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# Detection of human immunodeficiency virus Type 1 phylogenetic clusters with multidrug resistance mutations among 2011 to 2017 blood donors from the highly endemic Northern Brazilian Amazon

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**BACKGROUND:** This study describes transmitted drug resistance (TDR) in blood donors diagnosed with human immunodeficiency virus Type 1 (HIV-1) infection from 2011 to 2017 in three reference public blood centers from the Northern Brazilian Amazon.

**STUDY DESIGN AND METHODS:** This was a cross-sectional study on HIV-positive blood donors from HEMOAM, Manaus, Amazonas, AM (n = 198); HEMERON, Porto Velho, Rondônia, RO (n = 20); and HEMORAIMA, Boa Vista, Roraima, RR (n = 9). HIV-1 pol sequences (protease, reverse transcriptase) were analyzed for drug resistance mutations (DRMs) using the Calibrated Population Resistance tool (Stanford). TDR/DRM clusters were investigated by phylogenetic analysis after removing positions associated with drug resistance of Subtype B sequences from untreated and treated subjects from Northern Brazil.

**RESULTS:** Transmitted drug resistance/DRM in blood donors was 11% (25 of 227), all of them from HEMOAM. Most blood donors with TDR/DRM had multiple and similar DRMs. Nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations predominated (10.1%), followed by nucleoside reverse transcriptase inhibitor (NRTI) mutations (5.3%) and protease inhibitor mutations (0.4%). Dual-class NNRTI/NRTI mutations represented 4.8%. Three highly supported Subtype B monophyletic clades mostly composed by individuals from Amazonas with TDR/DRM mutations were identified. The largest transmission cluster contained 10 sequences, eight from HEMOAM and two sequences described previously (one from a treated subject from Amazonas and the other one from Roraima). This cluster was characterized by NRTI (D67N, T69D, T215S/F/L, K219Q) and NNRTI (K101H, K103 N, G190A) mutations. The other two transmission clades comprised only three and two sequences from HEMOAM sharing the E138A NNRTI mutation.

**CONCLUSIONS:** The identification of transmission clusters of multidrug-resistant viruses in blood donors from Amazonas highlight the need of continued monitoring of TDR/DRM and the importance of pretreatment genotyping in the highly endemic Amazonas state.

Despite significant improvements in the control of human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) epidemic in Brazil, its management in the highly endemic, remote, and isolated Northern region is challenging. The Brazilian HIV/AIDS epidemic is multifaceted and compartmentalized in its different geographic regions. In the recent decade, the national AIDS incidence rate decreased 9.4% and AIDS-related mortality declined 14.8%.[1] These data reflect the beneficial impact of the free universal access to antiretroviral drugs (ARV) implemented by the Ministry of Health since

**ABBREVIATIONS:** ARV = antiretroviral drugs; DRM(s) = drug resistance mutation(s); ML = maximum likelihood; NNRTI = nonnucleoside reverse transcriptase inhibitors; NRTI = nucleoside reverse transcriptase inhibitor; PR = protease; RT = reverse transcriptase; SDRM = Surveillance Drug Resistance Mutation; TDR = transmitted drug resistance; TDRM = transmission clusters of drug resistance mutations.

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mid-1990s, which since December 2013 has adopted the "test and treat policy."[1] In contrast, during the same period, a growing epidemic with 44.2% increase in AIDS incidence and rise in AIDS-related mortality has been reported in almost all states from the Northern region.[1] The widespread ARV use in countries as Brazil raises the public health concern on the selection of drug resistance mutations (DRMs) since transmitted drug resistance (TDR) has been associated with first-line antiretroviral failure.[2] Different rates of TDR to the main ARV drug classes nucleoside and nonnucleoside reverse transcriptase inhibitors (NRTI and NNRTI, respectively) and protease inhibitors (PI) have been reported in Brazil, mostly ranging from 5% to 15%.[3–12] In Northern Brazil, TDR rates ranging from 0% to 21% have been reported; however, studies from this region are still scarce.[8,13–17]

In the context of a highly endemic region for HIV/AIDS, apparently healthy blood donors recently diagnosed with HIV-1 infection represent important sentinel populations for the assessment of TDR/DRM. As part of the routine screening process of blood donors, it has been assumed that all HIV-1–positive blood donors were honest about not knowing their HIV-positive status and not taking any ARV that could otherwise readily select for DRM. Therefore, all HIV-positive blood donors identified here were considered ARV naive. Previous reports from HEMOAM, a reference public blood bank in Amazonas state, Northern Brazil, showed a significant number of HIV-1 infections in blood donors and a remarkable prevalence of Subtype B (M.E. Crispim et al., submitted for publication).[18,19] In this study we describe TDR/DRM rate and transmission clusters of drug-resistant viruses in these HIV-positive ARV-naive blood donors from Northern Brazil.

## MATERIALS AND METHODS

### Study area and study population

This cross-sectional study was conducted among 227 HIV-positive blood donors from any sex, age range, or first-time and repeat donor or coming for voluntary or replacement donations. Blood donors were diagnosed from 2011 to 2017 in three public blood centers located in the Northern Brazilian region: "Fundação de Hematologia e Hemoterapia do Amazonas, HEMOAM" (n = 198), located in Manaus, capital of Amazonas state, "Fundação Hematologia e Hemoterapia de Rondônia, HEMERON" (n = 20), located in Porto Velho, capital of Rondônia state; and "Hemocentro de Roraima, HEMORAIMA" (n = 09), situated in Boa Vista, capital of Roraima state. As recently reported, the majority of these blood donors was young (82% aged between 20 and 40 years), single (78%), and male (88%) and approximately half were repeat donors (49%) (M.E. Crispim et al., submitted for publication). HIV-1 subtype B was predominant (90.7%), followed by BF1 recombinants (5.3%), Subtype C (3.1%), and Subtype F1 (0.9%). The $B_{PANDEMIC}$ and $B_{CARIBBEAN}$ ($B_{CAR}$) lineages represented 78 and 22% of Subtype B infections, respectively (M.E. Crispim et al., submitted for publication).

### Genetic analysis

Residual plasma samples from blood donors were used for RNA extraction (QIAamp viral RNA mini kit, Qiagen), RNA was reverse transcribed into complementary DNA (Invitrogen) and used as the target for nested polymerase chain reaction (PCR). The HIV-1 protease (PR) and reverse transcriptase (RT) K1/K2 external primers and DP10/F2 internal primers[20,21] amplify the entire PR region (Positions 2253-2549 relative to HXB2 genome, GenBank Accession Number K03455) and a 750-bp fragment of RT region (Positions 2550-3299 relative to HXB2 genome). Amplicons were purified (QIAquick PCR purification kit, Qiagen GmbH) and genomic sequencing was performed (DYEnamic ET dye terminator kit, GE Healthcare; ABI Prism 3100 genetic analyzer, Applied Biosystems). All generated sequences were subjected to quality control analysis by HIV-1 4969 Quality Analysis Pipeline Tool (http://www.sanbi.ac.za) and were screened by visual inspection of the alignment (Bioedit software) to check for sample mix-ups and contamination.[22,23] GenBank Accession Numbers of the sequences presented in this study are MH673055 through MH67328.

### Antiretroviral drug resistance analyses

Transmitted drug resistance mutations were analyzed using the Calibrated Population Resistance tool employing the Stanford Surveillance Drug Resistance Mutation (SDRM) list, which represents sensitive and specific indicators of ARV selection pressure suggesting transmitted resistance (available at http://cpr.stanford.edu/cpr.cgi accessed on December 20, 2017). The Calibrated Population Resistance tool is a Web-accessible program that performs standardized genotypic estimation of transmitted HIV-1 drug resistance and is linked to the Stanford HIV drug resistance database. It can additionally perform viral genotyping and algorithmic estimation of resistance to specific ARV.[24] As previously described, the identification of SDRMs was based on four criteria: 1) SDRMs should be recognized as causing or contributing to drug resistance, defined as being present on three or more of five expert lists of DRMs (ANRS, HIVdb, IAS-USA, Los Alamos and Rega algorithm lists); 2) mutations should be nonpolymorphic and should not occur at highly polymorphic positions; 3) the mutation list had to be applicable to the eight most common HIV-1 subtypes; and4) the list should be parsimonious, excluding mutations resulting exceedingly rarely from drug pressure.[24–26] The use of this standard list of SDRMs allows the comparison of results of sequencing studies performed in different regions or at different times facilitating meta-analyses of surveillance data collected by different groups at different times.[26]

The prevalence of TDR was based on the number of RT/PR sequences containing at least one DRM to any drug class (NRTI, NNRTI, PI). Double resistance was defined by

the presence of mutations to two drug classes (NRTI + NNRTI or NNRTI + PI or NNRTI + PI) and triple resistance was defined by the presence of DRM to all three drug classes. TDR was calculated according to the presence of mutations associated with any level of drug resistance (low, intermediate, or high level) excluding sequences containing surveillance DRM classified as susceptible or potentially low-level resistant by the HIVdb program. Only major PI associated mutations were considered.

### Identification of TDR clusters

Human immunodeficiency virus Type 1 genetic subtype was defined by REGA automated genotyping tool (Version 2.0) and by phylogenetic inference.[27,28] To identify clusters of TDR/DRM, Subtype B sequences from antiretroviral therapy–naive and –treated subjects from Northern Brazil described here and previously[8,15,17,29,30] were aligned and subject to maximum likelihood (ML) phylogenetic analyses after removing positions associated with drug resistance. ML phylogenetic trees were reconstructed with the PhyML 3.0 program[31] under the best nucleotide substitution model, selected by the SMS (Smart Model Selection) software[32] integrated into the PhyML Web server. The SPR branch-swapping algorithm was selected for heuristic tree search and the approximate likelihood-ratio test (aLRT)[33] to estimate the reliability of the tree topology obtained. TDR/DRM clusters were defined as highly supported (aLRT > 0.90) monophyletic clades mostly (≥80%) or exclusively composed by untreated subjects with TDR mutations.

### Statistical analysis

A descriptive analysis of percentage and medians was performed using computer software (Prism 8, GraphPad).

### Ethics issues

The ethical issues of this study were reviewed by the HEMOAM review board that approved the use of residual plasma samples from blood donors (Comite de Ética em Pesquisa com Seres Humanos, Fundação de Hematologia e Hemoterapia do Amazonas, Protocol 31061814.6.0000.0009).

## RESULTS

### Prevalence of TDR mutations

Among 227 blood donors diagnosed with HIV-1, 11% (25 of 227) had TDR/DRM to any ARV class (Table 1). All 25 blood donors with DRM were from HEMOAM, Amazonas state (12.6% prevalence, 25 of 198). Blood donors with drug resistant strains were mostly male (n = 22) and repeat donors (n = 15), and the median age was 28 years (20-52 years range; Table 1). HIV-1 Subtype B was prevalent in drug-resistant sequences (n = 22), two females with TDR/DRM were infected with BF1 recombinants and one male had Subtype C infection (Table 1).

Mutations associated with resistance to NNRTI class predominated (10.1%), followed by NRTI mutations (5.3%) and PI mutation (0.4%) (Table 1). Ten different mutations associated with NNTRI were detected in 23 participants. The most prevalent NNRTI mutations were as follows: K103 N (n = 10), E138A (n = 9), K101H (n = 8), and G190A (n = 8; Fig. 1). Twelve of 23 donors with mutations to NNRTI (52.2%) harbored multiple mutations (ranging from two to four) and five isolates (BRAM_50, BRAM_133, BRAM_137, BRAM_159, BRAM_409) shared the same mutations and resistance profiles (Table 1).

Nucleoside reverse transcriptase inhibitor resistance mutations were seen in 12 sequences and nine of them harbored multiple similar mutations, ranging from four to seven; most drug-resistant sequences had four mutations (Table 1). The most prevalent mutations associated with NRTI were as follows: D67N (n = 9), T69D (n = 8), K219Q (n = 8), and T215S (n = 6; Fig. 1). Seven isolates shared the D67N, T69D, T215S/L, and K219Q NRTI mutations (BRAM_50, BR_AM133, BRAM_137, BRAM_159, BRAM_243, BRAM_250, BRAM_412; Table 1).

Just one male blood donor (first time, 24 years old) had a major PI associated mutation (M46I, isolate BRAM_377). Dual-class mutations were seen in 4.8% of the isolates (11 of 227): 10 isolates had mutations to NNRTI and NRTI (BRAM_50, BRAM_133, BRAM_137, BRAM_159, BRAM_243, BRAM_250, BRAM_268, BRAM_381, BRAM_409, BRAM_412) and one isolate had mutations to PI and NNRTI (BRAM_377; Table 1). Triple-class mutation was not detected.

### Investigation of transmission clusters among donors with TDR/DRM

The presence of several HIV-positive donors carrying multiple and similar ARV resistance mutations in their *pol* sequences led us to investigate the existence of possible transmission clusters associated with drug resistance. ML phylogenetic analysis of Subtype B sequences from untreated and treated subjects from Northern Brazil identified here and in previous studies were conducted after removing positions associated with drug resistance. This analysis showed three highly supported (aLRT > 0.90) monophyletic clusters mostly (≥80%) or exclusively composed by untreated subjects with TDR mutations in Amazonas (Fig. 2).

The largest cluster (transmission clusters of drug resistance mutations-I [TDRM-I]; Fig. 2) contained 10 non-pandemic Subtype B ($B_{CAR}$) sequences, eight from untreated subjects from Amazonas described here and two from treated subjects from Amazonas and Roraima described in previous studies. Most HIV-1 sequences from this cluster share a set of four NRTI mutations (D67N, T69D, T215S/F/L, K219Q) and three NNRTI mutations (K101H, K103 N, G190A; Table 2). Of note, the sequences from the treated subject from Amazonas (described previously) branched at the base of the TDR/DRM cluster (Fig. 2). Among the eight donors from HEMOAM

**TABLE 1. Characteristics of 25 ARV-naive blood donors from Amazonas with DRMs to ARV***

| Specimen ID | sex | Age (years) | Type of donor/donation | HIV-1 subtype | PI major DRMs (n = 1) | NRTI DRMs (n = 12) | NNRTI DRMs (n = 23) | ARV resistance profile Low | Intermediate | High |
|---|---|---|---|---|---|---|---|---|---|---|
| BRAM_30 | Male | 44 | Repeat/voluntary | BB | | | K101P, K103N | | | EFV, ETR, NVP, RPV |
| BRAM_50 | Female | 23 | Repeat/voluntary | BB | | D67N, T69D, T215S, K219Q | K101H, K103S, G190A | ABC, TDF, ETR, RPV | AZT | EFV, NVP |
| BRAM_58 | Male | 33 | Repeat/replacement | BB | | | E138A | RPV | | |
| BRAM_98 | Female | 27 | Repeat/replacement | BF | | | E138A | RPV | | |
| BRAM_102 | Male | 24 | First time/replacement | BB | | | K103 N | | | EFV, NVP |
| BRAM_121 | Male | 25 | Repeat/voluntary | BB | | | E138A, V179D | ETR, RPV | | |
| BRAM_133 | Male | 22 | First time/replacement | BB | | D67N, T69D, T215S, K219Q | K101H, K103 N, G190A | ABC, TDF, ETR, RPV | AZT | EFV, NVP |
| BRAM_137 | Male | 28 | Repeat/replacement | BB | | D67N, T69D, T215S, K219Q | K101H, K103N, G190A | ABC, TDF, ETR, RPV | AZT | EFV, NVP |
| BRAM_138 | Female | 40 | Repeat/replacement | BF | | | M230I | EFV, ETR | NVP, RPV | |
| BRAM_159 | Male | 33 | First time/replacement | BB | | D67N, T69D, T215S, K219Q | K101H, K103N, G190A | ABC, TDF, ETR, RPV | AZT | EFV, NVP |
| BRAM_225 | Male | 27 | Repeat/replacement | CC | | | E138A | RPV | | |
| BRAM_243 | Male | 30 | Repeat/replacement | BB | | D67N, T69D, T215S, K219Q | K101H, G190A, F227L | ABC, TDF, ETR, RPV | AZT | EFV, NVP |
| BRAM_250 | Male | 36 | First time/voluntary | BB | | D67N, T69D, T215L, K219Q | K101H, K103 N, G190A, F227L | ABC, TDF, ETR, RPV | AZT | EFV, NVP |
| BRAM_251 | Male | 43 | Repeat/replacement | BB | | | E138A | RPV | | |
| BRAM_268 | Male | 27 | First time/replacement | BB | | M41 L, E44D, D67N, T69D, L74I, L210W, T215D | K103N, V108I | | TDF | ABC, AZT, EFV, NVP |
| BRAM_269 | Male | 28 | Repeat/voluntary | BB | | | E138A | RPV | | |
| BRAM_274 | Male | 31 | First time/voluntary | BB | | T215D | | AZT | | |
| BRAM_299 | Male | 27 | Repeat/voluntary | BB | | | E138A | RPV | | |
| BRAM_342 | Male | 38 | Repeat/replacement | BB | | | E138A | RPV | | |
| BRAM_350 | Male | 22 | First time/voluntary | BB | | T215A | | AZT | | |
| BRAM_377 | Male | 24 | First time/replacement | BB | M46I | | V108I, E138A | NVP, RPV | | |
| BRAM_381 | Male | 43 | Repeat/voluntary | BB | | M184I | K103N | ABC | | FTC, 3TC, EFV, NVP |
| BRAM_402 | Male | 26 | First time/replacement | BB | | | K103N | | | EFV, NVP |
| BRAM_409 | Male | 32 | Repeat/replacement | BB | | D67N, T215S, K219Q | K101H, K103N, G190A | ABC, TDF, ETR, RPV | AZT | EFV, NVP |
| BRAM_412 | Female | 20 | First time/replacement | BB | | D67N, T69D, T215L, K219Q | K101H, G190A | ABC, TDF, ETR, RPV | AZT, EFV | NVP |

* ARV resistance profile: low, intermediate, and high according to the Stanford HIVdb Program Genotypic Resistance Interpretation Algorithm.
3TC = lamivudine; ABC = abacavir; AZT = zidovudine; EFV = efavirenz; ETR = etravirine; FTC = emtricitabine; NVP = nevirapine; RPV = rilpivirina; TDF = tenofovir.

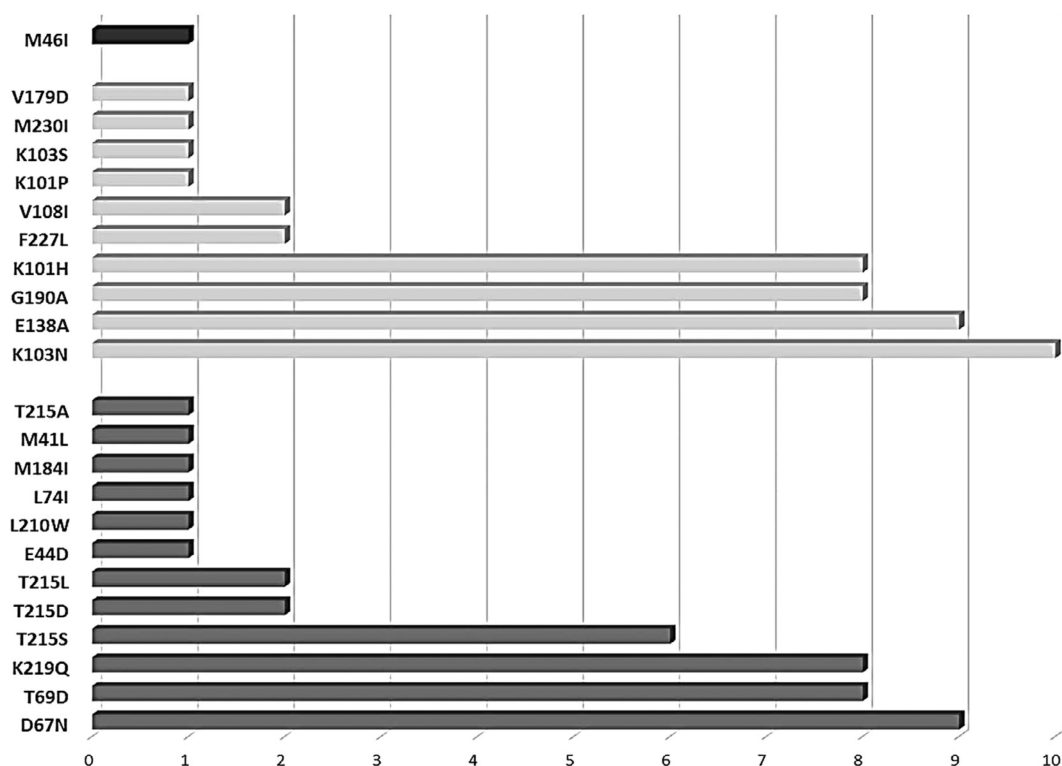


**Fig. 1. Number of HIV-1 sequences with DRMs detected among ARV-naive newly diagnosed HIV-1–infected blood donors from Northern Brazil. (■) PI mutation; (■) NNRTI; (□) NRTI.**

included in the TDRM-I, five came from Amazonas state, one from Para state (North region), one from Santa Catarina state (South region), and one from Minas Gerais state (Southeast region). The samples from this cluster were collected from 2011 to 2017 (2011 = 2, 2012 = 3, 2016 = 2, 2017 = 1).

The other two clusters (TDRM-II, TDRM-III; Fig. 2) comprise three and two $B_{PANDEMIC}$ sequences of blood donors from Amazonas described here and share the E138A NNRTI DRM (Table 2). Among the three donors in TDRM-II, two came from Amazonas and one from Para state; one sample was collected in 2013 and two in 2015. TDRM-III includes two donors, one from Para state collected in 2012 and one from Ceara state (Northeast region) collected in 2016. Analysis of a large number of HIV-infected individuals from Amazonas will be necessary to confirm whether these clades represent new emerging TDR/DRM clusters of larger size or transmission clusters restricted to a few closely related individuals.

## Features of HIV-1–infected repeat blood donors harboring TDR/DRM

The majority of blood donors with TDR/DRM (60%, 15 of 25) was repeat donors with a median of four previous donations and most sequences with DRM (52%, 13 of 25) belonged to one of the TDRM clusters (Table 2). In the transmission clusters of TDR/DRM, most sequences (62%, eight of 13) were from repeat blood donors, male (87.5%), and single (87.5%) and with median age of 28 years (Table 3). The time between the last seronegative donation and the index donation positive for HIV-1 ranged from 7 months (BRAM_121) to over 10 years (BRAM_251).

In this group of repeat donors with TDR/DRM, the number of previous donations ranged from two to 25 (Table 3). The male repeat donor with 25 previous donations (BRAM_299, Table 3, TDRM-II) was the oldest donor in the TDRM transmission clusters, was married, reported less than 8 schooling years, and worked as a professional cook. Half of the repeat blood donors that participated in the TDR/DRM transmission clusters had multiple resistance mutations (ranging from six to eight) to NNRTI and NRTI (Table 3).

## TDR among HIV-1–positive blood donors diagnosed during the immunologic window period

In June 2012, HIV nucleic acid testing (NAT) was implemented at HEMOAM, and up to 2017, four single male donors were diagnosed during the immunologic window period (21, 24, 25, and 27 years old; Table 4). These donors were all seronegative for HIV-1 and -2 and positive by HIV NAT only (data not shown). Two of these immunologic window cases were repeat donors (BRAM_121, BRAM_302) reporting two and three previous donations, respectively. HIV-1 Subtype B infections were identified in these four cases of recent infection. TDR was detected in the sequence BRAM_121 (E138A and V179D NNRTI mutations; Table 1) that belonged to the TDRM-II transmission cluster (Fig. 2, Table 2).

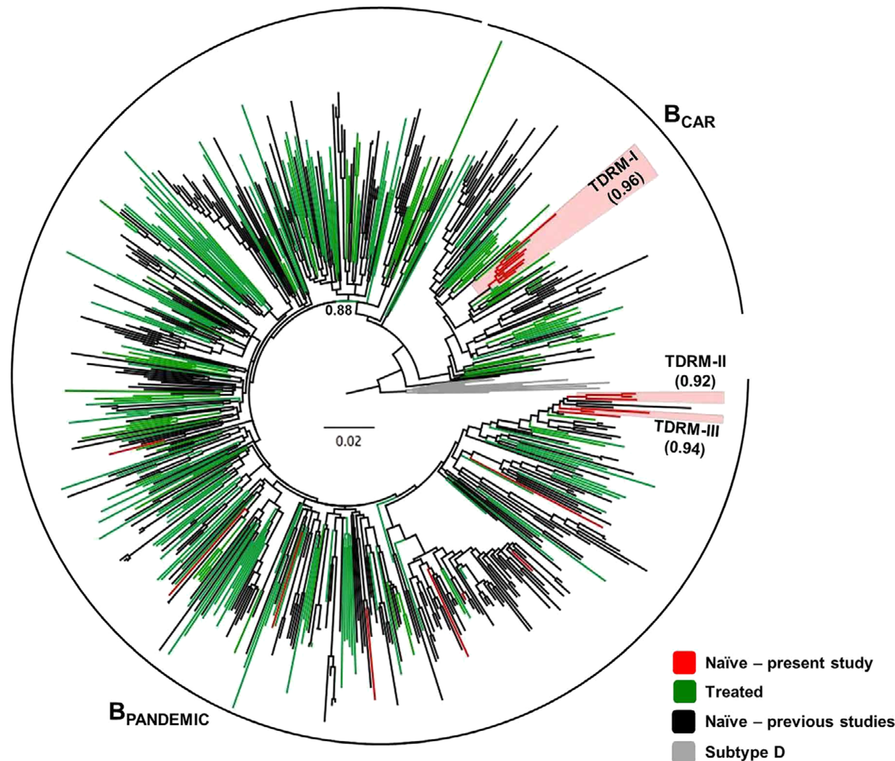


**Fig. 2. Maximum likelihood (ML) phylogenetic analysis of Subtype B sequences from untreated and treated subjects from Northern Brazil identified in the current investigation and in previous studies after removing positions associated with drug resistance. Three highly supported (aLRT > 0.90) clusters of transmission of TDR (TDRM-I, -II, -III) were detected among blood donors from Amazonas. TDRM-I = the largest cluster contains 10 Subtype B Caribbean/$B_{CAR}$ sequences, eight from untreated blood donors from Amazonas and two from treated subjects from Amazonas and Roraima described in previous studies. TDRM-II and TDRM-III comprise three and two $B_{PANDEMIC}$ sequences from blood donors from Amazonas described here. [Color figure can be viewed at wileyonlinelibrary.com]**

## DISCUSSION

Our study shows a very peculiar profile of TDR/DRM among ARV-naive, HIV-1–positive blood donors from Northern Brazil. Instead of singleton mutations, a significant proportion of donors with TDR/DRM presented multiclass drug resistance to NNRTI and NRTI. Similar mutations were also shared among donors from HEMOAM suggesting the existence of transmission clusters that were supported by phylogenetic analyses. The

**TABLE 2. Features of sequences that participate in each transmission cluster of TDR identified among blood from Amazonas state, Northern Brazil***

| Lineage | Sample ID | PI major mutations | NRTI mutations | NNRTI mutations |
|---|---|---|---|---|
| TDRM-I | AM.2011.24 AM110177 | | D67N, T69D, K70R, T215F, K219Q | K103 N, G190A |
| | RR.2013.ION94 | | D67N, T69D, K70R, M184V, T215F, K219Q | K101H, K103 N, G190A |
| | BRAM_50 | | D67N, T69D, T215S, K219Q | K101H, K103S, G190A |
| | BRAM_133 | | D67N, T69D, T215S, K219Q | K101H, K103 N, G190A |
| | BRAM_137 | | D67N, T69D, T215S, K219Q | K101H, K103 N, G190A |
| | BRAM_159 | | D67N, T69D, T215S, K219Q | K101H, K103 N,G190A |
| | BRAM_243 | | D67N, T69D, T215S, K219Q | K101H, G190A, F227L |
| | BRAM_250 | | D67N, T69D, T215L, K219Q | K101H, K103 N, G190A, F227L |
| | BRAM_409 | | D67N, T215S, K219Q | K101H, K103 N, G190A |
| | BRAM_412 | | D67N, T69D, T215L, K219Q | K101H, G190A |
| TDRM-II | BRAM_121 | | | E138A, V179D |
| | BRAM_269 | | | E138A |
| | BRAM_299 | | | E138A |
| TDRM-II | BRAM_251 | | | E138A |
| | BRAM_377 | M46I | | V108I, E138A |

* Samples IDs AM.2011.24 AM110177 and RR.2013.ION94 were described in previous studies.

**TABLE 3. Features of HIV-1–positive repeat blood donors who participated in transmission clusters of TDR/DRM**

| Sample ID | Gender/age (years) | Diagnosis month/year | Previous donation month/year | Number of donations | DRM class | Number of mutations |
|---|---|---|---|---|---|---|
| BRAM_50 | Female/23 | September 2012 | 09.2011 | 4 | NTRI + NNTRI | 7 |
| BRAM_121* | Male/25 | March 2013 | 08.2012 | 8 | NNRTI | 2 |
| BRAM_137 | Male/28 | September 2011 | 04.2008 | 2 | NTRI + NNTRI | 7 |
| BRAM_243 | Male/30 | July 2012 | 09.2009 | 4 | NTRI + NNTRI | 7 |
| BRAM_251 | Male/43 | July 2012 | 02.2002 | 2 | NNRTI | 1 |
| BRAM_269 | Male/28 | August 2015 | 01.2013 | 2 | NNRTI | 1 |
| BRAM_299 | Male/52 | December 2015 | 01.2014 | 25 | NNRTI | 1 |
| BRAM_409 | Male/32 | May 2016 | 04.2012 | 3 | NTRI + NNTRI | 7 |

* Diagnosis of HIV-1 infection was performed during the immunologic window period.

**TABLE 4. Features of blood donors diagnosed during the immunologic window period by HIV NAT only at HEMOAM (2012-2017)**

| Sample ID | Sex | Age (years) | Date of diagnosis | Donor type | Donation type | HIV-1 subtype | TDR | TDR *Cluster* |
|---|---|---|---|---|---|---|---|---|
| BRAM_99 | Male | 21 | Jun 21, 2013 | First time | Voluntary | B | No | No |
| BRAM_121 | Male | 25 | Mar 15, 2013 | Repeat | Voluntary | B | NNRTI | TDRM_ I |
| BRAM_156 | Male | 27 | Apr 23, 2015 | First time | Voluntary | B | No | No |
| BRAM_302 | Male | 24 | Aug 18, 2012 | Repeat | Voluntary | B | No | No |

largest cluster comprises eight donors identified here and two individuals under ARV treatment described previously indicating transmission of drug-resistant viruses from ARV treated to untreated and among untreated individuals. These findings suggest that a proportion of the HIV-1–infected blood donors from Amazonas belong to different high-risk transmission networks including infected individuals under ARV and harboring drug resistance.

Transmission clusters of TDR can be more difficult to be identified due to the decreased replication fitness of viruses with DRM. A study with more than 80,000 RT sequences obtained worldwide showed that sequences containing DRM were less likely to participate in a cluster compared to wild type viruses.[34] Studies have also shown that sequences from recently infected individuals are more likely to cluster with sequences of other recently infected individuals, which is compatible with high viral loads and high transmissibility during acute infection.[35–37] We can speculate that our study population includes recent infection with HIV-1, a hypothesis that was corroborated by the four immunologic window cases found. One of them (BRAM_121) had DRM and participated in one of the TDR/DRM transmission clusters. The existence of a significant number of HIV-1–infected individuals among blood donors from HEMOAM represents per se a risk for blood recipients. In the 2017 national AIDS incidence rate, Manaus, Amazonas, ranked fifth among 26 capitals.[1] Therefore, in the context of a ramping AIDS epidemic, in an isolated geographic setting with moderate TDR rate, active transmission clusters of DRM highlight the potential risk of increasing TDR rate overtime.

From the public health perspective, these results raise concerns, as these blood donors were apparently healthy individuals who denied any risk factor during predonation screening and probably have HIV-1–infected sexual partners under ARV and having DRM. These donors with DRM have probably started insufficiently strong first-line ARV regimens that may have rendered them more vulnerable to treatment failure. When genotyping tests were performed in 2017, the majority of patients had already started ARV therapy. Although interesting, the clinical follow-up and outcome of donors with TDR/DRM was out of the scope of this study. While our analyses were based on the assumption of honest ARV-naive blood donors who were not aware of their HIV status, we cannot exclude the possibility of undisclosed HIV diagnosis and previous exposure to ARV therapy. However, the investigation of ARV drugs in samples was not part of this study as this possibility is a well-defined exclusion criterion for donation.

Except for one mutation associated with PI, all other antiretroviral mutations detected here were associated with NNRTI and NRTI. The most prevalent mutations to NNRTI (K103 N, E138A, G190A, and K101H) and to NRTI (revertant mutations T215S/L/D/A, D67N, T69D, and K219Q) are compatible with the extensive use of these classes of drugs in the first-line ARV regimen and with the greater persistence of these mutations in the absence of selective drug pressure.

Other Brazilian multicenter and site-specific studies using bulk viral population sequencing methods (detects variants that represent >20%) have reported TDR ranging from 5% to 15%.[3–12] These studies included recently diagnosed individuals from the general population and blood donors, mainly from more industrialized and populous regions. In Northern Brazil few studies have reported TDR prevalence: a moderate rate was shown in Tocantins (11.5%)[8] and Roraima (8.3%)[15]

states while a very low rate was reported in Amapá state (1%).[17] In Manaus, 21.4% TDR was recently described among 117 newly diagnosed ARV-naive children, in which 28.8% was previously exposed to mother-to-child ARV prophylaxis.[13] A recent nationwide study with 1,558 patients showed 9.5% TDR and the 10.2% rate reported in the Northern region is similar to our findings.[12] In 128 ARV-naive, chronically infected blood donors (from Sao Paulo, Minas Gerais, Pernambuco, and Rio de Janeiro states), a high prevalence of TDR was detected in near full-length HIV-1 genomes obtained by ultradeep sequencing, which increases the detection of DRM.[38] Therefore, the TDR rate detected here by bulk sequencing may be underestimated if compared to next-generation sequencing of full-length or near-full-length genomes that detects minority viral populations and DRM in other genomic regions. Nevertheless, the clinical impact of DRM in minority viral populations is currently controversial.[39,40] The potential impact of the recent Venezuelan migration into the local AIDS epidemic in Northern Brazil is unknown but should be monitored as it has increased poverty, malnutrition, commercial sex, and compromised the public health system in Roraima and Amazonas. In the last national AIDS incidence rank, Roraima had the highest rate in the country.[1] Our results reinforce the need to monitor TDR/DRM rates in highly endemic states as Amazonas and Roraima.

One advantage of our study was the use of residual plasma samples from donations, which eliminates the bias associated to donor return. However, we acknowledge limitations in our study such as the small sample size from Rondônia and Roraima; however, data from these states are scarce with a limited number of HIV-1 sequences of ARV-naive patients available at GenBank. The lack of information about the probable duration of the HIV-1 infection is also a constraint, but the finding of multiple mutations in a significant proportion of donors suggests recent infection. Without the pressure of ARV treatment, resistant mutants tend to revert to wild-type viruses so that TDR decays over time; however, the persistence of transmitted and acquired drug-resistant mutations in the absence of ARV drugs has been reported.[3]

Our results provide a much needed baseline data on TDR/DRM in ARV-naive individuals from a highly endemic and geographically isolated region in Northern Brazilian Amazon. Within the sentinel population of apparently healthy, HIV-1–infected blood donors from public reference hemocenters a moderate rate of TDR/DRM and transmission clusters of drug-resistant viruses were found. These findings underscore the need to monitor TDR locally and to perform pretreatment genotyping to assure the efficacy of first-line ARV regimens. Therefore, continued monitoring of TDR/DRM in this region is key for preventive strategies.

## CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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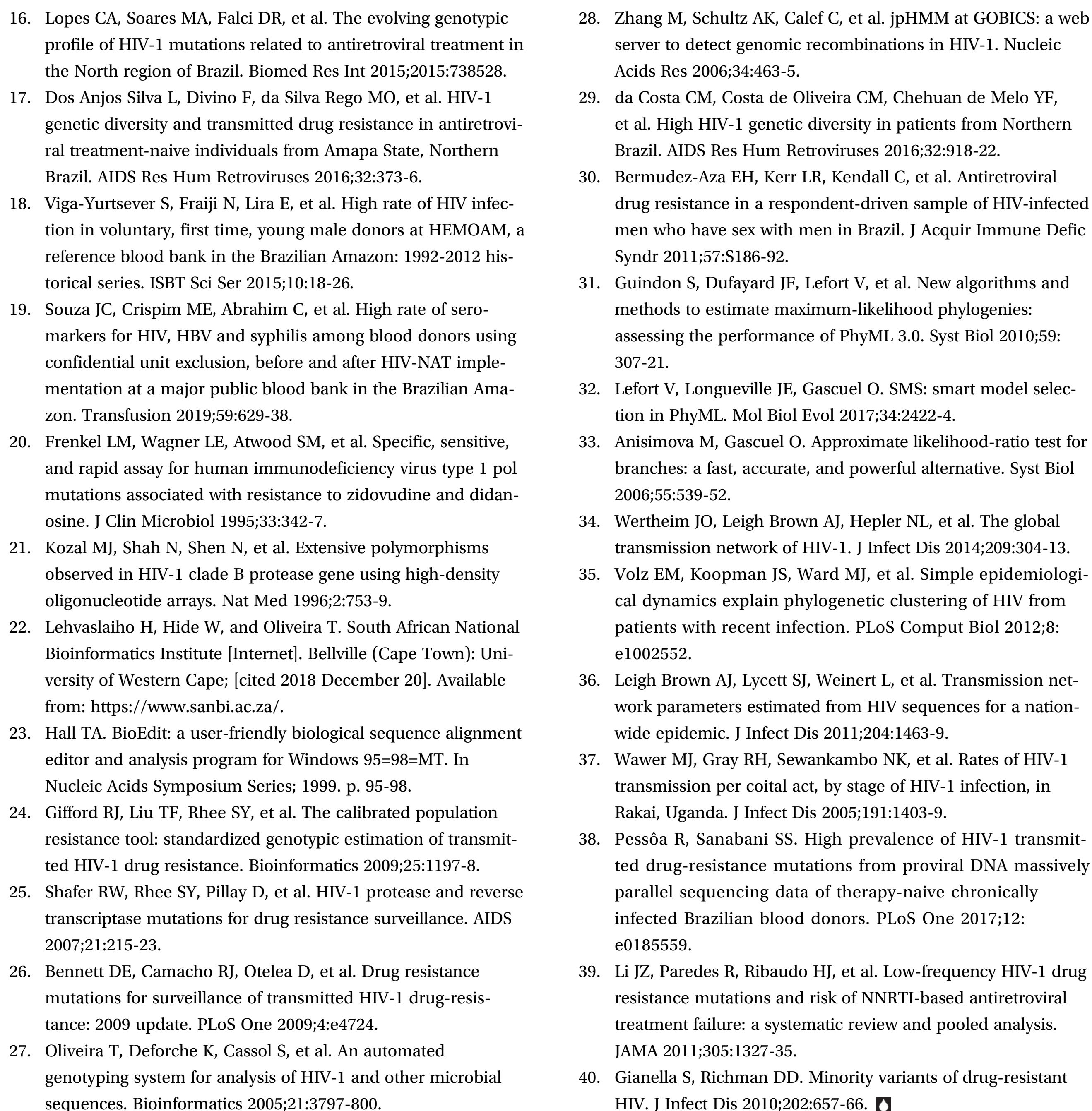


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