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Evaluation of 16S rRNA qPCR for detection of *Mycobacterium leprae* DNA in nasal secretion and skin biopsy samples from multibacillary and paucibacillary leprosy cases

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ABSTRACT

Mycobacterium leprae bacilli are mainly transmitted by the dissemination of nasal aerosols from multibacillary (MB) patients to susceptible individuals through inhalation. The upper respiratory tract represents the main entry and exit routes of *M. leprae*. Therefore, this study aimed to evaluate the sensitivity and specificity of real-time quantitative polymerase chain reaction (qPCR) in detecting *M. leprae* in nasal secretion (NS) and skin biopsy (SB) samples from MB and paucibacillary (PB) cases. Fifty-four NS samples were obtained from leprosy patients at the Dona Libânia National Reference Centre for Sanitary Dermatology in Ceará, Brazil. Among them, 19 MB cases provided both NS and SB samples. Bacilloscopy index assays were conducted and qPCR amplification was performed using specific primers for *M. leprae* 16S rRNA gene, generating a 124-bp fragment. Primer specificity was verified by determining the amplicon melting temperature (T_m = 79.5 °C) and detection limit of qPCR was 20 fg of *M. leprae* DNA. Results were positive for 89.7 and 73.3% of NS samples from MB and PB cases, respectively. SB samples from MB patients were 100% positive. The number of bacilli detected in NS samples were 1.39×10^3–8.02×10^5, and in SB samples from MB patients were 1.87×10^3–1.50×10^6. Therefore, qPCR assays using SYBR Green targeting *M. leprae* 16S rRNA region can be employed in detecting *M. leprae* in nasal swabs from leprosy patients, validating this method for epidemiological studies aiming to identify healthy carriers among household contacts or within populations of an endemic area.

KEYWORDS

Mycobacterium leprae; quantitative real-time PCR; nasal cavity; biopsy; paucibacillary leprosy

Introduction

Leprosy is a chronic infectious disease caused by the acid-fast bacilli *Mycobacterium leprae*. Variations in susceptibility to *M. leprae* and clinical manifestations of the infection are attributed to the pattern of the host immune response. *M. leprae* bacilli mainly invade the Schwann cells in the peripheral nerves, leading to nerve damage and development of physical disabilities [1,2]. Even with the implementation of multidrug therapy, leprosy continues to be a public health concern that has not yet been eliminated. The number of new cases reported globally in 2016 was 214,783. Brazil is the second most affected country, where 28,761 new cases were observed in 2015, accounting for 13% of all new cases detected worldwide [3]. Northeast Brazil is considered a highly endemic area for leprosy, and in 2016, leprosy detection rate in the overall population of the state of Ceará was 18.9/100,000 [4].

Leprosy diagnosis is based on clinical examinations, bacilloscopy of slit-skin smears, and histopathology of skin biopsies; however, paucibacillary (PB) forms are not easily detected by the latter two methods [5]. Despite its low sensitivity, detection by bacilloscopy of slit-skin smears is recommended as the 'gold-standard' by the Brazilian health authorities, as it is cheap and non-invasive compared with skin biopsies [6]. The direct detection of acid-fast bacilli in slit-skin smears has a high specificity but a low sensitivity, as approximately 50% of all leprosy patients are slit-skin smears negative [5,6]. Moreover, the bacilloscopy index is not sensitive enough for the diagnosis of subclinical infections, including household contacts of leprosy cases [7–9]. Molecular-based approaches using conventional and quantitative PCR (qPCR) have

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Supplemental data for this article can be accessed here.

already been demonstrated as having higher sensitivities than the sensitivity observed for bacilloscopy of skin biopsies and slit-skin smears [8,10–12].

The upper respiratory tract of a susceptible person is considered to be the main entry and exit route of *M. leprae* [13], and individuals with active disease - multibacillary (MB) cases in particular - are the main sources of infection [14]. In addition, several studies based on nucleic acid amplification have demonstrated that nasal cavities are mainly responsible for the transmission of bacilli [13–17]. Thus, nasal secretion (NS) [7,13,18,19] and skin biopsy (SB) [11,20,21] samples have been widely investigated by both conventional and qPCR, the latter proving much more sensitive. The aim of this work was to evaluate the sensitivity and specificity of qPCR for the detection of *M. leprae* in NS and SB samples from MB and PB cases.

Materials and methods

Collection and processing of clinical material

NS and SB specimens were obtained from patients at the Dona Libânia National Reference Centre for Sanitary Dermatology, Ceará, Brazil. Untreated leprosy cases were included and confirmed by clinical skin examinations, skin smears, and biopsies. They were classified using the Ridley-Jopling [22] criteria based on histology and bacilloscopy indices (BI) and according to the World Health Organization (WHO) [23] as PB or MB cases. Recruitment of cases was random. A total of 54 NS samples from both nostrils were obtained from patients with different clinical forms, 39 MB (20 lepromatous leprosy [LL] and 19 borderline-borderline [BB]) and 15 PB (14 tuberculoid [T] and one indeterminate [I]). In addition, 19 SB specimens (paired with NS samples) were obtained from MB cases (10 BB and 9 LL). Dried slit-skin smear slides were stained by the Ziehl-Neelsen carbol-fuchsin procedure as described previously [6]. Stained slit-skin smears were examined by optical microscopy and the bacilloscopy index (BI) was calculated according to the Ridley scale. MB patients (LL and BB) exhibited BIs ranging from +1 to +6.0, while all PB cases (including T and I forms) had bacilloscopy-negative [6].

During examination and after the confirmation of a new leprosy case, these patients were invited to participate in the study. The research was approved by the Institutional Ethical Committee (protocol number 011/07) and all participants signed an informed consent form and authorized the collection of samples.

NS samples were obtained from all participants by gently rubbing a nasal swab, previously wetted with Tris-EDTA buffer (pH 8.0), in the vestibule on each side of the nose. After collection, each swab was immersed in a labelled sterile tube and stored at −20 °C until processing. DNA was extracted as described by Lima et al. [19] and was later eluted in dH_2O and stored at −20 °C until amplification.

Skin biopsies were collected using a 6-mm diameter punch (Kolplast, Brazil) to obtain tissues from new skin lesions of untreated leprosy cases. DNA extraction was performed using the DNeasy® blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

qPCR

Primers were designed using the Primer3Plus software [24] based on the nucleotide sequence of the 16S ribosomal RNA of *M. leprae* (GenBank accession number: X53999.1). A 124-bp region was amplified using the primers 16S2_For rRNA (5′-AGTGGCGAACGGGTGAGTAA-3′) and 16S2_Rev rRNA (5′-CGCAAAAAGCTTTCCACCAC-3′). Both forward and reverse primers had the same melting temperature (T_m) of 62.9 °C. The PCR amplification mixture contained 10 µL of Power SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA), 40 ng µL of sample DNA (template), and 100 nM of each primer in a total volume of 20 µL. For each qPCR assay, a positive control of 20 pg genomic *M. leprae* DNA was included, as was a negative control without the target DNA. Amplification was performed using a CFX96 Touch™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). PCR cycling conditions consisted of initial denaturation at 95 °C for 7 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 min.

Data were analyzed by the CFX™ Manager software (version 3.0; Bio-Rad Laboratories) to assess the mean quantitative cycle (Cq). Samples were considered negative when there was no increase in fluorescence signals until 45 cycles (Cq = 45). A standard curve ranging from 2 ng to 0.2 fg was generated by serial dilution of the plasmid pIDT16SrRNAMleprae (pIDT Blue; Integrated DNA Technologies, Coalville, IA) containing a 171-bp region of the 16S rRNA gene of *M. leprae* [25]. To quantify the number of colony-forming units/mg of each sample tested, the mean Cq values obtained (of each sample tested in duplicate for each batch) were interpolated from the constructed standard curve.

Amplification efficiency and limit of detection (LoD) of qPCR

Amplification efficiency curves were determined for three different assays performed on different days. For this purpose, the linearity of each assay was determined using serial 10-fold dilutions of 2 µL of each DNA sample at the following concentrations: 10, 10^2, 10^3, 10^4, 10^5, and 10^6 IU/mL. At each concentration, three replicates were tested in a single run. The following data were determined as estimators of the amplification efficiency:

slope, coefficient of determination (R^2), and efficiency parameters. We determined the qualitative LoD using diluted samples that were no longer showing a high amplification efficiency by repeating the qPCR amplification reaction 10 times. The LoD was defined as the concentration at which amplification was detected before 37 cycles 95% of the time.

Sensitivity and specificity of qPCR

To ensure specificity of the PCR products, we conducted a melting curve analysis, in which the reaction temperature was increased by 0.5 °C every 20 s, beginning at 60 °C and ending at 95 °C. Throughout the curve construction process, the changes in fluorescence were measured, and the data acquired using the iQ™5 Optical System software (version 2.0, Bio-Rad Laboratories) were processed to verify if a single peak was obtained. Subsequently, PCR products were electrophoresed using a 2% agarose gel stained with ethidium bromide was performed and analyzed using an ImageQuant™ 300 biomolecular imager (GE Healthcare, Little Chalfont, UK).

The sensitivity and specificity of the 16SrRNA assays were assessed by testing *M. tuberculosis* ATCC 697-7, *Mycobacterium* sp., and *Streptococcus pneumoniae* ATCC 49,619. The number of genome equivalents (GEs) was calculated according to the following equation, number of GEs = Avogadro constant (GEs g^{-1} mol^{-1}) × DNA concentration (C) (ng/µL)/Genome length in bp × molecular mass of 1 bp (g mol^{-1} bp^{-1}) × 1.0 × 10^9 (ng). Assays were determined by testing serial decimal dilutions of genomic DNA in triplicate ranging from 1.0 × 10^8 (approximately 2.0 × 10^7 GEs) to 1.0 × 10^1 fg (approximately 2 GEs).

Table 1. Bacilloscopy index, frequency of qPCR positivity, and mean number of M. leprae DNA copies and Cq values in nasal samples of leprosy cases, according to the operational and Ridley-Jopling classifications.

Operational classification	BI	Positive 16s qPCR, *N* (%)	Mean of Cq values	Mean of bacilli copy number (range)
	Mean (±SD)			
PB *N* = 15	0	11 (73.3)	35.7	1.1×10^4 (1.4×10^3 to 6.1×10^4)
Tuberculoid *N* = 14	0	10 (71.4)	35.7	1.1×10^4 (1.4×10^3 to 6.1×10^4)
Indeterminate *N* = 1	0	1	34.7	1.8×10^4
MB *N* = 39	3.6 (±1.5)	35 (89.7)	35.5	3.7×10^4 (5.6×10^2 to 8.0×10^5)
Borderline *N* = 19	2.6 (±1.5)	17 (89.4)	35.6	2.0×10^4 (9.0×10^2 to 1.7×10^5)
Lepromatous *N* = 20	4.5 (±0.7)	18 (90)	35.4	5.2×10^4 (5.6×10^2 to 8.0×10^5)
Total *N* = 54		46 (85.2)	35.5	2.9×10^4 (5.6×10^2 to 8.0×10^5)

BI, bacilloscopy index; SD, standard deviation; Cq, quantification cycles; MB, mutibacillary; PB, paucibacillary.

Statistical analysis

A standard linear regression analysis of DNA from *M. leprae* or the number of copies of the *M. leprae* curve vs. Cq values was calculated automatically by the CFX Manager™. Differences in the Cq mean values between the MB and PB groups were analyzed by the Mann-Whitney U test using Prism software (version 6.01; GraphPad Software Inc., La Jolla, CA). In addition, the correlation between the Cq values in paired NS and SB samples from MB cases was analyzed using Excel 2013 (Microsoft, Redmond, WA). For all tests, *p*-values < 0.05 were considered significant.

Results

To validate the sensitivity of the primer set used for qPCR analysis, we analyzed DNA samples extracted from NS and SB samples from patients with leprosy. The primer pair amplified a specific gene fragment from the 16S ribosomal RNA of *M. leprae*. Ten-fold serial dilutions of the DNA samples ranging from 2 ng to 0.2 fg were prepared and tested in triplicate. The same melting temperature (T_m = 79.5 °C) was observed for all dilutions tested. The assay showed a LoD of less than 20 fg of *M. leprae* DNA with a Cq value ≤ 37 cycles. All negative control samples were negative for *M. leprae* DNA detection.

The specificity of each probe-primer set was evaluated by testing DNA samples from other bacteria. The 16S rRNA assays correctly detected all *M. leprae* sample cases. The assay also amplified the DNA of *M. tuberculosis* ATCC 697-7 and *Mycobacterium* sp., but the T_m values of their PCR products differed from that of *M. leprae* DNA.

Of the 54 *M. leprae* NS samples, 46 tested positive by the qPCR assay (85.2%), while all SB samples tested positive. Of the 15 nasal samples from PB and 39 from MB patients, 11 (73.3%) and 35 (89.7%) tested positive, respectively (Table 1). As expected, all PB cases had negative BI while the average BI for MB was 3.6 (± 1.5). A higher BI was observed in LL cases (4.5 ± 0.7) compared with borderline cases (2.6 ± 1.5). Among the *M. leprae* NS samples, the number of bacilli ranged from 5.6 × 10^2 to 8.0 × 10^5 and the Cq values ranged from 27.5 to 38.2 cycles (mean of 35.5 cycles). As expected, low Cq values were found for MB cases (mean of 35.5 cycles) compared with PB cases (mean of 35.7 cycles). The tuberculoid cases had the highest Cq (mean of 35.7 cycles) and the lowest bacilli load mean (1.1 × 10^4 copies). As shown in table 2, only one NS sample from the 19 MB paired samples was not found positive by the qPCR assay.

To obtain a standard curve for the absolute quantitation of *M. leprae* DNA, 10-fold dilutions of the plasmid

Table 2. Frequency of qPCR positivity in detecting the 124-bp region of the 16S rRNA gene of M. leprae in 19 paired multibacillary (MB) leprosy patient skin biopsies (SB) and nasal samples (NS).

Operational classification	Positive 16S rRNA qPCR		Total of cases
	NS	SB	
MB	18	19	19
Borderline	9	10	10
Lepromatous	9	9	9

NS, nasal secretion; SB, skin biopsy; MB, multibacillary.

pIDT16SrRNAMleprae were used as template. The standard curve that was generated showed a linear relationship from 10^3 to 10^6 DNA copies (Supplementary figure). Linear regression analysis yielded an R^2 of 0.98. The slope value for the plasmid was −3.460.

The bacterial load of the 19 SB samples ranged from 30 to 1.5×10^6 copies and the Cq ranged from 26.5 to 42.6 cycles. NS from the same patients ranged from 2.3×10^2 to 8.8×10^6 bacilli and the Cq values ranged from 27.5 to 39.6 cycles (Table 3).

Furthermore, a significant association between the bacilloscopy index values of slit-skin smear samples and the number of *M. leprae* genomic copies was found while analyzing paired SB and NS samples from MB patients (Figure 1). Higher logarithmic copy numbers of *M. leprae* bacilli were associated with high bacilloscopy index values in SB samples ($R^2 = 0.2131$; $p = 0.001$). However, no correlation was observed between the log copy number and bacilloscopy index values of NS samples ($R^2 = 0.019$; $p > 0.005$).

Table 3. Bacilli copy number and Cq values in nasal and skin biopsy samples of 19 multibacillary paired cases.

		Skin biopsy		Nasal secretion[a]	
Sample ID	BI	Cq	*M. leprae* copy number	Cq	*M. leprae* copy number
336	4	31.0	7.5×10^4	36.3	2.1×10^3
372	1.8	33.0	2.0×10^4	37.6	9.0×10^2
381	0	36.5	1.9×10^3	36.6	1.7×10^3
386	5	29.2	2.7×10^5	35.5	3.8×10^3
387	5.5	26.5	1.5×10^6	32.9	2.0×10^4
389	4	33.5	1.4×10^4	27.5	8.8×10^6
393	1	37.4	9.4×10^2	35.4	3.8×10^3
398	4.5	39.4	2.6×10^2	36.5	1.8×10^3
400	2.8	30.6	9.4×10^4	35.6	3.3×10^3
401	1.8	33.9	3.1×10^4	32.5	7.6×10^4
403	4.8	39.2	2.9×10^2	29.8	1.7×10^5
422	2.8	28.3	4.9×10^5	37.3	1.1×10^3
423	5	28.5	4.1×10^5	36.5	1.9×10^3
429	4.8	28.4	4.5×10^5	36.6	1.7×10^3
431	3.7	37.4	1.0×10^3	37.2	1.2×10^3
437	3.5	42.6	3.0×10^1	39.6	2.3×10^2
441	1.6	37.2	1.2×10^3	0.0	–
443	3.8	34.3	8.0×10^3	38.2	5.6×10^2
463	3.6	34.9	5.5×10^3	38.7	4.3×10^2
Mean	3.4	33.8	1.8×10^5	35.6	5.1×10^5
Range	0 to 5.5	26.5 to 42.6	30 to 1.5×10^6	27.5 to 39.6	2.3×10^2 to 8.8×10^6

[a]The sample ID 441 was not considered for the mean and range calculation.
BI, bacilloscopy index; Cq, quantification cycles; SD, standard deviation.

Discussion

In the present study, we identified and quantified *M. leprae* in nasal secretion samples and biopsies of leprosy cases. Although several studies have reported the detection of *M. leprae* by real-time PCR in samples from skin biopsies [11,26–28], this is the third study published in the literature that quantified *M. leprae* in nasal secretion samples [13,18]. The first study investigated 31 nasal secretion samples of leprosy patients without quantifying the number of bacilli [18]. The second study employed qPCR for DNA detection of *M. leprae* using nasal swabs, nasal turbinate biopsy, and peripheral blood of 113 leprosy cases and 104 household contacts [13]. The same study obtained a 66.4% positive rate of *M. leprae* DNA detection using TaqMan qPCR [13], whereas our study used the SYBR Green assay and revealed 85.2% positivity of *M. leprae* DNA in nasal swabs. In addition, our qPCR assay exhibited a higher positivity for PB (11/15, 73.3%) and MB (35/39, 89.7%) compared with the previous report [13] (PB 14/32, 43.8% and MB 61/81, 75.3%) and we found a lower mean of bacilli number per reaction in PB cases (1.1×10^4). In trials where *M. leprae* DNA was detected by conventional PCR, detection rates of the disease in the general population varied between 9.1 to 84.9% [8,12,14,17,29,30].

Compared with the detection of *M. leprae* using conventional PCR in leprosy cases, the rate of qPCR positive detection of the 16S rRNA region in nasal secretions (in accordance with the operational classification of positive results) was 73.3% for PB cases, which is far superior to other values reported that included 5.3, 13.3, and 36.4% [8,29,31]. With regards to detection rates of MB cases, we also obtained high results (89.7%) similar to what was found in Minas Gerais (91.9%), Southeastern Brazil [8]. Compared to a study conducted in São Paulo [12], which used qPCR on different biopsy samples, our study had a higher rate of positive results for PB cases and lower rates for MB cases.

Analytical sensitivity refers to the minimum number of PCR amplicons of a sample that can be measured accurately in one assay, whereas clinical sensitivity is the percentage of individuals with a particular disease that the assay identifies as positive for that condition [32]. Our qPCR assay accurately detected 20 fg of *M. leprae* DNA, equivalent to four bacilli. However, the highest dilution of the standard curve where quantitative analysis of the samples was possible corresponded to Cq ≤ 37. More than this Cq, precise quantification is not possible, and the samples can only be analyzed qualitatively (positive or negative). These findings are similar to those in studies performed with biopsies of patients from Khon Kaen Province, Thailand and from Rio de Janeiro, Brazil [11,20]. However, our study revealed a clinical sensitivity of 73.3% for PB cases, individuals whose diagnosis is more difficult because of the reduced number of bacilli.

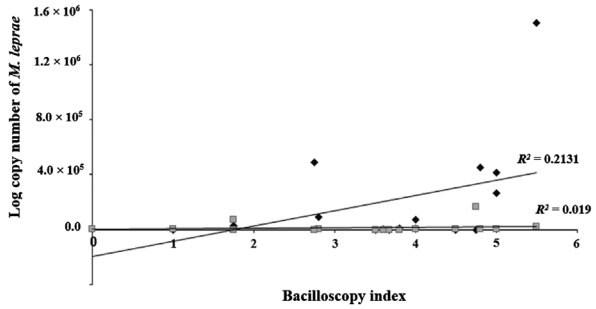

Figure 1. Linear range quantification of *M. leprae* DNA of 18 paired multibacillary (MB) leprosy patients from skin biopsies and nasal samples. The bacilloscopy index (BI) of skin biopsies (◆) and nasal samples (■) was plotted against the logarithmic count number of *M. leprae* bacilli.

The Cq values of the two groups, MB and PB, were not significantly different ($p > 0.05$). This is probably because the number of PB cases in the study was low. Among the 11 positive cases, only five were Cq ≤ 37 and only these could be analyzed. Although PB cases had fewer bacilli (1.39×10^3–1.42×10^4 copies) in nasal secretions than in MB cases (1.42×10^3–8.02×10^5 copies), it is important to note that PB patients are carriers of the bacillus bacteria and are potential transmitters to susceptible individuals.

In the 19 cases of paired biopsies and nasal secretion samples from leprosy patients, the positive results obtained by the bacilloscopy index were confirmed positive by real-time PCR. Biopsies from patients with higher BI values were deemed positive for bacteria earlier in the amplification cycle, as seen by the lower Cq values and high copy numbers of bacilli. However, the correlation between higher copy number of *M. leprae* and higher BI values of NS samples were not significantly different ($p > 0.05$), whereas in biopsy specimens the copy number and BI values differed ($p = 0.001$). The lack of correlation between the 16S rRNA genomic region detection in NS samples and the BI demonstrate that nasal swabs are not suitable to for leprosy diagnosis. Similar results were also seen in other studies [13,27,29].

Primers specific for the 16S rRNA region displayed 100% specificity for *M. leprae* with a single PCR product at a melting temperature of 79.5 °C. However, the amplification of *M. tuberculosis* DNA ATCC 697-7 and *Mycobacterium* sp. occurred at a different T_m than that of *M. leprae*. The amplification of *M. leprae* and *Mycobacterium* sp. regions could not be distinguished in assays with the same primers using conventional PCR. A previous comparative study targeting several genomic regions of *M. leprae* performed with skin biopsies reported that real-time PCR targeting of the 16S rRNA region was more sensitive in determining viable *M. leprae* [27].

There are several methodologies available for real-time qPCR, of which TaqMan and SYBR Green are the most common. Real-time PCR has been used for the diagnosis of several diseases. In general, the SYBR Green methodology costs less and is relatively easier compared with the TaqMan assay, which requires probe design and synthesis. The specificity of the TaqMan method is based on the design of labelled oligonucleotide probes and the exonuclease activity of the Taq polymerase enzyme [33], whereas the SYBR Green assay is based on the attachment of a fluorophore to the dsDNA – any nonspecific product may bind to the primers and give false positive results [34]. Therefore, primer design is the most important step of a PCR assay. In this study, we used the Primer3Plus software for primer design and produced a single fragment [24]. We also performed a BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against all microbial nucleotide databases of the 124-bp 16S rRNA fragment generated from *M. leprae*, and confirmed 100% specificity to the *M. leprae* genome. Lower similarities were also found for other species of the *Mycobacterium* genus. Real-time PCR assays using DNA from other bacterial species demonstrated specificity for *M. leprae*, except for *Mycobacterium* sp., which generated an amplification product with a different T_m than *M. leprae*. These optimizations ensured that the SYBR Green methodology had a similar specificity to that of TaqMan assays.

In endemic regions, detection of *M. leprae* DNA from nasal secretions does not differentiate contacts from cases [16,19,35]; therefore, quantification of *M. leprae* DNA can be used in epidemiological studies to identify healthy carriers among household contacts or within populations of an endemic area [7,36].

Conclusions

Our results suggest that the qPCR assay employing SYBR Green and the *M. leprae* 16S rRNA target region can be utilized for the detection of bacillus in nasal secretion samples from leprosy patients. This validates the method for use in epidemiological studies aiming to identify healthy carriers between leprosy household contacts or within populations of an endemic area.

Geolocation information

Available at: https://goo.gl/maps/uLepvoDvdr42.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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