Characterization of HIV-1 CRF90_BF1 and putative novel CRFs_BF1 in Central West, North and Northeast Brazilian regions

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Abstract

The Brazilian AIDS epidemic has been characterized by an increasing rate of BF1 recombinants and so far eight circulating recombinant forms/CRFs_BF1 have been described countrywide. In this study, pol sequences (protease/PR, reverse transcriptase/RT) of 87 BF1 mosaic isolates identified among 828 patients living in six Brazilian States from three geographic regions (Central West, North, Northeast) were analyzed. Phylogenetic and bootscan analyses were performed to investigate the evolutionary relationship and mosaic structure of BF1 isolates. Those analyses showed that 20.7% of mosaics (18 out of 87) were CRFs-like isolates, mostly represented by CRF28/CRF29_BF-like viruses (14 out of 18). We also identified five highly supported clusters that together comprise 42 out of 87 (48.3%) BF1 sequences, each cluster containing at least five sequences sharing a similar mosaic structure, suggesting possible new unidentified CRFs_BF1. The divergence time of these five potential new CRFs_BF1 clusters was estimated using a Bayesian approach and indicate that they probably originated between the middle 1980s and the middle 1990s.

DNA was extracted from whole blood and four overlapping fragments were amplified by PCR providing full/near full length genomes (FLG/NFLG) and partial genomes. Eleven HIV-1 isolates from Cluster # 5 identified in epidemiologically unlinked individuals living in Central West and North regions provided FLG/NFLG/partial genome sequences with identical mosaic structure. These viruses differ from any known CRF_BF1 reported to date and were named CRF90_BF1 by the Los Alamos National Laboratory. This is the 9th CRF_BF1 described in Brazil and the first one identified in Central West and North regions. Our results highlight the importance of continued molecular screening and surveillance studies, especially of full genome sequences to understand the evolutionary dynamics of the HIV-1 epidemic in a country of continental dimensions as Brazil.
Introduction

Human Immunodeficiency Virus-1 (HIV-1) is a highly polymorphic and fast evolving pathogen [1]. Worldwide HIV-1 can be classified into groups (M, N, O and P), and the pandemic group M is classified in subtypes (A-D, F-H, J and K) and sub-subtypes (A1-A4, F1-F2) [2,3]. While mutation rates are similar to other RNA viruses, HIV-1 has a high recombinogenic capacity and intersubtype recombination events are frequent in coinfected or superinfected individuals from areas where two or multiple variants cocirculate [4]. Recombinant strains exhibiting identical mosaic patterns identified in at least three epidemiologically unlinked individuals have been classified as circulating recombinant forms (CRFs), while the ones displaying unique mosaic structures or only infecting individuals with epidemiological link are known as unique recombinant forms (URFs) [5,6]. Recombination has been recognized as a driving force in shaping the diversity of HIV-1 globally since the mid 90’s [7]. Currently, 88 CRFs have been assigned and 81 of them have been published with public data available at the Los Alamos HIV database [http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html]. CRFs together with URFs are estimated to account for at least 20% of HIV-1 infections worldwide [8].

The Brazilian AIDS epidemic is characterized by the cocirculation of multiple HIV-1 subtypes. Subtype B predominates in most regions followed by subtypes F1, C, and recombinants among these subtypes [9–15]. The first Brazilian BF1 mosaics were identified in the early 90’s in the Southeast region which is considered the epicenter of the epidemic [16,17]. Among the 14 CRFs_BF1 described so far, eight originated in Brazil (CRF28_BF, CRF29_BF, CRF39_BF, CRF40_BF, CRF46_BF, CRF70_BF, CRF71_BF and CRF72_BF) [18–22]. The importance of BF1 recombinants in Brazil is further corroborated by the description of countless URFs in all country regions [23–26]. Previous studies from our research group in different study populations in Central West, North and Northeast Brazilian States showed variable prevalence of BF1 recombinants in the pol subgenomic fragment: (Goiás: 3.7–18.1%, Mato Grosso: 11.9%, Mato Grosso do Sul: 8.2–25.9%, Tocantins: 7.7%, Maranhão: 7.5%, Piauí: 4.5% [11,27–37].

In this study, previously produced pol sequences of BF1 mosaic isolates circulating in Central West, North and Northeast Brazil were reclassified into possible CRFs or URFs. Full/near full-length genome (FLG/NFLG) and partial genome sequences were obtained for the most representative potential CRF detected. These analyzes allowed the identification of the novel CRF90_BF1 that is circulating in Central West and North Brazil, away from the epicenter of the epidemic. Other putative novel CRFs_BF1 are also described. The median time of origin of these mosaics was also estimated. The detailed molecular characterization of recombinant forms circulating countrywide contributes to the mapping of HIV-1 diversity in Brazil.

Material and methods

Study population

Previous studies from our group recruited from 2003 to 2013 a total of 828 individuals infected with HIV-1 residing in six Brazilian States located in three geographic regions (Central West: Goiás/GO, Mato Grosso/MT, Mato Grosso do Sul/MS; North: Tocantins/TO; Northeast: Maranhão/MA, Piauí/PI) (S1 Table) [11,27–37]. These studies have identified a total of 87 (10.5%) BF1 recombinant isolates based on sequencing of pol subgenomic fragment covering the protease (PR) and partial reverse-transcriptase (RT) (positions 2253–3251 relative to HXB2 genome). The related research protocols were approved by the institutional Ethics Committee review boards (Goiás: protocols #073/05, #003/2008, #163/2010 at CEPMHA/HC/UFU, Mato Grosso: protocol #435/07 at Universidade Federal do Mato Grosso/UFMT, Mato Grosso do
Sul: protocol #1143 at Universidade Federal do Mato Grosso do Sul/UFMS, Piauí; protocol #022/2011 at Universidade Estadual do Piauí/UESPI, Maranhão: protocol #16/2011 at Hospital de Doenças Tropicais Dr Natan Portela). All patients signed an informed consent form before blood collection for HIV-1 molecular studies.

**Amplification of HIV-1 PR/RT**

RNA extraction, reverse transcription into complementary DNA (cDNA) and amplification by nested polymerase chain reaction (nested-PCR) of the PR/RT regions were previously described [11,27–37].

**Amplification of HIV-1 full length genomes**

Genomic DNA was extracted from whole blood samples (QIAamp® DNA Blood Mini Kit/ QIAGEN, Qiagen, Hilden, Germany). The complete HIV-1 genome was amplified by nested-PCR employing Platinum Taq DNA polymerase enzyme (Invitrogen, Carlsbad, CA) into four overlapping fragments using HIV-1 specific primers, as following: fragment 1- SCAOSD/LR51 external primers and SCANS/DIP11 internal primers (408–2594), fragment 2- DP10/SCCNAS external primers and DP16/SCCOAS internal primers (2253–4830); fragment 3- MMINT8/ED14 external primers and MMINT3/ED12 internal primers (4653–7811); fragment 4- ED5/SCDOAD external primers and JH44/LTR2 internal primers (6954–9625) (S2 Table) [38–40], all positions were relative to HXB2 genome. Isolates with all four fragments completely sequenced were considered full length genomes (FLG); isolates with three complete fragments were considered near full length genomes (NFLG), and isolates with one or two fragment sequences were referred as partial genomes.

**DNA sequencing**

The amplified DNA fragments from the nested-PCR products were separated by gel electrophoresis, purified (kit QIAquick® PCR Purification Kit/QIAGEN, Qiagen, Hilden, Germany) and sequenced with the Big Dye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA) in an automated ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA). Chromatograms were analyzed and edited using the SeqMan software from the package DNASTAR Lasergene (MA, USA).

**Phylogenetic and recombination analyses**

Sequences were aligned using Clustal X 2.0 implemented in BioEdit 7.2.0 program [41]. Reference sequences of HIV-1 group M subtypes (A–D, F–H, J and K) and CRF-BF1 sequences were obtained from the Los Alamos HIV database (http://hiv.lanl.gov/). Phylogenetic trees were generated using the neighbor-joining (NJ) method [42] under the Kimura two-parameter model [43] using MEGA 6.0 software [44]. Bootstrap values (BP, 1,000 replicates) above 70% were considered significant. Recombination analyses were performed in all viral isolates using bootscan implemented in Simplot v3.5.1 software with the following parameters: 200nt or 300nt window, 20nt increments, NJ method under Kimura’s two-parameter correction with 100 bootstrap replicates [45]. In this study the parameters used for bootscan analyses of recombinant viruses differed for smaller and larger fragments: for the analyses of pol fragments (998nt) a smaller sliding window of 200nt was used whereas for larger fragments of near full-genomes (>6670nt) a larger sliding window of 300nt was adopted. To better characterize the recombination breakpoints suggested in the previous analyses, the putative recombinants were subjected to informative site analyses as described elsewhere [39]. For this purpose, consensus
sequences from Brazilian HIV-1 subtypes B and F were generated in the DAMBE program [46]. Fragments of sequences assigned to specific HIV-1 subtypes were finally confirmed by separate NJ phylogenetic analysis as described above.

Representative samples from the HIV-1 BF1 Brazilian clusters herein identified were submitted to a Basic Local Alignment Search Tool (BLAST) analysis in order to recover other Brazilian sequences with high similarity (>95%) and probably similar recombination profile. The BLAST analysis was done sequences using sequences obtained from the Los Alamos HIV database (http://hiv.lanl.gov/).

Evolutionary analyses of BF1 recombinants

The time of the most recent common ancestor (T_{MRCA}) of HIV-1 BF1 clades was estimated using a Bayesian Markov Chain Monte Carlo (MCMC) approach implemented in BEAST v1.8 [47,48] with BEAGLE to improve run-time [49]. Analyses were performed using the GTR+I +G nucleotide substitution model, a Bayesian Skyline coalescent tree prior [50] and a relaxed uncorrelated lognormal molecular clock model [51] with an informative uniform prior interval (1.0–3.0 x 10^{-3} nucleotide substitutions per site per year). One MCMC chain was run for 1x10^{7} generations. Convergence and uncertainty of parameter estimates were assessed by calculating the effective sample size (ESS) and the 95% highest probability density (HPD) values, respectively using Tracer v1.6 [52]. The maximum clade credibility (MCC) tree was summarized with TreeAnnotator v1.8 and visualized with FigTree v1.4.0.

Data availability

All HIV-1 sequences generated in this study were deposited in the GenBank database (KY628215-KY628225).

Results

Phylogenetic and evolutionary analyses of BF1 pol recombinants

Initial phylogenetic analyses of 87 HIV-1 isolates previously characterized as BF1 recombinants in the PR/RT region (S1 Table) classified 18 (21%) sequences as CRF_BF-like (14 CRF28/CRF29_BF-like, two CRF17_BF-like, one CRF12_BF-like and one CRF47_BF-like) and 27 (31%) sequences as URFs_BF (Fig 1). The remaining 42 (48%) sequences were distributed in five clusters comprising between five and 22 sequences, sharing the same mosaic structure and were classified as potential news CRFs_BF1 (Fig 1). Clusters # 1, 3 and 4 displayed high supports (BP ≥ 99%) at initial analysis. For Clusters # 2 and 5, however, high supports were obtained only after exclusion of the URFs_BF MS251, BRGO3127 and BRGO4162 sequences (Fig 1). Cluster # 1 had six sequences, from three different States (two from Goiás, three from Maranhão and one from Piauí). Cluster # 2 had five sequences, all from Goiás State. Cluster # 3 comprised four sequences from two States (two from Mato Grosso and two from Goiás). Cluster # 4 had five sequences from three States (one from Goiás, three from Maranhão and one from Piauí). Cluster # 5 contained 22 sequences from three States (20 from Goiás, one from Mato Grosso and one from Tocantins).

A Blast search analysis was performed to identify sequences similar to the five potential new CRF_BF1 Brazilian clusters. The recovered sequences were included in the phylogenetic and recombinant analysis, bootstrap values higher than 87% and similar mosaic profiles compared to those previously classified in Clusters # 3, 4 and 5 was verified (Fig 2). Eighteen sequences branching within Cluster # 3 were recovered from patients recruited in four States from the North region (seven from Amazonas, five from Rondônia, three from Roraima and one from...
Fig 1. Phylogenetic analysis of 87 pol sequences of B/F1 HIV-1 isolates presenting five highly supported clusters and the mosaic pattern of recombination in each cluster (neighbor-joining method, Kimura 2-parameters evolutionary model/1000 replicate bootstrap values). Bootscanning analyses of BF1 inter-subtype recombinant clusters (#1–5) are represented. The five clusters identified in our study are indicated by different colors: Cluster #1: purple, Cluster #2: blue, Cluster #3: pink, Cluster #4: green and Cluster #5: red. Bootscan analysis was performed in a 200nt sliding window advanced in 20nt step size increments (1,000 replicates). All CRF_BF depicting recombination breakpoints in pol region were included in the analysis. In the mosaic structure representations of BF1 isolates, the breakpoint positions according to HXB2 genome numeration are shown on the right and left sides of the clusters, blue stands for subtype B and green stands for subtype F.

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Acre) along with two sequences from the South region (Paraná) (Fig 2). Two sequences from the North region (Amapá) classified in Cluster # 4 and three sequences classified in Cluster # 5 were recovered from patients from the North region (Rondônia) (Fig 2).

The Bayesian MCC tree displayed the same topology of the NJ tree, thus confirming the five BF1 phylogenetic clusters initially described (Fig 3). According to this analysis, the median $T_{MRCA}$ of the five potential new Brazilian CRFs_BF identified was estimated between the middle 1980s and the middle 1990s (Fig 3).
Analysis of FLG, NFLG and partial genomes

Phylogenetic (Fig 4) and bootscan analyses of six full length genomes (BRGOAP801, BRGO6043, BRTO10_66, BRGO4141, BRGO3145 and BRGO3047) obtained from isolates classified in Cluster # 5 allowed the description of a new recombinant lineage designated CRF90_BF1 by the Los Alamos HIV Sequence Database (Los Alamos National Laboratory) according to the standardized nomenclature [2]. We also obtained one NFLG and four partial genomes for isolates from this Cluster that share the same mosaic structure (Fig 5). The mosaic structures inferred from the analyses of these FLG, NFLG and partial genomes showed a genome predominantly of subtype B, which can be divided into seven subregions alternating subtypes B and F1. These seven subregions were named I (626–2,661), II (2,662–2,971), III (2,972–4,295), IV (4,296–4,759), V (4,760–8,671), VI (8,672–9,492) and VII (9,493–9,612) all positions relative to HXB2 genome. Subregion NJ analyses also confirmed the putative parental HIV-1 subtype (Fig 5). Fully coincident intersubtype breakpoint locations at I-III sub regions were also observed in the NFLG of BRGO4188 isolate and in the partial genome sequences of BRMT508, BRGO3027, BRGO3059 and BRGO6048 isolates (Fig 5 and Table 1).

The epidemiological features of the 11 patients presenting the newly described CRF90_BF1 lineage included six females (four of them pregnant) and five males (two of them prisoners) (Table 1). The prevailing risk category was heterosexual sex reported by nine patients while one prisoner patient reported intravenous drug use. Six patients were ARV naïve and five had been exposed to ARV drugs either as highly active antiretroviral therapy (HAART) or temporary mother-to-child-transmission (MTCT) prophylaxis. Most patients were from the Central West region (Goiás State: isolates BRGO3027, BRGO3047, BRGO3145, BRGO3059, BRGO4188, BRGO4141, BRGO6048, BRGOAP801 and BRGO6043; Mato Grosso State: isolate BRMT508) and one patient lived in the North region (Tocantins State: isolate BRTO10_66).

Discussion

In this study, we report the characterization of a novel HIV-1 CRF_BF1, named CRF90_BF1 based on six FLG, one NFLG and four partial genome sequences. These isolates shared identical mosaic structures and were identified in individuals without any epidemiological link that live in two distinct geographic regions in Brazil (Central West and North) located around 800–900 km apart. These criteria fulfill the requirements to define a new CRF, which is circulating in distant interior urban areas in Brazil. This novel CRF is the 9th CRF involving subtypes B and F1 described in Brazil and the 14th reported in South America. The estimated frequency of the CRF90_BF1 in our sample set was 1.3% (11/828), with predominant detection in the Central West region. However, the actual prevalence of this new CRF in these geographic regions cannot be accurately estimated since there is limited molecular data on HIV-1 isolates especially from the States of Mato Grosso, Mato Grosso do Sul and Tocantins.

The CRF12_BF, the first CRF identified in the Americas was described in 2001 in patients from Argentina and Uruguay and its origin was estimated around the early 80s [53,54], while BF1 recombinants were first reported in Brazil in the early 90’s [16,17]. Patients harboring the CRF90_BF1 were diagnosed between 2002 and 2011. The median estimated TMRCA of the CRF90_BF1 and of other putative CRF_BF1 clusters identified in our study is not recent and ranges from middle 80’s to middle 90’s, similar to that previously estimated for Brazilian CRF28_BF and CRF29_BF [55]. These estimates indicate that CRFs_BF1 have been probably circulating in Brazil for three to four decades.

Besides its early generation, we have evidences, as shown by blast search analyses, that the CRF90_BF1 and also the other putative CRFs_BF1 clades identified here have a wide geographic circulation (Fig 6). The CRF90_BF1 that we identified in Central West (Goiás and
Fig 4. Phylogenetic analyses on the full length/near-full-length genome sequences of HIV-1 BF1 isolates from cluster # 5 belonging to a new CRF90_BF1 identified in patients from Goiás and Tocantins States in the Central West and North Brazilian regions. The HIV group M reference sequences of subtypes were obtained from the Los Alamos database. The scale bar represents 0.02 nucleotide substitutions per site. Phylogenetic analyses were constructed with Mega software version 6.0.

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Fig 5. Mosaic structure of the new CRF composed by subtypes B and F1. Breakpoint positions according to HXB2 genome numbering system are indicated. The phylogenetic trees for each of the seven mosaic segments (I-VII) were constructed with Mega software v6.0 and the trees were midpoint rooted. The stability of each node was confirmed by bootstrapping with 1,000 replicates and
Mato Grosso) and North Brazil (Tocantins) is probably also circulating in Rondônia, another State in the North region which borders Bolivia in the South/East. HIV-1 BF1 isolates with the same recombination pattern of isolates from Cluster # 3 detected in Central West were also identified in several North Brazilian States (Amazonas, Rondônia, Roraima and Acre), and in the South State of Paraná. Isolates with similar recombination profile of isolates from Cluster # 4 were also identified in the North region (Amapá State) besides the Central West (Goiás) and Northeast Brazil (Maranhão and Piauí). These results suggest the existence of novel CRFs_BF1 circulating in Brazil.

CRF28_BF and CRF29_BF described in the Southeast in 2006 (Santos, São Paulo State) represent the first Brazilian CRFs, and their origin date to 1988–1989 [18,55]. Studies have shown a low prevalence of CRF28_BF and CRF29_BF [14,56], outside São Paulo except in Salvador, Bahia State, Northeast where prevalence ranged from 10%-21% [57,58]. Among all BF1 isolates identified in our study we have found a moderate rate of CRF28/CRF29_BF-like isolates (16.1%, 14 out of 87) and an overall rate of 1.7% (14 out of 828) which represent one of the highest frequencies of these CRFs identified outside São Paulo State.

Despite the predominance of subtype B in most geographic Brazilian regions, except in the South where subtype C prevails, studies have shown that the prevalence of non-B subtypes, particularly URFs_BF1 and URFs_BC has increased in the last decade [15,25,40,59,60]. Our studies have shown a significant percentage of recombinant BF1 forms (3.7–25.9%) in the Central West, North and Northeast Brazilian regions [11,27–37]. The most recently described Brazilian CRFs_BF1 (CRF70_BF1 and CRF71_BF1) were identified among blood donors from Pernambuco State, Northeast region [22]. The CRF72_BF1 was identified among blood donors from five public blood banks in Minas Gerais State, Southeast region [21]. These recent data point out the increasing generation and spread of CRFs, especially involving subtypes B and F1 which play an important role in the Brazilian AIDS epidemic. However, the number of complete genome sequences available is still limited, especially sequences from areas away from the epicenter, as our study areas (Central West, North and Northeast) suggesting that only significant bootstrap values >70% are shown at the corresponding nodes. The genetic distance corresponding to the length of the branches is shown by the line at the bottom. The red color represents the CRF90_BF1 identified in this study.

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Table 1. Demographic characteristics of study subjects infected by HIV-1 CRF90_BF1.

| Sample ID | ARV status | Gender | Age | Risk Group | HIV Diagnosis (Year) | Sample Collection (Year) | HXB2 location (nt) | Accession Number |
|-----------|------------|--------|-----|------------|----------------------|-------------------------|--------------------|-----------------|
| BRGOAP801 | HAART      | M      | 59  | Heterosexual | 2007                 | 2010                    | 407–962            | KY628223        |
| BRGO6043  | Naïve      | F      | 30  | Heterosexual | 2011                 | 2011                    | 407–962            | KY628221        |
| BROTO10_66| Naïve      | F      | 27  | Heterosexual | 2009                 | 2009                    | 407–9616           | KY628225        |
| BRGO4141  | Prophylaxis| F      | 24  | Heterosexual | 2010                 | 2010                    | 408–9615           | KY628219        |
| BRGO3145  | HAART      | F      | 35  | Heterosexual | 2004                 | 2007                    | 407–9612           | KY628218        |
| BRGO3047  | HAART      | M      | 47  | NA         | 2002                 | 2007                    | 474–9589           | KY628216        |
| BRGO4188  | Prophylaxis| F      | 27  | Heterosexual | 2006                 | 2010                    | 407–7080*          | KY628220        |
| BRGO3027  | Naïve      | M      | 27  | IDU        | 2002                 | 2007                    | 453–5924*          | KY628215        |
| BRMT508   | Naïve      | M      | 41  | Heterosexual | 2009                 | 2009                    | 414–5919*          | KY628224        |
| BRGO3059  | Naïve      | M      | 51  | Heterosexual | 2007                 | 2007                    | 407–4778*          | KY628217        |
| BRGO6048  | Naïve      | F      | 30  | Heterosexual | 2009                 | 2012                    | 454–4122*          | KY628222        |

M: Male; F: Female; Naïve: antiretroviral naïve patients; HAART: Patients under highly active antiretroviral therapy; Prophylaxis: mother-to-child-transmission antiretroviral prophylaxis (MTCT ARV prophylaxis); IDU: intravenous drug user; N.A.: not available

* isolates with partial genome sequences; Isolates are listed according to the size of sequenced fragments. nt: nucleotide position

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the actual proportion of HIV-1 recombinant forms in the Brazilian pandemic is probably underestimated.

**Conclusions**

In summary, we identified the novel CRF90_BF1 among heterosexual patients living in two geographic regions in Brazil, away from the epicenter of the epidemic. This is the 9\(^{th}\) CRF_BF1 described in Brazil indicating that continued molecular screening and surveillance are necessary to fully understand the evolutionary dynamics of the HIV-1 epidemic in such a country of continental dimensions. Our results also underscore the importance of full-length genome sequencing of HIV-1 isolates obtained from patients infected by different transmission routes and in different country regions to fully understand the diversity and complexity of the HIV-1 epidemic in Brazil.
Supporting information

S1 Table. Prevalence of BF1 recombinants identified in previous studies among patients from six Brazilian States: Goiás/GO, Mato Grosso/MT, Mato Grosso do Sul/MS, Tocantins/TO, Piauí/PI and Maranhão/MA. Pregnant: women infected with HIV-1 attending a regional antenatal care; Naïve: antiretroviral naïve patients; HAART: Patients under highly active antiretroviral therapy. * Ref 29: the study group (n = 27) comprises prisoner patients recruited in Goiania/GO (n = 7) and in Campo Grande (n = 20).

(DOCX)

S2 Table. List of HIV-1 primers used in the present study for full length genome amplification. * Some primers had their original sequence modified based on the alignment of subtypes B, C and F1 HIV sequence compendium (2005) from HIV Los Alamos Database.

(DOCX)

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Author Contributions

Conceptualization: MMAS.
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Formal analysis: MNGR, MLG, GB, MMAS.
Funding acquisition: MMAS, MLG.
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Project administration: MMAS.
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Supervision: MMAS.
Validation: MMAS, MNGR, GB, MLG.
Visualization: MMAS, MNGR, GB, MLG.
Writing – original draft: MMAS, MNGR.
Writing – review & editing: MMAS, MNGR, GB, MLG.

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