First evidence for continuous circulation of hepatitis A virus subgenotype IIA in Central Africa

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Abstract

Although a high seroprevalence of antibodies against hepatitis A virus (HAV) has been estimated in Central Africa, the current status of both HAV infections and seroprevalence of anti-HAV antibodies remains unclear due to a paucity of surveillance data available. We conducted a serological survey during 2015-2017 in Gabon, Central Africa, and confirmed a high seroprevalence of anti-HAV antibodies in all age groups. To identify the currently circulating HAV strains and to reveal the epidemiological and genetic characteristics of the virus, we conducted molecular surveillance in a total of 1007 patients presenting febrile illness. Through HAV detection and sequencing, we identified subgenotype IIA (HAV-IIA) infections in the country throughout the year. A significant prevalence trend emerged in the young child population, presenting several infection peaks which appeared to be unrelated to dry or rainy seasons. Whole-genome sequencing and phylogenetic analyses revealed local HAV-IIA evolutionary events in Central Africa, indicating the circulation of HAV-IIA strains of a region-specific lineage. Recombination analysis of complete genome sequences revealed potential recombination events in Gabonese HAV strains. Interestingly, Gabonese HAV-IIA possibly acquired the 5'-untranslated region (5'-UTR) of the rare subgenotype HAV-IIB.
1 | INTRODUCTION

Hepatitis A virus (HAV), which is one of the major causative agents of acute hepatitis, poses a major public health concern worldwide. The Global Burden of Disease Study estimated that approximately 170 million cases of acute hepatitis A occurred globally in 2017. HAV is mainly transmitted faecal orally, through person-to-person contact and through consumption of HAV contaminated water and food. The incidence of HAV infection is closely related to socioeconomic conditions that impact the quality of sanitation and access to clean water. Among HAV endemic regions, sub-Saharan Africa is estimated to be highly endemic with anti-HAV IgG detection in over 90% of individuals at 10 years of age. Within sub-Saharan Africa, Central Africa shows the highest HAV seroprevalence rate in the population under 5 years of age; however, epidemiological data for this region are available only from before 1995 due to lack of surveillance studies.

Until now, genotypes I–VI have been identified for HAV; genotypes I–III infect humans and are further divided into subgenotypes A and B, while genotypes IV–VI are of simian origin. Studies on genotype distribution showed that genotype I is the most prevalent worldwide. Genotype III also follows a global distribution pattern, with HAV subgenotype IIB (HAV-IIB) mainly reported in Japan. In spite of a large number of genotype prevalence studies worldwide, genotype II has been rarely reported so far. Limited information on full-length coding sequences (CDS) of HAV-IIA and IIB is available in public databases. HAV-IIA strains have been sporadically detected in Europe. These sporadic infections included cases of returning travellers, suggesting that HAV-IIA originated from West and/or Central Africa, although little direct evidence has been reported from the region to date. Concerning HAV-IIB, epidemiological and genetic data have been largely unavailable since the first identification in 2002, rendering it an elusive subgenotype.

With limited molecular surveillance data, whether HAV genotype II strains still exist and circulate locally in Africa and globally remains to be elucidated.

The aim of this study was to investigate the currently circulating HAV strains and to reveal the epidemiological and genetic characteristics of the virus in Central Africa, through molecular surveillance in patients who developed fever during 2015-2017. This study also aimed to examine the seroprevalence of anti-HAV antibodies and the present existence of genotype II strains in the region from which genotype II was assumed to be originated.

2 | MATERIALS AND METHODS

2.1 | Study population

A total of 1007 febrile patients (≥37.5°C body temperature) who visited the Centre de Recherches Médicales de Lambaréné (CERMEL) and the Albert Schweitzer Hospital in Lambaréné were recruited in a surveillance study between January 2015 and June 2017. In the present study, the age of the participants was restricted to ≥1 year. Demographic information (age and sex) of recruited participants was also collected.

2.2 | Detection of anti-HAV IgG antibodies using enzyme-linked immunosorbent assay (ELISA)

A commercial ELISA kit was used to detect anti-HAV IgG antibodies in serum samples according to the manufacturer’s instructions (human hepatitis A virus IgG antibody ELISA kit, MBS3800852; MyBioSource). We set the detection limit of the kit as a cut-off value.

2.3 | Viral RNA extraction and detection by reverse transcription–quantitative PCR (RT-qPCR)

Viral RNA was extracted from 140 μL of each serum sample with a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. RT-qPCR was performed in a 20 μL reaction using a One Step PrimeScript III RT-qPCR Mix (Takara Bio). Each reaction mixture contained 10 μL 2 × One Step PrimeScript RT-qPCR Mix, 0.6 μmol/L of each primer, 0.25 μmol/L TaqMan probe, 0.4 μL ROX Reference Dye, 2 μL RNA template and RNase-free water up to 20 μL. RT-qPCR assays were carried out with a StepOnePlus instrument (Thermo Fisher Scientific) under the following conditions: 5 minutes at 52°C, 10 seconds at 95°C, and 45 cycles of 5 seconds at 95°C and 35 seconds at 60°C. Primers and the probe were designed using the sequences previously reported. Data collected from the RT-qPCR assays were analysed using software included in the StepOnePlus system. RT-qPCR assays were performed in duplicates, and samples reaching threshold cycle (Ct) values under 40 were set as positive.
2.4 | Sequencing of the VP1-P2B region for genotyping of detected HAV strains

Amplification of the VP1-P2B region (481 nucleotides [nt]) of HAV genome was performed with PrimeScript II High-Fidelity One Step RT-PCR Kit (Takara Bio) using previously reported primers. After agarose gel purification with Qiaquick Gel Extraction Kit (Qiagen), the PCR products were processed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and analysed with an ABI3500 capillary sequencer (Thermo Fisher Scientific) to obtain sequence data. Sequenced fragments were assembled using CLC Main Workbench 8 software (Qiagen) and consensus sequences were extracted. The genotypes of HAV strains detected in this study were determined by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 | Whole-genome sequencing

Multiplex PCR was performed to efficiently sequence full-length coding regions with a next-generation sequencer. First, primer sets for multiplex PCR were designed by the online program Primal Scheme (http://primal.zibaproject.org/), setting the amplicon size at 450 bp and the overlap at 50 bp. The template sequences were derived from the V18500348/USA strain (accession no. MH77314) for HAV-IB and the CF53/France strain (AY644676) for HAV-IIA, and twenty-two sets of primers were designed for each subgenotype. Primer sets were divided into two pools as described previously. Extracted viral RNA was reverse-transcribed by SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) using 8 μL of each RNA sample combined with random hexamers according to the manufacturer's protocol. Reverse-transcribed complementary DNA (cDNA) was purified with Agencourt AMPure XP (Beckman Coulter) and eluted in 30 μL RNase-free water. Then, multiplex PCR was performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs) in a 25 μL mixture containing 5 μL Q5 Reaction Buffer, 0.5 μL dNTP Mixture (10 mmol/L each), 0.25 μL Q5 High-Fidelity DNA Polymerase, 0.015 μmol/L of each primer, 15 μL purified cDNA and RNase-free water up to 25 μL, under the following conditions: 30 seconds at 98°C, 40 cycles of 15 seconds at 98°C and 4 minutes at 65°C. Multiplex PCR products were purified using Agencourt AMPure XP (Beckman Coulter) and eluted in 30 μL RNase-free water, followed by quantitation with a Qubit 2.0 Fluorometer and a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

Libraries were prepared using 3-250 ng of each multiplex PCR product and a Nextera DNA Flex Library Prep Kit (Illumina) in combination with Nextera DNA CD Indexes (Illumina) according to the manufacturer’s instructions. After a quality and quantity inspection of each library using an Agilent 2100 Bioanalyzer (Agilent Technologies) with a High Sensitivity DNA Kit (Agilent), sequencing was performed using a 300-cycle High Output Kit (Illumina) on a MiSeq sequencer (Illumina). Mapping of the paired-end reads was performed on CLC Genomics Workbench 11.0.1 software (Qiagen) using whole-genome sequences of the V18500348/USA strain (HAV-IB) and the CF53/France strain (HAV-IIA) as templates. Consensus sequences were extracted and aligned with reference strains on BioEdit 7.0.5.3 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.6 | Phylogenetic analysis

To infer phylogeny of the VP1-P2B region of HAV strains, a maximum-likelihood analysis was performed with reference sequences that include all VP1-P2B sequences of African HAV strains using MEGA 7 software (https://www.megasoftware.net/). The analysis was performed using a general time-reversible model and gamma distribution of the substitution rate, as described previously. A total of 5000 bootstrap replicates were generated. To compare with data from countries outside of sub-Saharan Africa, reference strains were widely selected from each continent and from various time points of the collection period. For the phylogenetic analysis of complete genome sequences, reference sequences with more than 7300 nt of submitted genome length were obtained from the Virus Pathogen Resource (https://www.viprbrc.org/). Consensus sequences of the whole genome of HAV strains detected in this study and reference sequences were aligned and checked manually for gaps to be removed. Laboratory strains were also removed from the analysis. Finally, 101 sequences were analysed using a maximum-likelihood method as described above. To infer the phylogeny of VP1-P2B sequences in HAV-IIA strains, all VP1-2B sequences of HAV-IIA strains that include a fragment of a 304 nt (nucleotide position in AY644676: 2890-3193) were obtained and aligned. A Bayesian analysis was performed with time-stamped reference sequences on BEAST v2.6.0 program (https://www.beast2.org/) using the HKY model, the relaxed lognormal clock and 400 million generations. The effective sample size (ESS) values were verified to show more than 1000. For better visualization, the phylogenetic tree was modified using FigTree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree).

2.7 | Recombination analysis

Potential recombination events and identification of possible recombination breakpoints were analysed using the six methods embedded in the Recombination Detection Program (RDP) v3.44 package (http://web.cbl.rochester.edu/~darren/rdp.html), using the default settings for each method. Subsequently, whole-genome sequences were used as reference strains: HAV-IA, LA (GenBank accession no. K02990) and GBM (X75215); HAV-IB, HM-175 (M14707) and MBB (M20273); HAV-IIA, CF53/Berne (AY644676); HAV-IIIB, SLF88 (AY644670); HAV-IIIA, HAJNG04-90F (AB279732); HAV-IIIB, HAJ85-1F (AB279735). A potential recombination event was determined by detection with at least four methods, while P < .05 and Bonferroni correction for multiple comparisons were applied. The results of the Bootscan analysis were exported and visualized using GraphPad Prism 7 software (GraphPad Software).
2.8 | Statistical analysis

Statistical data analysis was performed using GraphPad Prism 7 software (GraphPad Software). Welch’s t tests and Fisher’s exact tests were used to determine significant differences in comparisons of general categorical variables. Results were considered to be statistically significant with P values under .05.

2.9 | Sequence data availability

We deposited the sequences of HAV strains obtained in this study in GenBank, under the accession numbers LC515196-LC515208.

2.10 | Ethical considerations

This study was approved by the Institutional Review Boards of CERMEL and Nagasaki University (approval no. CEI-007 and 170921177, respectively). Written informed consent was obtained from all participants or their parents.

3 | RESULTS

3.1 | Seroprevalence of anti-HAV antibodies and HAV genome detection in samples of febrile patients

To investigate the age-specific seroprevalence of anti-HAV IgG antibodies and the current situation of HAV endemicity in Gabon, Central Africa, serum samples were collected from patients with fever (>37.5°C body temperature) from January 2015 to June 2017. A total of 1007 febrile patients, with a mean age of 10.83 ± 14.4 years (±SD) and an age range of 1-82 years, were enrolled. Sixteen patients provided no information on age and were thus excluded from epidemiological analyses concerning age. First, ELISA tests were performed with 596 samples selected across the whole range of age. The result showed that 98.8% of tested samples were positive for anti-HAV IgG antibodies (Table 1).

Consistent with the estimation in the past, all age groups showed a high seroprevalence, even in the population under 5 years of age (Table 1).

Then, the current situation of HAV endemicity was investigated using all of 1007 serum samples of patients with fever. Samples were screened for HAV by RT-qPCR as previously described. We detected HAV in 13 child samples (Table 2). Sample collection dates of HAV-positive samples were distributed over the year 2016, from January to December (Table 2). As febrile participants were recruited actively from November 2015 to December 2016 (Figure S1 A), we analysed seasonality of HAV infections within this period. The peaks of HAV positivity were observed in January, May, August and October 2016, exhibiting no correlation of HAV infections with rainy or dry seasons in Gabon (Figure S1 B). Outside this active recruitment period, no HAV positive patients were recorded. Next, we analysed the participants’ demographic information to reveal epidemiological factors of acute hepatitis A incidence in Gabon. Most of HAV positive patients were under 5 years of age (positivity rate of under-5-year patients, 76.9%) (Table 2). Comparison of mean age showed significant differences between the HAV-positive and HAV-negative populations (Table S1). However, there was no statistical significance in HAV positivity rates between female and male populations (Table S2).

3.2 | Genotyping of detected HAV strains

To determine the genotypes of HAV strains detected in this study, we obtained the sequence of the VP1-P2B region from all HAV positive samples (481 nt, GenBank accession nos. LC515196-LC515208) using a method as described previously. BLAST analysis revealed that 84.6% (11 in 13 positive samples) of HAV strains detected in this study belonged to subgenotype IIA (HAV-IIA) and two strains to HAV-IB (Table 2). Sequence homology between the Gabonese HAV-IIA strain and the CF53/Berne strain (GenBank accession no. AY644676; reference strain of HAV-IIA) was 96.26%-96.88%, whereas the homology between the Gabonese HAV-IB strain and the HM-175 strain (M14707; reference strain of HAV-IB) was 95.22%. For the genetic characterization of HAV strains, phylogenetic analysis was performed using VP1-P2B sequences, showing

| Age group | No. of samples positive/tested (positivity %) | Total |
|-----------|-----------------------------------------------|-------|
| F         | M                                             |       |
| 1-4       | 121/122 (99.2) | 119/121 (98.3) | 240/243 (98.8) |
| 5-9       | 95/95 (100)   | 95/95 (100)    | 190/190 (100)  |
| 10-19     | 32/34 (94.1)  | 41/41 (100)    | 73/75 (97.3)   |
| 20-29     | 14/14 (100)   | 13/14 (92.9)   | 27/28 (96.4)   |
| 30-39     | 8/8 (100)     | 8/8 (100)      | 16/16 (100)    |
| 40-59     | 18/18 (100)   | 15/16 (93.8)   | 33/34 (97.1)   |
| 60-76     | 4/4 (100)     | 6/6 (100)      | 10/10 (100)    |
| Total     | 290/295 (99.0)| 291/301 (98.7)| 581/596 (98.8) |

Abbreviations: F, female; M, male.
that two HAV-IB strains detected in this study were genetically close
to the Ghana-2010 strains (KC632110, KC632113, and KC632115)
(Figure 1). Even though the Gabonese HAV-IIA strains formed a large
cluster with the strains detected in Cameroon and Italy, the number
of reference strains was insufficient to reveal the details of phylog-
eny in genotype II (Figure 1). To infer detailed genetic relationships
between Gabonese and other HAV-IIA strains, we conducted a time-
scaled Bayesian analysis based on collection dates using shorter se-
quencies of the VP1-P2A region (304 nt) that were identified in most
HAV-IIA strains. The Bayesian analysis revealed three main clusters
of HAV-IIA strains: a Central African cluster including the strains of
Gabon and Cameroon; a West African cluster with the strains origin-
ating from Benin, Togo and Mauritania; and a French cluster with
strains originating from France (Figure 2). Two Gabonese strains
(SYMAV-D02 and D03) formed a cluster with Cameroonian strains,
whereas all other Gabonese strains formed one Gabon cluster. The
Bayesian tree also showed that an Italian strain detected in 2012
was derived from the Central African lineage, indicating a possible
import case of a HAV-IIA strain by a returning traveller (Figure 2).

3.3 Whole-genome sequencing of Gabonese HAV strains

Whole-genome sequence information of HAV enables genotyping
of a higher resolution as compared to sequencing of short genomic
fragments, such as the VP1-P2B region. Therefore, we attempted
to sequence the complete genome of the HAV strains detected in
this study by next-generation sequencing. Nearly complete genome
sequences (7381-7413 nt) were obtained from seven samples col-
clected between January and December 2016, including six HAV-IIA
(D01, D03, D05, D10, D11 and D13) and one HAV-IB (D12) strains
(GenBank accession nos. LC515196-LC515202). Whole-genome se-
quence homology between Gabonese HAV-IIA and the CF53/Berne
strain (AY644676) was 96.25%-96.48%, whereas homology be-
tween the Gabonese HAV-IB and the HM-175 strain (M14707) was
95.43%. Phylogenetic analysis was performed with whole-genome
sequences of HAV strains, which have a genome larger than 7300 nt
in public databases, except of laboratory strains. The result indicated
a similar branching pattern to the tree constructed from VP1-P2B
sequence homology (Figure S2).

3.4 Genetic characterization of the complete genome of Gabonese HAV strains

We investigated sequence similarity across the entire genomes of
Gabonese and reference HAV strains for each subgenotype. Interes-
tingly, all of the six Gabonese HAV-IIA strains whose whole
genomes were sequenced in this study had possibly experienced
a significant recombination event with the SLF88 HAV-IIB refer-
cence strain (AY644676) was 96.25%-96.48%, whereas homology be-
tween the Gabonese HAV-IB and the HM-175 strain (M14707) was
95.43%. Phylogenetic analysis was performed with whole-genome
sequences of HAV strains, which have a genome larger than 7300 nt
in public databases, except of laboratory strains. The result indicated
a similar branching pattern to the tree constructed from VP1-P2B
sequence homology (Figure S2).

Table 2: Demographic and laboratory data of patients infected with HAV

| Sample ID | Age (years) | Sex (M/F) | Ct value\(^a\) | Collection Date | Subgenotype\(^b\) |
|-----------|-------------|-----------|----------------|-----------------|-----------------|
| 01        | 5           | M         | 32.92          | 8 Jan 2016      | IIA             |
| 02        | 1           | F         | 37.94          | 21 Jan 2016     | IIA             |
| 03        | 6           | F         | 37.88          | 28 Jan 2016     | IIA             |
| 04        | 1           | F         | 36.88          | 5 Feb 2016      | IIA             |
| 05        | 1           | F         | 33.93          | 3 May 2016      | IIA             |
| 06        | 1           | F         | 37.91          | 29 Jul 2016     | IIA             |
| 07        | 3           | M         | 37.92          | 1 Aug 2016      | IIA             |
| 08        | 3           | M         | 39.88          | 8 Aug 2016      | IIA             |
| 09        | 3           | F         | 33.96          | 19 Oct 2016     | IB              |
| 10        | 1           | M         | 31.96          | 3 Nov 2016      | IIA             |
| 11        | 1           | F         | 31.86          | 9 Nov 2016      | IIA             |
| 12        | 14          | F         | 28.44          | 13 Dec 2016     | IB              |
| 13        | 4           | F         | 35.90          | 27 Dec 2016     | IIA             |

Abbreviations: F, female; M, male.
\(^{a}\)Threshold cycle (Ct) values were the average of the duplicated detection by RT-qPCR.
\(^{b}\)Subgenotypes were determined using the VP1-P2B sequence.
Amino acid substitutions in viral proteins of Gabonese HAV strains

Several studies have reported possible antigenic sites in the capsid proteins of HAV. Comparison of the amino acid sequences of Gabonese and reference strains showed complete compatibility in the antigenic sites (the VP1 immunodominant site, the glycophorin A binding site in the VP1 protein and the putative receptor binding site). However, a variety of amino acid substitutions were detected by comparison of the reference and Gabonese HAV-IIA (38 substitutions and 2 insertions) or HAV-IB strains (12 substitutions), suggesting that changes may have occurred in the functions of some viral proteins, such as the 2C and 3D proteins, in the sequence of which a remarkable number of amino acid substitutions were identified (Table S4 and S5). Further analysis is required to determine the effect of these amino acid substitutions on viral replication and/or pathogenicity.

DISCUSSION

Sub-Saharan Africa is one of the most highly HAV endemic regions. Especially in Central Africa, >95% of children aged under 5 years are estimated to be positive for the anti-HAV antibody, even though surveillance studies in the area have been limited. Systematic reviews describing hepatitis A occurrence in Africa estimated the seroprevalence in Central Africa using only limited data published before 1995, reflecting insufficiency in data availability.

After initial identification of HAV-IIA in 1979, only a single epidemiological study of HAV-IIA conducted in France was published in 2010, including data from autochthonous and import cases from Africa in 2004 and 2007-2009. This report significantly contributed to the identification of HAV-IIA endemic regions and the prediction of clinical features of HAV-IIA infections. However, most infections were detected in the adult population, aged 27.6 ± 16.9 years.
The current study yielded the first results suggesting that HAV-IIA has been circulating in the under-5-year population in Central Africa (Table 2). This trend in HAV infection is similar in other endemic regions such as South Asia. Furthermore, HAV-IIA was fortuitously detected in Cameroon, in 2014, in a study of gut virome analysis, solidifying the evidence on current HAV-IIA circulation in Central Africa. Most Gabonese HAV-IIA strains formed a Gabonese phylogenetic cluster, whereas two Gabonese strains (SYMAV-D02 and D03) were categorized among Cameroonian strains, indicating that the D02 and D03 strains have been imported from Cameroon. As HAV-IIA infections represented 84% of all HAV cases in Gabon, it can be suggested that HAV-IIA is endemic and highly prevalent in Central Africa (Figure S5).

Whole-genome sequence information facilitates accurate inference of viral strain relatedness and supports epidemiological studies worldwide, enabling molecular tracing of hepatitis A outbreaks and HAV transmission routes. Even though the sequence of the VP1-P2B region was sufficient to successfully determine the genotypes of detected HAV strains, genetic analysis of the complete genome sequence revealed unexpected inter-(sub)genotypic recombination events in Gabonese HAV. A BLAST analysis of the 5′-UTR sequence indicated that HAV-IIA may have obtained the 5′-UTR of HAV-IIB in Central Africa and that, therefore, there is a possibility of undetected HAV-IIB circulation in the area (Table S3). As HAV-IIB is very rare, with only one sequence having been available so far, it would be challenging to investigate current HAV-IIB circulation in Central Africa, but also an attractive objective for future molecular surveillance studies. Evidence supporting our findings has been provided in a previous report describing a natural recombination event.

**FIGURE 2**  Phylogenetic analysis of the short VP1-P2B sequence of HAV-IIA using a time-scaled Bayesian maximum clade credibility tree. Virus lineages are shown on the right. An entire illustration of the phylogeny, including 95% CI bars at each node, is provided in Figure S4.
between HAV-IIB and HAV-IB in an African isolate. A short fraction of the 3D polymerase of the Gabonese HAV-IB strain appeared to be exchanged with the corresponding fragment in the GBM HAV-IA strain. A BLAST analysis of the fraction identified recent African HAV-IB strains as close homologues (Uganda—2013, South Africa—2011 and Egypt—2014), indicating that the African HAV-IB acquired a HAV-IA genetic fraction, at least before 2011, and that it has been circulating in the African continent since then retaining the recombinant fraction (Table S3). As the GBM strain is one of the oldest HAV-IA strains, the African HAV-IB might have acquired the fraction long before 2011. Until now, there have been a small number of studies investigating inter-(sub)genotypic recombination events in HAV, possibly due to the limited amount of whole-genome information available in the database. Further research is required to enrich public databases with the complete genomic sequences of HAV strains and to decode the complex scene of recombination events in the HAV genome.

In conclusion, the present HAV molecular surveillance conducted on febrile populations revealed epidemiological and genetic characteristics of HAV strains in Central Africa and supports the continuous circulation of HAV-IIA. This is the first report providing direct evidence for HAV-IIA circulation at a fixed study site through several years of molecular surveillance. Whole-genome sequencing and detection of possible recombination events elucidated a distinctive feature of African HAV strains, suggesting the present existence of HAV-IIB in Central Africa. These findings inform our understanding of the potential HAV transmission route and the necessity of an appropriate vaccination programme in Central Africa. However, continuous surveillance is required to determine the need for immediate alerting and planning for infection control.

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CONFLICT OF INTERESTS

None of the authors has any conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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