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Genotyping comparison of *Mycobacterium leprae* isolates by VNTR analysis from nasal samples in a Brazilian endemic region

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ABSTRACT

This study analyzed the genetic diversity by MIRU-VNTR of *Mycobacterium leprae* isolates from nasal cavities and related to epidemiological and clinical data. The sample consisted of 48 newly diagnosed leprosy cases that tested positive for *M. leprae* PCR in nasal secretion (NS) attending to the National Reference Center of Dermatology Dona Libania (CDERM), Fortaleza, Brazil. Total DNA was extracted from NS of each patient and used for amplification of four *M. leprae* VNTR loci. Four clusters of *M. leprae* isolates were formed with identical genotypes. In the spatial analysis, 12 leprosy cases presented similar genotypes organized into 4 clusters. The most common genotypes in the current study was AC8b: 8, AC9: 7, AC8a: 8, GTA9: 10, which may represent a genotype of circulating strains most often in Ceará. A minimum set of four MIRU-VNTR loci was demonstrated to study the genetic diversity of *M. leprae* isolates from NS.

KEYWORDS

Leprosy; *Mycobacterium leprae*; Brazil; nasal cavity; genotype; genetic marker; VNTR loci; cluster analysis

Introduction

Despite global efforts to eradicate leprosy, Brazil is still considered as and endemic country, with 213,899 new cases reported globally in 2014 [1]. Brazil, India and Indonesia accounts for 81% of all reported new cases. In Brazil, during the period from 2012 to 2014, the areas of greatest risk for leprosy were concentrated in pockets of the Central region and its neighborhood in the North and Northeast regions. These three areas concentrate only 14% of the population of the Brazilian country and accounts for 44% (13,597/31,044) of the new cases diagnosed in 2014 [1]. In the same year, the State of Ceará, Northeast Brazil, reported 2.069 new cases, with a coefficient of case detection rate (CDR) of 24/100.000. It is also observed that 148 (80.5%) municipalities in Ceará diagnosed new leprosy cases in 2014, 34 (18.4%) of them with more than 10 new cases per 100,000 inhabitants and 23 (12.5%) municipalities were reported as hyper-endemic with a CDR of more than 40/100,000 inhabitants [2].

The conventional epidemiology of leprosy has be improved by strain genotyping tools. The isolates differentiation approach using molecular markers are useful to distinguish different strains of the leprosy bacilli. Variable number tandem repeat (VNTR) provides data about the pattern of variation in the *Mycobacterium leprae* genome [3–5]. The VNTR typing tool is based on the number of repetitive sequences in polymorphic micro- and mini-satellite regions of the bacteria [6]. Some polymorphic loci are suitable for identifying genotypes according to the discriminatory capacity, stability, and reproducibility. There is considerably more variation in repeat numbers at VNTR locus than in non-repetitive DNA sequences, because length-altering mutations due to slipped-strand mispairing occur at a much higher rate than the inherent DNA substitution or mutation frequency of DNA polymerase [7,8].

Extensive research on different loci has been done on strain typing of the leprosy bacilli and it was achieved the standardization of 17 loci optimized with *M. leprae* DNA from the strain NHDP63 obtained from infected armadillo tissue [9]. Since *M. leprae* cannot be cultivated in axenic media, there are several challenges to its molecular typing, including the difficult to obtain sufficient amounts of genomic DNA from clinical samples. In addition, samples from leprosy patients are often of poor quality, and often contaminated with other cultivable agents [10].

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Molecular typing of *M. leprae* through VNTR can establish leprosy transmission chains within populations and allow the evaluation of genomic markers for differentiating bacillus strains. Nasal route is considered as the main entrance and exit of the leprosy bacilli and collection of nasal secretion are not invasive and painless. In this way, genotyping of the bacteria harbored in nasal cavity can provide insights about the circulating strains in the community and active transmission can be correlated. Thus, this study analyzed the genetic diversity by MIRU-VNTR of *M. leprae* isolates from nasal cavities and related to epidemiological and clinical data.

Materials and methods

Subjects

A cross-sectional study was conducted in Reference Center on Dermatology Dona Libania (CDERM), Fortaleza, capital of Ceará state, northeastern region of Brazil. Nasal samples (NS) from 48 new leprosy cases were collected from June 2009 to December 2010. Cases were diagnosed by clinical evaluation, slit skin smear and biopsy samples. They were classified according to Ridley-Jopling criteria based on histological study and bacilloscopic index (BI) [11]. A well structured questionnaire in combination with a review of medical records were used to collect socio-demographic data.

Ethical considerations

Written consent was obtained from the participants and authorized the collection of samples. The study was approved by the CDERM Ethics Committee and guidelines of the National Ethical Committee were followed to conduct the research.

Sampling collection and DNA extraction

NS were collected from all participants using sterile cotton perinasal swabs pre-moistened with Tris-EDTA buffer (pH 8.0). Nasal specimens were obtained by rotating in each nostril a single swab over the lateral anterior conchae. Swabs were then placed into a sterile and labeled tube and stored at −20 °C until processing. DNA extraction procedure was conducted as previously described [12].

Detection of M. leprae DNA

DNA from NS of participants was searched for a 238-pb fragment of RLEP2 (GenBank accession NC002677) in a semi-nested PCR reaction following the protocol previously described [12]. The PCR products were submitted to the ABI Prism 3730 automated DNA sequencer using the ABI PRISM BigDye Terminator v 3.0 sequencing kit (Applied Biosystems, Foster City, CA). The sequences were identified using SecScape software v.2.7 (Applied Biosystems, Foster City, CA).

MIRU-VNTR genotyping

To assess the genetic diversity of *M. leprae* among individuals, DNA obtained from NS were analyzed by VNTR analysis targeting 4 loci: AC8b, AC9, AC8a, and GTA9. To determine how many short tandem repeat segments are present in each sample allele, we made a comparison with the positive control NHDP63 strain of *M. leprae*. The NHDP63 VNTR and amplicon size were verified through gene sequencing. The amplicon sizes at each locus for the positive control were 384 bp to AC8b, 207 bp to AC9, 125 bp to AC8a and 307 bp to GTA9 [4,9].

PCR amplifications were performed using using the HotStartTaq Master Mix (Qiagen, Hilden, Germany), 0.2 µM of each primer pair and 2 µl of template DNA. Thermocycling conditions used were: initial denaturation at 95 °C for 15 min followed by 40 cycles of 94 °C for 30s, 60 °C for 90s, 72 °C for 90s followed by a final elongation step f 72 °C for 10 m [4,9].

Analysis of fragments

Fluorescently-labeled primers were used in each reaction, being amplified in a total of four reactions. PCR products were diluted 30–60 times in water and 1 µl was mixed with 0.3 µl of GeneScan™ 500 LIZ® Size Standard molecular weight marker (Applied Biosystems, Foster City, CA) and 8.7 µl deionized formamide was added. Allele copy number was determined by denaturation of amplicons and capillary gel electrophoresis on the sequencer ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Capillary electrophoresis (length 50 cm, polymer POP-7) was conducted at 15 kV over 60 °C with a running time of 45 min [4,9]. Copy number definition of each of the four allele were defined using the Peak Scanner software (Applied Biosystems, Foster City, CA).

Calculation of allelic diversity and discriminatory power

The Hunter – Gaston discrimination index (HGDI) was used as a numeric parameter VNTR discriminatory power. The HGDI was calculated as recommended by Hunter and Gaston [13] by using the formula: $h = 1 - \sum x_i^2 \left[n/(n-1)\right]$ where x_i is the frequency of the allele i at locus, n is the number of isolates). The sample is considered to be highly discriminatory polymorphic if $h \geq 0.6$; moderately discriminatory if $0.3 \leq h \leq 0.6$ and weakly discriminatory if $h \leq 0.3$ [13].

Cluster definition and genotype comparison

Definition of clustering was based on comparison of the copy number of the VNTRs, considering identical

genotypes those that presented identical copy number for all four alleles.

Spatial analysis

The home addresses of the new leprosy cases were georeferenced using Google Earth (https://www.google.com/earth/) to define latitude and longitude. The spatial database was visualized using the QuantumGis Geographic Information System 18.1.0® licensed by General Public License) (http://www.qgisbrasil.org). The cartographic bases used were obtained from the Brazilian Institute of Geography and Statistics [14]. Data were used to generate graphics showing the distribution of the georeferenced individuals in Fortaleza by VNTR typing.

Statistical analysis

For evaluation of the association of the demographic, clinical and environmental/behavior variables and having a clustered or a unique *M. leprae* genotype, we used squared and the Fisher exact two-tailed test. A P value of ≤ 0.05 was considered statistically significant. Mann-Whitney test was used to evaluate the differences between a single characteristic between individuals with clustered genotypes or unique patterns. STATA version 12.0 (Stata Corp., College Station, TX, USA) was also used to analyze the data.

Results

Genotyping

The differentiating power of the four allele for the 48 *M. leprae* NS isolates from leprosy new cases were analyzed, and moderate discriminatory index was found for the chosen VNTRs loci. Variations in copy number ranged from 6 to 8 for AC8b locus with $h = 0.47$, from 7 to 9 for AC9 locus with $h = 0.51$, from 8 to 11 for AC8a locus with $h = 0.57$, and from 8 to 16 for GT9 locus with $h = 0.57$. The most common genotypes in the current study were AC8b with 8 or 7 copies, AC9 with 7 or 8 copies, AC8a with 8 or 9 copies and GTA9 with 10 or 9 copies (Table 1).

Among the four loci, three (AC9, GTA9 and AC8a) showed absence of PCR products for some isolates and were thus subsequently excluded from the final selection. In addition, other isolates have not enough demographic data and were also excluded. Thus, a remaining 24 NS provided enough information for the genotypic comparison and descriptive analysis (Table 2). By using the 24 remaining isolates, four clusters of identical genotypes were formed resulting in a HGDI of 0.73.

Among the 24 isolates, 12 had unique genotypes and the remaining 12 isolates form four clusters of genotypes. Cluster 1 to 3 with two isolates each, and cluster 4 with 6 isolates (Table 3).

Spatial analysis and clusters

The distribution of the 24 cases was plotted in the map of the city of Fortaleza and spatial analysis according the

Table 2. VNTRs from 48 nasal secretions.

Sample ID	AC 8b	AC 9	AC 8a	GTA 9
84267*	8	8	8	10
85.886	7	8	9	9
86187*	8	7	8	10
86.190	7	8	9	9
86.340	7	7	8	9
86.362	7	8	9	9
86.394	7	7	8	–
86.498	7	7	8	16
86.555	7	8	9	10
86610*	6	–	8	9
86.645	7	–	9	–
86776*	7	8	8	9
86.829	7	–	8	9
86.855	8	7	8	10
86962*	8	7	8	10
87126*	8	7	8	10
87184*	7	8	9	–
87212*	7	8	9	8
87.214	8	7	8	10
87330*	7	7	10	8
87401*	8	7	8	10
87.419	7	–	9	–
87.549	8	7	9	10
87.560	7	8	9	9
87914*	8	7	8	10
88.276	8	–	–	–
88689*	8	7	8	10
88742*	7	8	9	10
88935*	7	8	9	12
89.115	7	8	9	9
89160*	7	8	8	10
89167*	7	8	9	9
89.167	7	8	9	9
90148*	8	7	9	10
90373*	7	7	9	10
90.730	8	–	10	–
90.780	8	–	–	10
91.371	8	–	–	10
91.796	7	8	9	9
91845*	7	8	10	10
91.979	8	7	9	10
92008*	7	8	9	10
92070*	7	9	11	10
92114*	7	7	8	10
92.275	7	–	–	10
92662*	7	8	8	9
92.642*	7	7	8	10
–	8	7	8	12

*24 samples used for descriptive analysis.

Table 1. Allelic diversity of the 4 loci of VNTRs.

	Number of allele								Allelic diversity (*h*)	Conclusion	Total samples
VNTR	6	7	8	9	10	11	12	16			
AC 8b	1	30	17						0.47	Moderately discriminatory	48
AC 9		19	19	1					0.51	Moderately discriminatory	39
AC 8a			20	20	3	1			0.57	Moderately discriminatory	44
GTA 9			2	14	24		2	1	0.57	Moderately discriminatory	43

clustering was performed. Although it was observed that cases were spread over the city, the Fortaleza administrative regions V and I were associated with the clusters. Cluster 4, with five of the six cases distributed in three neighborhoods of the administrative region V, 2 cases in Bonsucesso, 1 in Granja Portugal and 2 in Granja Lisboa (Figure 1).

In all four clusters, the 12 leprosy cases reported to have a previous contact with at least one leprosy case. Group 1 was composed of two individuals with lepromatous leprosy, BCG scar, aged 51 and 62, born in the same countryside of Ceará, and living there for 12 years. They were diagnosed in a difference of 17 months (Table 2). Group 2 was composed of two individuals with borderline leprosy, BCG scar, aged 59–60 years, diagnosed in 2010 with a difference in date of diagnosis of 4 months. Despite they were born in different cities of Ceará, they had moved to Fortaleza in 1995 and 1970, respectively. Group 3 was composed of two individuals aged 34 and 32 years and diagnosed within ten months of each other. The first was born and lived in Fortaleza, was diagnosed with borderline leprosy, and presented with a BCG scar. The second, a lepromatous leprosy without a BCG scar, was born in São Paulo, and living in Fortaleza since 1997, and had. Group 4 was composed of six participants with ages ranging from 12 to 71 years, all six cases were diagnosed from February to August 2009. Four cases had borderline leprosy and the two had lepromatous leprosy (Table 4).

The distribution of cases according to belonging to a cluster of genotype and not belonging to any cluster were related to age, to time in months between symptoms and diagnosis and to bacilloscopic index was analyzed. The age of non clustered cases ranged from 17 to 34 years, while to the clustered ranged from 26 to 64 years old, being the overall mean age of the non clustered (25.5 years), much lower than the clustered (44.6 years; $p < 0.0110$) ones. Despite not significant, the mean time between beginning of symptoms and leprosy diagnosis among the clustered isolates (20.3 months) was lower than those with unique genotype (22.6 months). No differences were seen among bacilloscopic index of the groups of isolates (Table 5).

Table 3. Groups of samples with identical *M. leprae* genotype.

Sample ID	Group	AC 8b	AC 9	AC 8a	GTA 9
86.776	1	7	8	8	9
92.662	1	7	8	8	9
92.642	2	7	7	8	10
92.114	2	7	7	8	10
88.742	3	7	8	9	10
92.008	3	7	8	9	10
87.401	4	8	7	8	10
86.187	4	8	7	8	10
87.126	4	8	7	8	10
87.914	4	8	7	8	10
86.962	4	8	7	8	10
88.689	4	8	7	8	10

Discussion

Genotyping of *M. leprae* bacilli has been reported in several countries, such as India [15], Indonesia [16], China [17] and Brazil [18]. However, most of the studies are based on *M. leprae* isolates from biopsy samples. A recent study conducted by our group had demonstrated that NS also can be used to genotype approaches [8].

The difficulty in analyzing the VNTRs from NS in the current research was also observed in Hyderabad, India [19], in which only three loci from samples of different tissues from the same patient were studied. A previous study also conducted in Ceará, Brazil, discuss lack of amplification in NS compared to biopsy samples [8]. A study conducted in Rio de Janeiro, Brazil, with skin biopsy

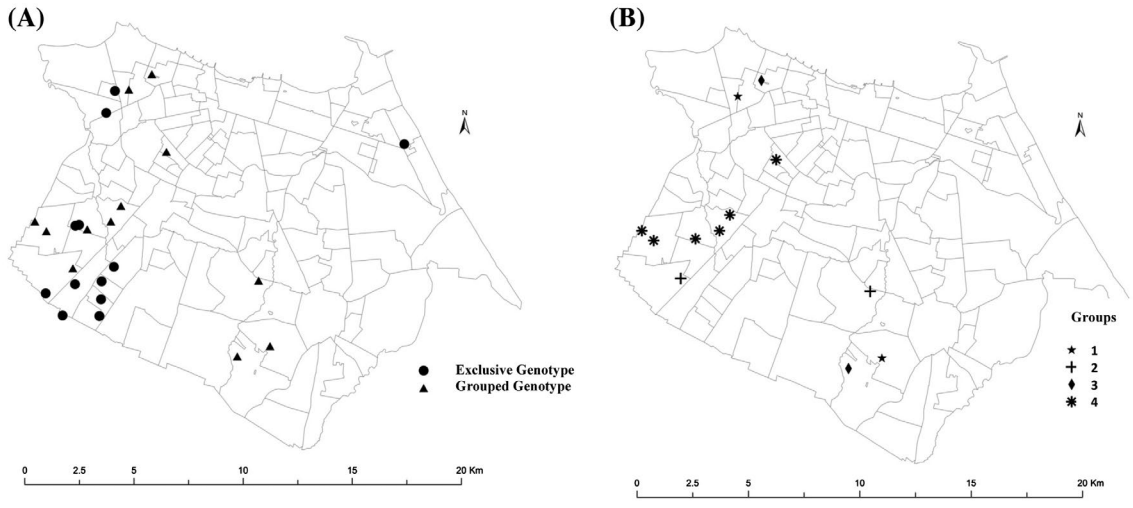

Figure 1. Maps of the Municipality of Fortaleza with the dwelling of the individuals with the genotypes of the *M. leprae* strain analyzed.

Source: (A) Location of patients' home according to those with strains of exclusive genotypes and strains of grouped genotypes. (B) Location of the patients' dwelling according to the groups formed from the strains with similar genotypes.

Table 4. Main characteristics of individuals belonging to the groups formed.

Sample ID	Group	Date of diagnosis	Clinical form	City of birth	Birth date	Age	BCG Scar
86776	1	11/03/2009	Lepromatous	Aracati/CE	29/11/1946	62	Yes
92662	1	19/08/2010	Lepromatous	Aracati/CE	08/10/1958	51	Yes
92642	2	18/08/2010	Borderline	Missão Velha/CE	18/08/1950	60	Yes
92114	2	29/04/2010	Borderline	Morada Nova/CE	16/04/1951	59	Yes
88742	3	09/09/2009	Borderline	Fortaleza/CE	16/06/1975	34	No
92008	3	21/06/2010	Lepromatous	Sao Paulo/SP	12/12/1977	32	No
87401	4	25/04/2009	Lepromatous	Uruburetama/CE	23/05/1964	45	Yes
86187	4	01/02/2009	Borderline	Canide/CE	01/08/1937	71	No
87126	4	01/04/2009	Lepromatous	Capistrano de Abreu/CE	29/10/1955	53	No
87914	4	01/07/2009	Borderline	Fortaleza/CE	19/03/1966	43	No
86962	4	01/03/2009	Borderline	Fortaleza/CE	22/05/1995	13	Yes
88689	4	28/08/2009	Borderline	Sao Joao da Se/PI	01/07/1997	12	Yes

Table 5. Comparison of characteristics of patients with samples exclusive genotypes and grouped genotypes by the Mann-Whitney test.

	Samples	*N*	Mean	sd	Median	*p*
Age	Exclusive Genotype	12	25.500	8.163	23.5	0.0110
	Grouped genotypes	12	44.583	18.807	48.0	
Time (months) between symptoms and diagnosis	Exclusive Genotype	12	22.625	17.391	24.8	0.7728
	Grouped genotypes	12	20.304	17.505	13.1	
Bacilloscopic index	Exclusive Genotype	9	4.239	0.302	4.0	0.9670
	Grouped genotypes	10	4.100	1.312	4.3	

sd, Standard Deviation.

samples had used four short tandem repeats (GAA GTA9, AT17 and TA18), had also demonstrated failure to amplify all loci [20]. *M. leprae* DNA extracted from NS is often of poor quality, in insufficient amounts, and demonstrates higher contamination with microorganisms derived from the community and from the environment.

The chosen four genetic markers (AC8b, AC9, AC8a, GTA9) used in this study to analyze the NS have been well studied for their stability potential and polymorphic diversity [8,9,19,21,22]. Genetic epidemiological analysis are considered appropriate when demonstrating a discrimination index of at least 90% [14]. In the present study, we report an HGDI of 73% for four VNTRs loci, but for this calculation, only 24 samples were used. In the current study, AC8b presented an h index of 0.47, in contrast to another study conducted in Brazil with samples from the states of São Paulo and Rio de Janeiro, with an HGDI of 0.05 [18]. In our study, this locus showed 6–8 repetitions, 7 being the most common, similar to the study conducted in São Paulo and Rio de Janeiro [18]. In studies conducted in Thailand, Indonesia, Korea and Japan, AC8b were often present with 8 repetitions [6,23].

In our study, AC9 presented an h index of 0.51, higher than reported in previous studies conducted in Brazil (h = 0.28 on average) [18]. Regarding the number of alleles for this genetic marker, it was observed in our study of 7 and 8 alleles as the most frequent. Although with small sample sizes, other studies found 10 alleles at this locus [6,23,24]. Ten AC9 repetitions are quite rare and not yet reported in samples from Brazil.

Thus, despite the small number of samples analyzed in this study, AC9 and AC8b loci showed greater diversity in samples from Fortaleza compared to isolates from states of São Paulo and Rio de Janeiro, but with a similar number of alleles [18].

In this study, AC8a presented an h index of 0.57, with 8 to 11 alleles, but in several reports, this locus showed more variability in the number of alleles [6,18,23–25]. Similarly, GTA9 showed an h index of 0.57, with 8 to 16 alleles. Other published studies showed more variability in this allele number, from 7 to 45 [18,23–25]. Contrary to these studies, our isolates report 10 alleles for this genetic marker.

The high genetic diversity of isolates in Brazil is demonstrated in the present study with only 24 leprosy cases, and only 4 clusters. This genetic diversity is similar to that reported in India [26]. Brazil and India alone account for 69% of all new leprosy cases detected worldwide. Thus, this high genetic variability of *M. leprae* would be proportional to the number of cases in these two countries, therefore suggesting the existence of different *M. leprae* strains in Fortaleza [27].

Our spatial analysis on genotype distribution based on these four loci demonstrated a distribution of clustering similar from disease distribution in Fortaleza in general. A previous study already had demonstrated the concentration of leprosy cases in areas with low socioeconomic status (SES) in Fortaleza [12]. The administrative regions V and I are hyperendemics (40–300/10.000) for leprosy in general population and in children less than 15 years of age (>10–55/10.000) [2]. Since the main route of infection of the bacilli is via nose, the *M. leprae* isolates from NS represent the truly genetic diversity of the active transmission of the disease in endemic community, such as Fortaleza.

The exact transmission source between individuals could not be established in the clusters with *M. leprae* isolates with similar genotypes, however transmission insights can be addressed. Given that our samples are derived from a single city, variations in a few VNTR loci

are quite significant for the formation of clusters, as also demonstrated in villages of North Maluku, Indonesia [16]. Other studies have also studied the molecular epidemiology of leprosy with few loci of VNTRs [20,21,28].

Primers specific for the RLEP2 region displayed 100% specificity for the only *M. leprae* species with a single PCR product. Despite *M. leprae* and *M. lepromatosis* are highly related mycobacterial species, sharing 93% of nucleotide sequence identity and now referred to as the Leprosy Complex [29], our primers did not show homology to *M. lepromatosis*. These two species share 75–90% sequence identity in segments of the four families of repetitive DNA [29].

Several studies discuss the ideal number of genetic markers and which loci fulfill the criteria of sufficient variability with required stability and robustness [4,30]. In addition, it was raised the concern that some markers did not yield consistent outcomes for different samples that originated from the same patient [31]. Thus, choosing appropriate markers is of outmost importance for the reliability of genotyping results. In the same way, there are a few information about the genetic diversity among *M. leprae* isolates from Brazil, in particular in the Northeast region, which is still endemic for the disease. Despite the cure rate in Brazil in 2016 was 81.8% [32], the distribution of leprosy is unevenly, with highly endemic regions in the North, Northeast and Central-West. SNP analysis in previous work revealed large variability in genotypes in Brasil, such as the prevalence of SNP genotype 3 in São Paulo, Rio de Janeiro and Rondônia, and a high prevalence of SNP genotype 4 in Ceará and Pernambuco in states of the Northeast Brazil [3].

In conclusion, we have demonstrated with a minimum set of four MIRU-VNTR loci, the genetic diversity of *M. leprae* isolates from NS. Since *M. leprae* isolates from nasal cavity are representative of the transmission within the local neighborhood, more studies are needed to provide insights about the leprosy dynamic. Furthermore, studies using a higher number of VNTR loci are encouraged, including the addition of other reliable and high-resolution molecular markers for evaluating *M. leprae* transmission.

Geolocation information

https://goo.gl/maps/ymqaQZnNVYH2

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Disclosure statement

The authors declare that they have no competing interests.

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