

A genotyping study of human immunodeficiency virus type-1 drug resistance in a small Brazilian municipality

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In Brazil, surveillance studies on antiretroviral drug resistance among drug-naïve and treatment-experienced patients have focused primarily on patients living in large urban centers. As the epidemic spreads towards small municipalities and the innermost parts of the country, it will be essential to monitor the prevalence of antiretroviral drug resistance in these areas. We report the first survey on the prevalence of antiretroviral drug resistance in a small Brazilian municipality. Between July 1999 and March 2005, 72 adult human immunodeficiency virus type-1 (HIV-1)-infected patients received care at the Municipal HIV/AIDS Program of the small, southeastern municipality of Miracema, state of Rio de Janeiro. A genotyping study of antiretroviral drug resistance was performed in 54 patients. Among 27 samples from treatment-experienced patients, 9 (33.3%) harbored strains with reduced drug susceptibility. Among these, 6 had reduced susceptibility to reverse transcriptase (RT) inhibitors and 3 to both RT and protease inhibitors. No primary antiretroviral drug resistance was recorded among 27 drug-naïve subjects. The relatively low prevalence of resistance mutations in the Miracema cohort argues against the concern that resource-poor settings should not implement widespread accessibility to standard of care antiretroviral combinations due to the possibility of sub-optimal adherence leading to the emergence and spread of drug-resistant strains.

Key words: antiretroviral resistance - genotyping - human immunodeficiency virus type-1 - small municipalities - subtypes - Brazil

Continued human immunodeficiency virus type 1 (HIV-1) replication in the presence of selective pressure of drugs targeting the reverse transcriptase (RT) and protease (PR) viral enzymes leads to the emergence of specific point mutations in the RT and PR genomic regions of the polymerase (*pol*) gene and poses one of the major obstacles to the long-term efficacy of antiretroviral therapy. Since strains harboring resistance-associated mutations to a single or to multiple antiretroviral agents can be transmitted both horizontally (Hecht et al. 1998) and vertically (Siegrist et al. 1994), it is essential to monitor the prevalence of resistant strains in the community.

In Brazil, surveillance studies on the prevalence of mutations conferring antiretroviral drug resistance are being reported both from national (Brindeiro et al. 1999, 2003, Tanuri et al. 2002) and regional (Dumans et al. 2002, Pires et al. 2004, Couto-Fernandez et al. 2005, Rodrigues et al. 2005) studies based on large urban centers. However, as the Brazilian epidemic spreads from the large urban centers towards small municipalities and the innermost parts of the country (Szwarcwald et al. 2000), appropriate studies on the features of HIV-1 infection in relatively small Brazilian communities are urgently needed. We report the results of the first survey on antiretroviral drug resistance in a small Brazilian municipality: the southeastern Miracema, in the state of Rio de Janeiro. Since Miracema is located in northwestern Rio de Janeiro, a re-

gion where municipalities are known to have low human development index values (IPEA 2003), the present study is an opportunity to analyze the prevalence of HIV-1 drug resistance in a resource-poor setting with widespread availability of standard of care highly active antiretroviral therapy (HAART) regimens.

MATERIAL AND METHODS

Patients and setting - Miracema is a small municipality in northwestern state of Rio de Janeiro (21°24'50"S; 42°11'52"W), 280 km far from the city of Rio de Janeiro, at the border of the state of Minas Gerais. A detailed clinical and epidemiological characterization of the cohort is presented elsewhere (Eyer-Silva et al. 2005). Monthly medical appointments are offered at a local ambulatory facility with a physician based in the city of Rio de Janeiro. Antiretroviral agents are freely supplied to patients when clinically indicated, as part of the national AIDS Program of the Ministry of Health. Patients were staged according to the 1993 Revised Classification System of the Centers for Disease Control and Prevention (CDC 1992). The study protocol was reviewed and approved by the Ethics Review Board at Instituto de Pesquisas Clínicas Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro.

Nucleotide sequencing - After obtaining signed informed consent, blood samples were collected from adult patients between December 2001 and March 2005. Total genomic DNA was extracted from 200 µl of buffy coat using a QIAmp Blood Kit (Qiagen Inc., Chatsworth, CA, US), according to manufacturer's instructions. DNA samples (± 1 µg) were PCR-amplified by a nested protocol in a Perkin Elmer 480 or 9600 Thermal Cycle. A fragment of the HIV-1 *pol* gene, spanning both PR and RT regions,

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was amplified in the following mixture: 5 µl of extracted DNA, 5 µl of 10× *Taq* buffer, 0.6 µl of 25mM (each) deoxynucleoside triphosphates, 5 µl of MgCl₂ 25mM, 1 µl (25 pM) of each PCR-primer, 33 µl of H₂O, and 0.5 µl of *Taq* DNA polymerase (Amersham Pharmacia Biotech Inc.). An initial cycle was performed with a denaturation temperature set at 95°C (3 min), annealing set at 55°C (1 min) and extension set at 72°C (1 min), followed by 35 cycles with denaturation at 95°C (1 min), annealing at 55° (45 s) and extension at 72°C (1 min). A final extension of 10 min was set at 72°C. For second-round PCR, a 5 µl aliquot of the first-round PCR mixture was used. Oligonucleotides DP10 (5'-TAACTCCCTCTCAGAAGCAGGAGCCG-3') and RT12 (5'-ATCAGGATGGAGTTCATAACCCATCCA-3') were used as sense and antisense outer primers, respectively. Oligonucleotides DP16 (5'-CCTCAAATCACTCTTTGGCAAC-3') and RT4 (5'-AGTTCATAACCCATCCAAAG-3') were used as sense and antisense nested primers, respectively. Amplification results were checked on agarose gel electrophoresis and ethidium bromide staining. The PCR products were then purified with the commercial Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, US) and sequenced in an automated ABI 310 or 3100 automated sequencer (Applied Biosystems, Foster City, CA, US) using primers DP16, RT4, LR49 (5'-CAATGGCCATTGACAGAAGA-3') and L51 (5'-TGTGGTAT CTAATTGAACTTCCC-3') for *pol* fragments. Sequencing reaction mixtures were assembled with BigDye Terminator v.3.0 Cycle Sequencing Reaction Kits (Applied Biosystems). Chromatogram sequencing files were inspected with Chromas 1.45 (Technelysium Ltd., Queensland, Australia) and *contigs* were assembled by using SeqMan II, included in the DNASTAR software package (Promega) (Burland 2000). Sequences were deposited on the GenBank database under the accession numbers AY929012 to AY929061 and DQ058780 to DQ058783.

Drug resistance genotyping - Previously described drug-resistance associated mutations in the PR and RT genes were sought and genotyping results were interpreted by using the drug resistance interpretation beta test from the HIV RT and PR Sequence Database, Stanford University, version 4.1 (Kantor et al. 2001).

Analysis of sequences and phylogenetic studies - For subtyping analysis, sequences were aligned against a set of reference strains from all known HIV-1 group M subtypes (gathered from the Los Alamos HIV Database: <http://hiv-web.lanl.gov>) and trimmed to equivalent lengths by using CLUSTAL X (Thompson et al. 1997). A SIVcpz sequence was used as outgroup. An alignment of 845 bp (corresponding to positions 2364 to 3198 relative to HXB2 genome, GenBank accession no. K03455) was obtained. Phylogenetic inferences were performed by the neighbor-joining (NJ) algorithm (Saitou & Nei 1987) based on a DNA distance matrix and using the F84 model of nucleotide substitution (Felsenstein 1984) implemented in PAUP* version 4.0b10 (Swofford 2002). The robustness of the trees was evaluated by bootstrap analysis (Felsenstein 1985) with 100 rounds of replication. The bootscanning method was used to detect and study re-

combination, as implemented in the SIMPLOT software, version 2.5 (Salminen et al. 1995). The analysis was performed on a sliding window of 400 nucleotides of the query sequences moving by increments of 20 nucleotides along an alignment of the reference sequences.

RESULTS

Between July 1999 and January 2005 a total of 72 HIV-1 infected adult patients (37 female) received care at the Municipal HIV-1/AIDS Program. Out of the 58 patients from whom a blood sample was available, *pol* sequences were obtained from 54 (we failed to obtain *pol* sequences from 4 samples, even after analysis of a second blood sample). Fifty samples were assigned subtype B, whereas strains M02, M08, M31, and M36 were BF1 mosaic forms (the first 3 sharing common intersubtype breakpoints). These BF1 recombinants will be further described elsewhere. No other subtypes were found.

The Table presents the demographic, epidemiological, clinical and virological data of 54 studied patients. As of sample collection, 27 patients were treatment-naïve, 23 had already been exposed to HAART and 4 were not on therapy but had been previously offered vertical transmission prophylaxis (VTP) with a combination of zidovudine, lamivudine and nevirapine. Among treatment-experienced patients, 10 were on a combination of 2 nucleoside RT inhibitors (NRTI) plus 1 non-nucleoside RT inhibitor (NNRTI), 9 were on a regimen of 2 NRTI plus 1 PR inhibitor (PI), and 4 were on a 2 NRTI plus 1 PI plus 1 NNRTI combination. A total of 29 patients were on an AIDS-defining CDC stage category (27 on stage C and 2 on stage A3).

Among the 27 treatment-exposed patients, 9 (33.3%) harbored strains with reduced susceptibility to anti-retroviral drugs. Among these, 3 had resistance mutations against NRTI and PI agents (patients M05, M11 and M12, all of whom had been exposed to PI-based regimens), 4 had mutations associated with NRTI resistance (patients M07, M14, M20, and M31), and 2 had NNRTI-associated mutations (patients M08 and M25). The most common mutations associated with NRTI were M184V (4 strains), and the thymidine-associated mutations D67N (2 cases), K70R (2 cases) and K219/E/Q (2 cases). Isolated cases of mutations T69S, M41L and L210W were also recorded. Resistance mutations to NNRTI were found in samples M08 (Y181C and G190A) and M25 (K103N). Mutations conferring reduced susceptibility to PI were found in samples M05, M11 and M12. Patients M05 and M12 had long treatment histories that included sequential use of 4 and 3 consecutive PI agents, respectively. Recorded resistance mutations in the PR gene were A71T and N88S/D (in all 3 cases). Among the 4 female patients who had previously received antiretroviral therapy for vertical transmission prophylaxis, none had reduced drug susceptibility, although sample M33 harbored the polymorphism V106I and the atypical mutation P225L, none of which are currently associated with resistance. Overall, the common polymorphisms M36I, L63P, and V77I were observed in 9 (16.6%), 34 (70%) and 13 (24%) samples, respectively. All strains recovered from the 27 treatment-naïve patients were considered susceptible to NRTI, NNRTI, and PI.

TABLE
Demographic, epidemiological, clinical and virological data from 54 adult human immunodeficiency virus type-1-infected patients followed at the Municipal HIV-1/AIDS Program of Miracema

Patient	Sex	Age	CDC stage	Year of sample	Time on therapy	Drugs used on treatment ^a	RT ^b	TR mutations	RT ^b	PR mutations	Subtype
M02	F	21	A3	2001	3.5 yr	DDI, AZT, 3TC, NFV, NVP	S	None	S	K63P, V77I	BF1
M03	F	20	A1	2005	VTP ^c	AZT, 3TC, NVP	S	None	S	L63P, V77I	B
M05	F	47	C3	2001	4.5 yr	AZT, DDI, SAQ, IND, RIT, 3TC, D4T, NFV	R	D67N, K70R, K219E	R	M36I, L63P, A71T, N88D	B
M06	F	26	C2	2001	3 mo	AZT, 3TC, NFV	S	None	S	M36I, L63P	B
M07	F	28	A2	2004	7 yr	DDI, AZT, 3TC, LOP	R	D67N, K219Q	R	L63P	B
M08	M	24	C3	2001	4.5 yr	AZT, IND, 3TC, D4T, NFV, NVP	R	Y181C, G190A	S	L10I, V77I	BF1
M11	M	27	C3	2001	2 yr	AZT, 3TC, NFV	R	M184V	R	K20I, M36I, L63P, A71T, N88S	B
M12	M	53	A2	2002	5 yr	AZT, SAQ, NFV, 3TC, D4T, LOP	R	T69S, K70R, M184V	R	M36I, L63T, A71T, N88D	B
M14	M	34	C3	2001	3 yr	AZT, DDI, D4T, 3TC, EFV	R	M41L, L210W	S	L63T, V77I	B
M16	F	35	A1	2001	VTP	AZT, 3TC, NVP	S	None	S	L63P	B
M17	M	31	C3	2001	5 yr	AZT, DDI, 3TC, D4T, EFV	R	None	S	L63P	B
M20	F	27	C3	2001	18 mo	AZT, 3TC, EFV	R	M184V	S	K20T	B
M21	F	26	A2	2001	naïve	—	S	None	S	L63P	B
M22	F	35	C3	2001	2.5 yr	DDI, AZT, 3TC, NFV	S	None	S	L63P	B
M23	F	42	A2	2001	naïve	—	S	None	S	L63T	B
M24	F	22	A1	2002	naïve	—	S	None	S	None	B
M25	F	24	A2	2003	6 yr	DDI, AZT, 3TC, D4T, NVP	R	K103N	S	L63P	B
M28	M	33	C3	2001	4 yr	AZT, DDI, SAQ, NFV, 3TC, D4T, LOP, EFV	R	None	S	L63P	B
M31	F	60	B2	2003	5 yr	AZT, DDI, RIT, D4T, 3TC, NFV, EFV	S	M184V	R	L63P, V77I	BF1
M33	F	27	A1	2001	VTP	AZT, 3TC, NVP	S	V106I, P225L	S	M36I, L63P	B
M34	F	16	A1	2004	VTP	AZT, 3TC, NVP	S	None	S	L63P	B
M36	F	38	A3	2002	1 yr	AZT, 3TC, EFV	S	None	S	M36I, D60E, V77I	BF1
M37	M	30	A1	2002	naïve	—	S	None	S	L63P	B
M38	F	57	B2	2002	naïve	—	S	None	S	L63P	B
M39	F	30	C3	2001	naïve	—	S	None	S	None	B
M40	F	24	A2	2001	naïve	—	S	None	S	None	B
M41	F	26	C3	2001	naïve	—	S	None	S	None	B
M42	M	32	C3	2001	2 mo	AZT, 3TC, EFV	S	V118L	S	L63P, I93L	B
M43	M	24	A1	2002	naïve	—	S	None	S	M36I, L63P	B
M44	F	33	A1	2002	naïve	—	S	None	S	M36I, L63P	B
M45	F	22	A1	2002	naïve	—	S	None	S	M36I, L63P	B
M46	M	37	C3	2002	6 mo	AZT, 3TC, EFV	S	None	S	L63P, A71T	B
M47	M	29	C2	2002	naïve	—	S	None	S	L33F, L63T, V77I	B
M48	M	41	C3	2002	3 mo	AZT, D4T, 3TC, EFV	S	None	S	L63P, V77I, I93L	B
M49	M	30	C3	2002	3 mo	AZT, 3TC, EFV	S	None	S	L63P, A71T	B
M50	F	20	B2	2002	naïve	—	S	None	S	L63T, V77I	B
M51	M	23	A2	2002	naïve	—	S	None	S	None	B
M52	M	25	C3	2002	naïve	—	S	None	S	None	B
M53	M	31	C3	2002	naïve	—	S	None	S	L63P, I93L	B ↓

Patient	Sex	Age	CDC stage	Year of sample	Time on therapy	Drugs used on treatment ^a	RT ^b	TR mutations	RT ^b	PR mutations	Subtype
M54	F	28	C3	2002	naïve	—	S	None	S	D60E, L63P	B
M55	M	38	C2	2003	naïve	—	S	None	S	L63P	B
M56	F	32	C2	2003	naïve	—	S	None	S	L10I, L63P, I93L	B
M57	F	25	A2	2003	naïve	—	S	None	S	L10V, D60E, L63P	B
M58	M	48	A1	2003	naïve	—	S	None	S	D60E, L63P	B
M61	F	26	A2	2004	naïve	—	S	None	S	L63P, V77I	B
M62	M	63	C3	2004	18 mo	AZT, D4T, 3TC, LOP	S	T69S	S	L63P	B
M63	M	31	C3	2004	1 yr	AZT, 3TC, LOP	S	None	S	D60E, L63P	B
M64	F	26	C3	2004	5 mo	AZT, D4T, 3TC, EFV	S	None	S	None	B
M65	M	33	A2	2004	naïve	—	S	None	S	None	B
M66	F	46	C2	2004	2 mo	AZT, 3TC, LOP	S	None	S	L63A	B
M67	F	27	C3	2004	naïve	—	S	None	S	L53A, A71T	B
M68	M	27	C3	2005	naïve	—	S	None	S	L63P, V77I	B
M69	M	43	C3	2005	naïve	—	S	None	S	V77I	B
M70	M	22	A2	2005	naïve	—	S	None	S	L63P, V77I, I93L	B

a: drugs used as of sampling time are displayed in boldface, italic type; AZT (zidovudine); DDI (didanosine); 3TC (lamivudine); D4T (stavudine); NVP (nevirapine); EFV (efavirenz); SAQ (saquinavir); IND (indinavir); RT (ritonavir); NFV (nelfinavir); LOP (co-formulation of lopinavir and ritonavir); *b*: drug resistance beta test interpretation result for the RT and RT genes: S (susceptible); R (reduced susceptibility); *c*: patients who were not on antiretroviral therapy as of sampling but who had been submitted to vertical transmission prophylaxis (VTP) with AZT, 3TC, and NVP.

DISCUSSION

Little is known about the features of HIV-1 infection in small Brazilian municipalities and rural areas. As the epidemic spreads from the large urban centers towards small municipalities and the innermost parts of the country (Szwarcwald et al. 2000, Brito et al. 2001), there is an urgent need to study the clinical, epidemiological and virological characteristics of HIV-1 infection in these settings. These studies will be of utmost importance to optimize the institution of adequate control measures, grasp the true magnitude of the problem, improve clinical recognition and management of HIV-1 infection and better allocate resources.

The present report is the first survey of antiretroviral drug resistance in a small Brazilian municipality. Like all similar studies in the field, it should be interpreted with the understanding that resistance testing is insensitive to minor drug-resistant variants and thus may fail to detect strains resistant to previously used drugs and to detect decayed resistant variants transmitted to untreated subjects.

Brazilian surveillance studies on the prevalence of resistance mutations in treatment-experienced patients have reported variable prevalence figures of reduced susceptibility to antiretroviral agents. Mutation M184V, for instance, associated with high-level resistance to the NRTI lamivudine, has been recently reported in 67.7, 64, and 18% of patients failing HAART in the states of Rio de Janeiro (Couto-Fernandez et al. 2005), São Paulo (Rodrigues et al. 2005), and the Federal District (Cerqueira et al. 2004), respectively. Prevalence figures of resistance mutations are to be interpreted taking into consideration current local prescribing patterns, as well as recommendations, eligibility and access to HIV resistance testing for patient management.

In the present Miracema cohort, we found an overall low prevalence of reduced susceptibility to antiretroviral agents among 27 treatment-exposed patients. We recorded 9 samples with reduced susceptibility against RT inhibitors and 3 against PI. The prevalence of thymidine-associated mutations (4 cases) and of the M184V mutation (4 cases) clearly reflects the frequency with which combinations that include thymidine analogues (zidovudine and stavudine) and lamivudine are prescribed. Both patients with NNRTI-associated mutations had been treated with the NNRTI nevirapine, not efavirenz. Rapid emergence of high-level resistance is known to be a potential drawback of nevirapine-including regimens due to the drug's low genetic barrier. No case of dual NRTI-NNRTI was recorded. Mutation T215Y/F, known to confer resistance to zidovudine and stavudine, was also not observed.

Among samples from 27 drug-naïve subjects, none had resistance mutations. These results corroborate findings from regional (Dumans et al. 2002) and national (Brindeiro et al. 1999, 2003) surveillance studies that reported a low prevalence of primary resistance in Brazil. Since antiretroviral drugs are readily available in Miracema since the early 1990s, our results seem to argue against the concern that resource-poor settings should not implement widespread accessibility to standard of care HAART

combinations due to the possibility of sub-optimal adherence leading to the emergence and spread of drug-resistant strains. Continued surveillance studies on the prevalence of drug resistant strains among treatment naïve and experienced patients will be necessary in large urban centers. However, as the Brazilian epidemic spreads towards the innermost parts of the country, additional investigations will also be needed in these settings.

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