving the bacterial or archaeal genome evolution [11]. The latter probably provide adaptive traits that enhance Pr 297 and Pr 269 fitness within clinical settings.

Genomic analysis of both *P. rettgeri* isolates also showed the presence of class 1 and class 2 integrons with gene cassette (GC) regions encompassing *arr-2* - *cmlA5* - bla_{OXA-10} - *ant (3'')-Ia* and *dfrA1* - *sat2* - *ant (3'')-Ia* – *orfX*, respectively. The class 2 integron was embedded in Tn*7* transposon structure (Fig. 1c). The same GC

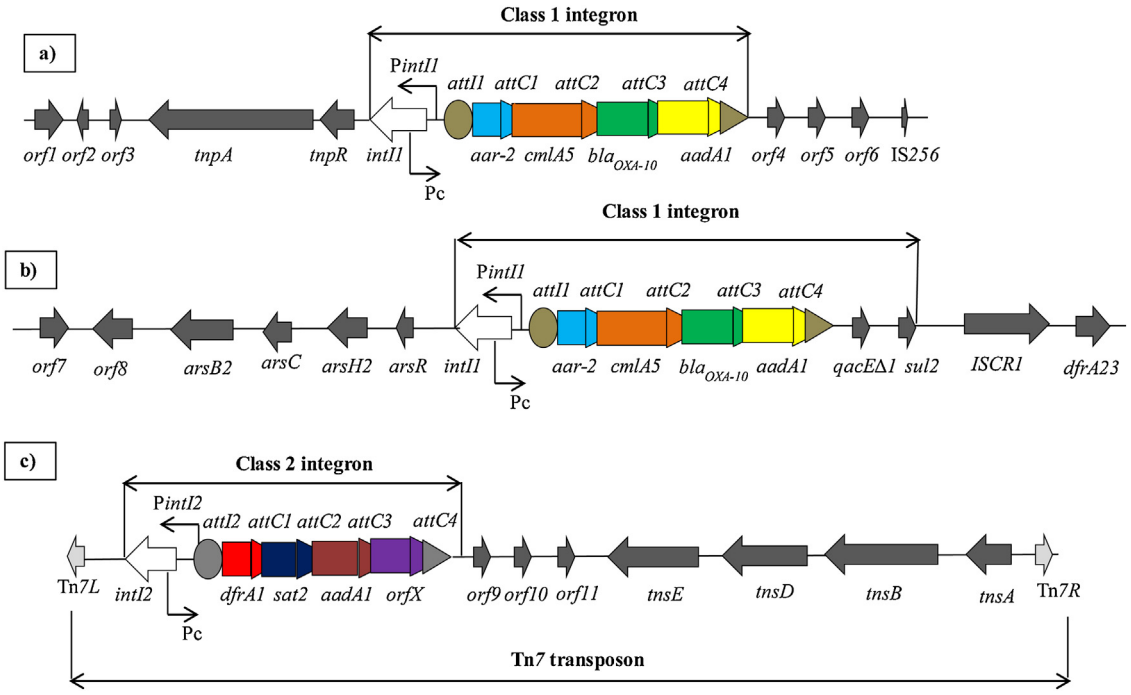

Fig. 1. Schematic representation of integrons and resistance-genomic islands, and their genetic environment described in this study. (a) and (b): Class 1 integrons described in *Providencia rettgeri* 269 and P. rettgeri 297, respectively. (c): Class 2 integron described in the two P. rettgeri.
Abbreviations: orf 1- orf12 genes encoded for hypothetical proteins; PintI, integrase promoter; Pc, promoter of cassettes; int, integrase gene; arr-2, cmlA5, blaOXA-10, aadA1, dfrA1, sat2, genes cassettes; attI1, attI2, attC1-4, recombination sites.

configurations have been described in *Escherichia coli* in Nigeria [12]; however, the genetic environment of the class 1 integron was different. The loci for arsenic resistance was found downstream in the *intI1* gene in Pr 297 (Fig. 1b), while the Tn*3* transposon module was present in Pr 269 (Fig. 1a). To date, integrons are key bacterial genetic elements for acquiring and disseminating antibiotic resistance through gene cassettes [10].

Regarding plasmid typing, IncA/C2, Col3M and ColE1-like type plasmids with lengths of 124 kb, 2.6 kb and 5.9 kb, respectively, were identified in this study (Fig. 2). IncA/C2, Col3M were found in both *P. rettgeri* isolates, while the ColE1-like plasmid was only present in *P. rettgeri* 269. The IncA/C2 plasmid designated herein as pPr297_269 (Fig. 2a) is a conjugative plasmid-borne AmpC-type β-lactamase bla_{CMY-30} and *sul2* genes. The bla_{CMY-30} gene is flanked upstream by an *ISEcp*1 providing a strong promoter [13]. AmpC β-lactamases are clinically important cephalosporinases produced in several Enterobacteriaceae, which exhibit an enhanced activity toward third-generation cephalosporins (cefotaxime, ceftazidime) and β-lactam-β-lactamase inhibitor combinations [14]. Sequence analysis of pPr297_269 plasmid showed a high identity percentage (> 95%) with the p972816 plasmid isolated from *Salmonella enterica* subsp. *enterica* SA972816 strain (GenBank accession no. CP007487). Further analysis also showed that the 75.5 kb region containing *ISEcpe1*- bla_{CMY-30} shared an identity of 100% with p2012EL-2176 plasmid from a *Vibrio cholera* 2012EL-2176 (GenBank accession no. CP007636) strain isolated in Haiti [15]. The IncA/C plasmids are widespread in Gram-negative bacteria and commonly confer multidrug resistance [10].

Quinolone resistance is usually mediated by point mutations in quinolone resistant-determining regions of gyrase (*gyrA, gyrB*) and topoisomerase IV genes (*parC, parE*), leading to a target modification, also by efflux encoded by the *qnr* genes, and by enzymatic modification encoded by AAC(6')Ib-cr [16]. In the current study, the resistance to nalidixic acid and ciprofloxacin exhibited by Pr 297 and Pr 269 was associated to mutations in *gyrA* (Ser83Ile and Asp87Glu), *parC* (Glu80Pro and Asp84Ile) topoisomerases and *qnrD* gene expression. The *qnrD* gene described in Pr 297 and Pr269 was enclosed in a small non-typeable and non-conjugative plasmid, matching a Col3M plasmid (Fig. 2c). It contained four putative ORFs and was identical to p356862 plasmid (GenBank accession no. MF062093) isolated in *Proteus vulgaris* 36852, except for Leu11Ser substitution in *qnrD* gene. As described by Guillard et al. the *qnrD* gene was expressed by a putative promoter containing -35 (TTTATA) and -10 (GTCTTAAAAT) hexamers sequences [17]. These results are in accordance with those found by Yaiche et al. with clinical *qnrD*-positive *Morganella morganii* isolates [18].

This study also identified a plasmid carrying the *aac(6')-Ib-cr-4* gene in the Pr 269 isolate, which mediated quinolone resistance. The acetyltransferase encoded AAC(6') Ib-cr4 is active on aminoglycosides (tobramycin and kanamycin) and fluoroquinolones [19]. The comparative analysis revealed that the ColE1-like plasmid described (Fig. 2b) in this study was 99.9% identical to the pMdT1 plasmid (GenBank accession no. JX457478), which was previously reported in a clinical *Salmonella enterica* serovar Typhimurium DT104B strain. One mutation (GTA/GTT) in the two RNAse genes present on the plasmid and four nucleotide substitutions on the plasmid sequence were found. The *aac(6 ′)-Ib-cr* gene is usually found in a cassette as part of an integron in a multi-resistance plasmid [16]. As previously reported, the *aac(6′)-Ib-cr4* gene was harboured on a ColE1-like plasmid (Fig. 2b). This gene was embedded in its classical gene cassette structure with the conserved *attC* recombination site downstream of the gene [19]. The mobilisation region composed to *mobA, mobB, mobC* and *mobD* genes showed significant identity to that of ColE1-like plasmids. These latter were described like small plasmid encoded colicin E1 (cea) and colicin immunity (imm) harbouring the basis of a mobility (bom) region, a cis-acting region required for plasmid mobility [10]. Two partially overlapping RNAs (RNAI and RNAII),

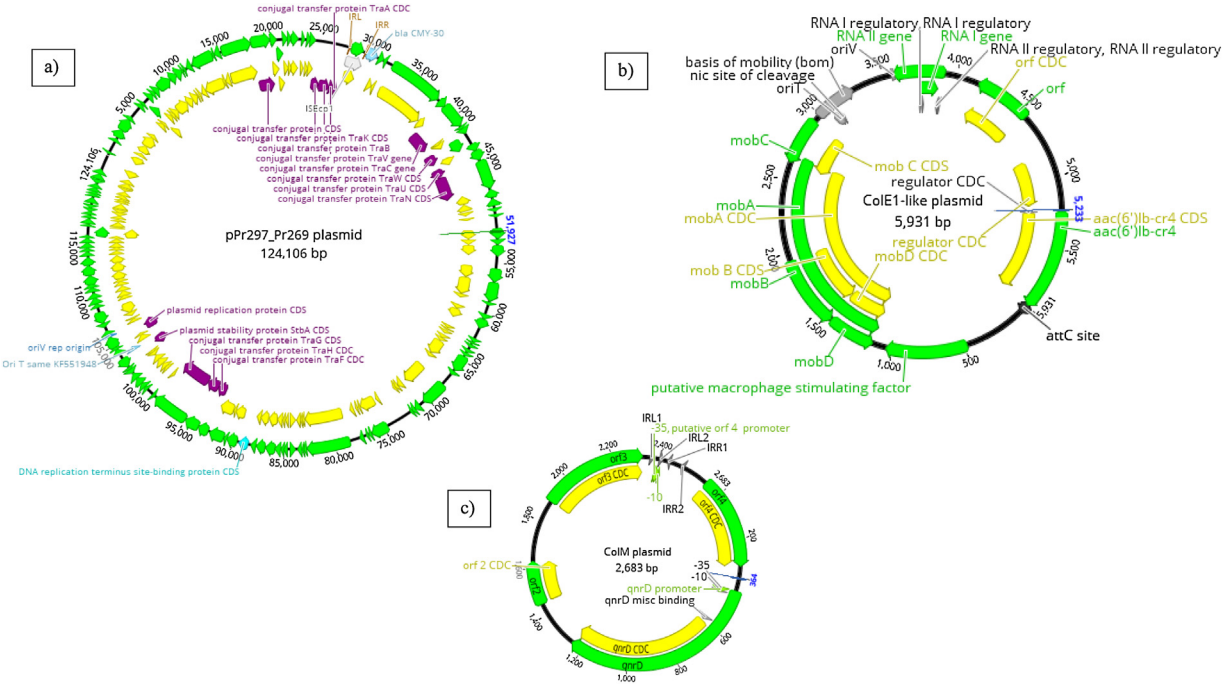

Fig. 2. Genetic organisation of pPr297_269 (IncA/C2), ColM and ColE1-like plasmids.
The pPr297_269 plasmid (a) harbours several genes, among them are 10 conjugal transfer genes (traK, traA, traB, traC, traF, traG, traK, traN, traU and traW) and ISEcp1-blaCMY-30 structure. The ColE1-like (b) is the plasmid backbone of aac(6')Ib-cr4 gene, four mobilisation genes (mobA, mobB, mobC and mobD) and RNAs genes (RNAI and RNAII). qnrD gene, which encodes a QnrD pentapeptide protein, has been described on the ColM plasmid (c).
Abbreviations: orf 2, orf3 and orf4 genes encoded for hypothetical proteins; oriV, origin of replication; oriT, origin of transfer.
Green arrows represent the genes. The yellow and purple arrows represent the coding sequences coding (CDS).

transcribed from the origin of vegetative replication (*oriV*), constituted the basic replication of the ColE1-like plasmids, which are necessary for replication and copy number of plasmids [20].

To further investigate the genetic relatedness of *P. rettgeri* 297 and *P. rettgeri* 269 with those described in the literature, a core genome phylogeny analysis was performed. The results revealed that the two analysed genomes were genetically identical and clustered more with *P. rettgeri* strains isolated from the USA and Spain (Fig. 3). Furthermore, the genomic comparison of Pr 297 and Pr 269 strains revealed a single-nucleotide substitution corresponding with one single-nucleotide polymorphism (SNP). It is important to point out that Joseph Ravoangy Andrianavalona hospital and the Military Hospital are 4.8 km apart. The genetic similarity between the two genomes suggests that MDR *P. rettgeri* isolates are circulating in the ICUs of these two hospitals.

Identification of ARG on mobile elements in these *P. rettgerii* isolates, previously described in other Gram-negative bacteria, suggests that these genetic elements were acquired through horizontal gene transfer, probably facilitated by antibiotic-selective pressure. In fact, a recent study reported that the consumption of antibiotics – including β-lactams, aminoglycosides and quinolones – was significantly higher in ICU than in other wards in

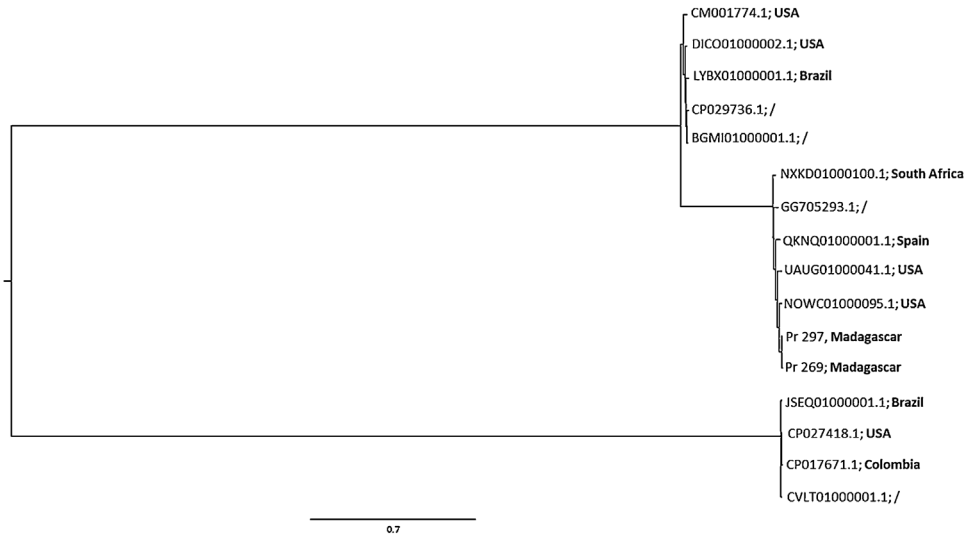

Fig. 3. Phylogenetic analysis of two *Providencia rettgeri* isolates described in this study. The phylogenetic tree was constructed with the Harvest suite for core genome alignment and visualisation.

Antananarivo Befelatanana University hospital [7]. Taking the current results into account, further molecular monitoring studies are needed to both understand the spread of MDR isolates and the evolution of genomic plasticity of these pathogens in Madagascan hospitals, especially in the ICU.

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Competing interests

None declared.

Ethical approval

Not required.

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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.2019.07.013.

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