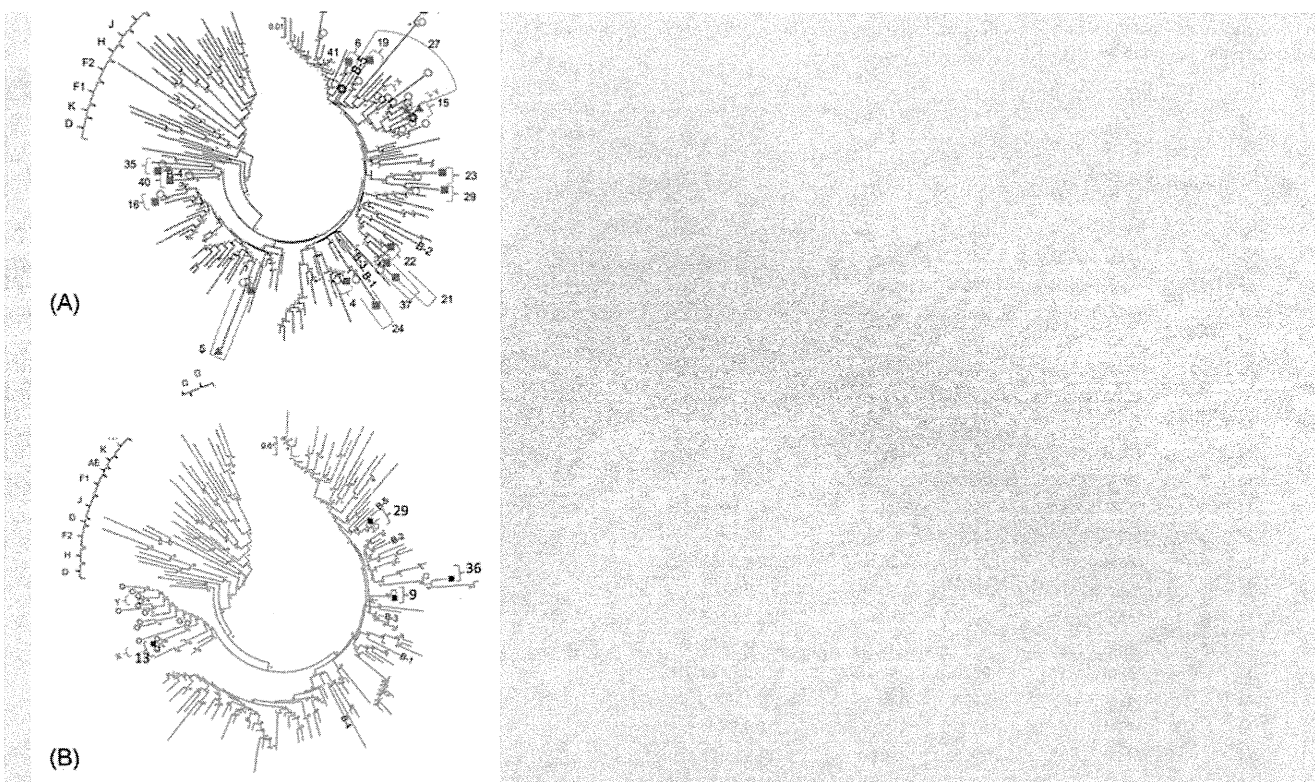

Figure 4. Phylogenetic tree of samples with or without minority variants of M46I/L or L90M drug resistance.
A neighbor-joining phylogenetic tree of all protease sequences using the Kimura 2-parameter model was generated in MEGA5. Numbers shown are IDs of patients with detectable minority drug resistance. Open circles (black color) are virus with M46I detected by bulk sequencing. A. Solid squares (red color) indicate sequences of M46I-specific amplicons and solid triangle (red color) indicate sequences of M46L amplicons; open circles (red color) indicate bulk sequences for persons with minority M46I/L mutations; X and Y are pairs of closely related transmitted M46I sequences. B. Solid squares indicate L90M-specific amplicon sequences; open squares indicate bulk sequences for persons with minority L90M mutations. Open circles (black color) are virus with M46I detected by bulk sequencing. Abbreviations of subtype B references: B-1; B.NL.00.671 00T36.AY423387, B-2; B.US.98.1058 11.AY331295, B-3; B.FR.83.HXB2 LAI IIIB BRU.K03455, B-4; B.TH.90.BK132.AY173951, B-5; B.US.98.15384 1.DQ853463.
doi:10.1371/journal.pone.0083150.g004

Discussion

In this study, we used a highly sensitive method to screen for minority drug-resistant populations in 149 cases of newly diagnosed HIV-infected patients. An additional six drug resistance mutations in the RT region and thirty drug resistance mutations in PR region were detected as minority-level drug resistance mutations. For the ten codons associated with resistance (RTI mutations: M41L, K65R, K70R, K103N, Y181C, M184V and T215F/Y, PI mutations: M46I/L and L90M) the prevalence of detectable drug resistance mutations increased from 15.4% to 26.8% using the highly sensitive assays. A previous surveillance study in Japan using bulk sequencing reported that in 2008 the prevalence of transmitted drug resistance was 8.3% [21]. Therefore, drug resistance mutation surveillance analyzed by bulk sequencing underestimates transmitted drug resistance, which potentially has both clinical and epidemiologic implications.

The epidemiologic implications of increased transmitted resistance may reflect prevention failures in persons who know they are infected and transmit HIV to their partners. The clinical implications of minority drug resistance center around the impact of these viruses on ART responses. Previous studies have reported that minority NNRTI-resistant variants are associated with increased risk of virologic failure in patients receiving first-line NNRTI-based ART regimens [20,26-32]. These findings are important because NNRTI resistance is the most commonly transmitted resistance in the US and Europe [33,34]. However, no evidence of either majority or minority NNRTI-resistance was found in this study, a unique finding that is explained by the historically infrequent use of NNRTIs in ART regimens in Japan. Instead, we note that the NRTI K65R and M184V mutations were both detected as minority populations. As major mutations K65R and M184V reduce the clinical efficacy of TDF and 3TC/FTC, respectively [35-37], however their clinical impact as minority mutations is not fully clear.

A previous study demonstrated no impact on therapy responses in patients who had minority-level K65R and M184V mutations when provided regimens that included protease inhibitors; however; because of the small number of patients representing different treatment regimens in that study, the bearing of these mutations could not be fully evaluated [29]. Additional studies are needed to assess clinically significant frequencies of different NRTI-resistant variants on various treatment regimens. In the present study, one of the 149 cases evaluated possessed six drug resistance mutations as minority variants (Table 2). Genotype interpretation by the Stanford HIV Drug Resistance Database showed that the patient possessed high-level resistance to NRTIs and some PIs. It was not possible to follow the clinical course of this patient to elucidate the significance of minority variants on subsequent cART.A major finding in this study was the high prevalence of transmitted PI resistance (20%) which accounted for about two-thirds the overall transmitted resistance. The high prevalence of transmitted PI resistance is supported both by detection at majority as well as minority variant levels, the latter comprising more than half of the transmitted PI cases. The high prevalence of PI resistance can be explained by the longstanding and predominant use of PI-based regimens in Japan, including darunavir and atazanavir in both first-line and second-line regimens

Genetic linkage analysis provided more insights into the composition of the viral population by showing that L90M, M46I and M46L in many patients existed on separate viral genomes. The capacity to identify linked mutations could be important for understanding the persistence [38] and clinical impact of mutant variants. A major factor that influences the persistence of drug-resistant mutants *in vivo* is their relative replicative capacity within the viral population. *In vitro* competition experiments conducted in the absence of drugs have shown that drug resistance mutations impair replicative fitness by different degrees. For instance, the M46I, the K70R, the 215 intermediate mutations have a lesser impact on fitness than L90M, K65R, and M184V [38-40], and, thus, such mutations are likely to persist longer *in vivo*. Moreover, accumulation of compensatory mutations such as L63P and A71V in protease have been demonstrated to increase or restore replicative fitness of PI resistant variants, and that once compensation has taken place reversion to wildtype is prohibited by a less fit intermediate [41]. This may explain, for example, the high prevalence of M46I we detected in this study as bulk and minority species.

Phylogenetic analysis showed the sequences of minority M46I/L and L90M-positive amplicons were closely related to their source bulk sequences, supporting that the

minority sequences detected were unique to the respective patients and were not the result of contamination. A few cases possessing the M46I mutation by bulk sequencing demonstrated a strongly supported identity which was not biased by including the resistance mutations in the analysis. However, with regard to minority M46I variants, we found they did not cluster closely with viruses from persons with majority-level M46I. This suggested that at least two pairs with majority-level M46I were phylogenetically related whereas those with minority M46I were scattered among the transmitted virus population, unrelated to any of the other cases in our analysis. While the sequence lengths used in the analysis might limit our ability to draw robust bootstrap values deeper in the tree nodes, the sequences for the pairs within the two clusters were identical with the exception of a few positions with mixed bases, and were further supported by high bootstraps.

The ability to conduct surveillance of minority-level drug resistance mutations is an important advancement to help understand transmission of HIV drug resistance in Japan. The finding from our select analysis of mutations of interest cannot be extrapolated to all codons associated with drug resistance; however, these results suggest that a substantial proportion of drug resistance-associated mutations are persist at low levels by the time HIV-infected persons are diagnosed and genotyped. Using an approach that can more broadly identify variants, such as next-generation sequencing [42-45], may identify other mutations that would further increase the prevalence of drug resistance. However, because the more commonly transmitted mutations are often targeted by AS-PCR, any additional increase in mutation prevalence identified by the more complex methods may be nominal. Hence, the lower cost and simplicity of AS-PCR offer advantages for routine surveillance, particularly when the sample burden may be high.

In conclusion, the relationship between minority drug resistance mutations and cART failure requires further exploration; nevertheless, the findings point to difficulties in getting infected persons diagnosed early and counseled to prevent forward spread of drug resistance.

Acknowledgments

Disclaimer: The findings and conclusions presented in this manuscript are those of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention. We thank William Switzer for assistance with the phylogenetic analysis.

Author Contributions

Conceived and designed the experiments: WS JJ WH. Performed the experiments: MN JH. Analyzed the data: MN JH ST WS. Contributed reagents/materials/analysis tools: JJ WH WS TM. Wrote the manuscript: MN JJ WH WS.

Supporting Information

Table_S1.doc

Table S1. Oligonucleotide sequence Proportion(RTI mutations)

	Primer	Oligonucleotide sequence	Proportion
Total copy reaction	ComFWD	5'-CTT CTG GGA AGT TCA ATT AGG AAT ACC	
	ComREV	5'-TGG TGT CTC ATT GTT TRT ACT AGG TA	
	Com 1P	5'-***FAM***-TGG ATG TGG GTG A"T"G CAT ATT TY T CAR TTC CCT TA	60%
	Com 2P	5'-***FAM***-TAC TGG ATG "T" GGG TGA TGC ATA TTT TTC ART TCC CTT A	40%
Mutation			
Protease			
L90M	Rev1	5'-GAA AAT TTA AAG TGC AAC CAA KTT GAG TGA T	
	Fwd	5'-AGA TCA CTC TTT GGC AAC GAC C	
	P1	5'-***FAM***-TAG GGG GAA "T" TG GAG GTT TTR TCA AAG TAA GAC AGT AT	
Reverse transcriptase			
M41L	F1	5'-AAT AAA AGC ATT ART RGA AAT YTG TRC AGC AT	35%
	F2	5'-AAT WAA AGC ATT ART RGA AAT YTG TRC WGC AT	10%
	F3	5'-AAA AGC ATT ART RGA AAT YTG TRC AGG AC	32%
	F4	5'-TAA AAG CAT TAR TRG AAA TYT GTR CAK GTC	13%
	F5	5'-AAG CAT TAR TRG AAA TYT GTR CAK GGC	10%
	Rev	5'-CCT AAT TGA ACT TCC CAG AAG TCT TG	
	41-70p	5'-***FAM***-TTG GGC CTG AAA A"T"C CAT ACA ATA CTC CAG TAT TT	
K65R	F1	5'- ACA ATA CTC CAR TAT TTG CCA TAA RCA G	
	Rev	5'-CCT GGT GTC TCA TTG TTT ATA CTA GGT	
	K65R-P1	5'-***FAM***- TCA GAG AAC "T" TAA TAA RAG AAC TCA AGA CTT CTG GGA	80%
	K65R-P2	5'-***FAM***-TCA GAG AAC "T" CAA TAA GAG AAC TCA AGA CTT CTG GGA	20%
K70R	Rev1	5'- GTT CTC TRA AAT CTA Y TA WTT TTC TCC CTC	70%
	Rev2	5'-TTC TCT RAA ATC TAY TAW TTT TCT CCC CC	30%
	Fwd	5'- AGA RAT TTG TAC AGA RAT GGA AAA GGA AG	
	41-70p	5'-***FAM***-TTG GGC CTG AAA A"T"C CAT ACA ATA CTC CAG TAT TT	

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Oligonucleotide sequence Proportion(RTI mutations).

Table S1.
Oligonucleotide sequence Proportion(RTI mutations).
doi:10.1371/journal.pone.0083150.s001
(DOC)

Table S2.
Oligonucleotide sequence Proportion(PI mutations).
doi:10.1371/journal.pone.0083150.s002
(DOC)

Table S3.
Assay ΔCt measures, cutoffs and sensitivities on clinical samples.
doi:10.1371/journal.pone.0083150.s003
(DOC)

References

1. Egger M, Hirschel B, Francioli P, Sudre P, Wirz M et al. (1997) Impact of new antiretroviral combination therapies in HIV infected patients in Switzerland: prospective multicentre study. Swiss HIV Cohort Study. BMJ 315: 1194-1199. doi:10.1136/bmj.315.7117.1194. PubMed: 9393221.
   View Article • PubMed/NCBI • Google Scholar

2. Sterne JA, Hernán MA, Ledergerber B, Tilling K, Weber R et al. (2005) Long-term effectiveness of potent antiretroviral therapy in preventing AIDS and death: a prospective cohort study. Lancet 366: 378-384. doi:10.1016/S0140-6736(05)67022-5. PubMed: 16054937.
   View Article • PubMed/NCBI • Google Scholar

3. Coffin JM (1995) HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science 267: 483-489. doi:10.1126/science.7824947. PubMed: 7824947.
   View Article • PubMed/NCBI • Google Scholar

4. Martinez-Picado J, Martínez MA (2008) HIV-1 reverse transcriptase inhibitor resistance mutations and fitness: a view from the clinic and ex vivo. Virus Res 134: 104-123. doi:10.1016/j.virusres.2007.12.021. PubMed: 18289713.
   View Article • PubMed/NCBI • Google Scholar

5. Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents. Available: http://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf.

6. Günthard HF, Wong JK, Ignacio CC, Havlir DV, Richman DD (1998) Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. AIDS Res Hum Retroviruses 14: 869-876. doi:10.1089/aid.1998.14.869. PubMed: 9671215.
   View Article • PubMed/NCBI • Google Scholar

7. Leitner T, Halapi E, Scarlatti G, Rossi P, Albert J et al. (1993) Analysis of heterogeneous viral populations by direct DNA sequencing. BioTechniques 15: 120-127. PubMed: 8363827.
   View Article • PubMed/NCBI • Google Scholar

8. Collins JA, Thompson MG, Paintsil E, Ricketts M, Gedzior J et al. (2004) Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. J Virol 78: 603-611. doi:10.1128/JVI.78.2.603-611.2004. PubMed: 14694092.
   View Article • PubMed/NCBI • Google Scholar

9. Ghosn J, Pellegrin I, Goujard C, Deveau C, Viard JP et al. (2006) HIV-1 resistant strains acquired at the time of primary infection massively fuel the cellular reservoir and persist for lengthy periods of time. AIDS 20: 159-170. doi:10.1097/01.aids.0000199820.47703.a0. PubMed: 16511408.
   View Article • PubMed/NCBI • Google Scholar

10. Goudsmit J, De Ronde A, Ho DD, Perelson AS (1996) Human immunodeficiency virus fitness in vivo: calculations based on a single zidovudine resistance mutation at codon 215 of reverse transcriptase. J Virol 70: 5662-5664. PubMed: 8764084.
    View Article • PubMed/NCBI • Google Scholar

11. Halvas EK, Aldrovandi GM, Balfe P, Beck IA, Boltz VF et al. (2006) Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. J Clin Microbiol 44: 2612-2614. doi:10.1128/JCM.00449-06. PubMed: 16825395.
    View Article • PubMed/NCBI • Google Scholar

12. Schuurman R, Brambilla D, de Groot T, Huang D, Land S et al. (2002) Underestimation of HIV type 1 drug resistance mutations: results from the ENVA-2 genotyping proficiency program. AIDS Res Hum Retroviruses 18: 243-248. doi:10.1089/088922202753472801. PubMed: 11860670.
    View Article • PubMed/NCBI • Google Scholar

13. Stürmer M, Morgenstern B, Staszewski S, Doerr HW (2002) Evaluation of the LiPA HIV-1 RT assay version 1: comparison of sequence and hybridization based genotyping systems. J Clin Virol 25 Suppl 3: S65-S72. doi:10.1016/S1386-6532(02)00190-7. PubMed: 12467779.
    View Article • PubMed/NCBI • Google Scholar

14. Villahermosa ML, Beck I, Pérez-Alvarez L, Contreras G, Frenkel LM et al. (2001) Detection and quantification of multiple drug resistance mutations in HIV-1 reverse transcriptase by an oligonucleotide ligation assay. J Hum Virol 4: 238-248. PubMed: 11907381.
    View Article • PubMed/NCBI • Google Scholar

15. Halvas EK, Wiegand A, Boltz VF, Kearney M, Nissley D et al. (2010) Low frequency nonnucleoside reverse-transcriptase inhibitor-resistant variants contribute to failure of efavirenz-containing regimens in treatment- experienced patients. J Infect Dis 201: 672-680. PubMed: 20102272.
    View Article • PubMed/NCBI • Google Scholar

16. Hance AJ, Lemiale V, Izopet J, Lecossier D, Joly V et al. (2001) Changes in human immunodeficiency virus type 1 populations after treatment interruption in patients failing antiretroviral therapy. J Virol 75: 6410-6417. doi:10.1128/JVI.75.14.6410-6417.2001. PubMed: 11413308.
    View Article • PubMed/NCBI • Google Scholar

17. Johnson JA, Li JF, Morris L, Martinson N, Gray G et al. (2005) Emergence of drug-resistant HIV-1 after intrapartum administration of single-dose nevirapine is substantially underestimated. J Infect Dis 192: 16-23. doi:10.1086/430741. PubMed: 15942889.
    View Article • PubMed/NCBI • Google Scholar

18. Jourdain G, Ngo-Giang-Huong N, Le Coeur S, Bowonwatanuwong C, Kantipong P, et al. (2004) Intrapartum exposure to nevirapine and subsequent maternal responses to nevirapine-based antiretroviral therapy. N Engl J Med 351: 229-240. doi: 10.1056/nejmoa041305
    View Article • PubMed/NCBI • Google Scholar

19. Shi C, Eshleman SH, Jones D, Fukushima N, Hua L et al. (2004) LigAmp for sensitive detection of single-nucleotide differences. Nat Methods 1: 141-147. doi:10.1038/nmeth713. PubMed: 15782177.
    View Article • PubMed/NCBI • Google Scholar

20. Johnson JA, Li JF, Wei X, Lipscomb J, Irlbeck D et al. (2008) Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. PLoS Med 5: e158. doi:10.1371/journal.pmed.0050158. PubMed: 18666824.

View Article • PubMed/NCBI • Google Scholar

21. Hattori J, Shiino T, Gatanaga H, Yoshida S, Watanabe D et al. (2010) Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: nationwide surveillance from 2003 to 2008 in Japan. Antiviral Res 88: 72-79. doi:10.1016/j.antiviral.2010.07.008. PubMed: 20692295.
View Article • PubMed/NCBI • Google Scholar

22. Bennett DE, Camacho RJ, Otelea D, Kuritzkes DR, Fleury H et al. (2009) Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PLOS ONE 4: e4724. doi:10.1371/journal.pone.0004724. PubMed: 19266092.
View Article • PubMed/NCBI • Google Scholar

23. Johnson JA, Li JF, Wei X, Lipscomb J, Bennett D et al. (2007) Simple PCR assays improve the sensitivity of HIV-1 subtype B drug resistance testing and allow linking of resistance mutations. PLOS ONE 2: e638. doi:10.1371/journal.pone.0000638. PubMed: 17653265.
View Article • PubMed/NCBI • Google Scholar

24. Boltz VF, Maldarelli F, Martinson N, Morris L, McIntyre JA et al. (2010) Optimization of allele-specific PCR using patient-specific HIV consensus sequences for primer design. J Virol Methods 164: 122-126. doi:10.1016/j.jviromet.2009.11.025. PubMed: 19948190.
View Article • PubMed/NCBI • Google Scholar

25. Tamura K, Peterson D, Peterson N, Stecher G, Nei M et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731-2739. doi:10.1093/molbev/msr121. PubMed: 21546353.
View Article • PubMed/NCBI • Google Scholar

26. Geretti AM, Fox ZV, Booth CL, Smith CJ, Phillips AN et al. (2009) Low-frequency K103N strengthens the impact of transmitted drug resistance on virologic responses to first-line efavirenz or nevirapine-based highly active antiretroviral therapy. J Acquir Immune Defic Syndr 52: 569-573. doi:10.1097/QAI.0b013e3181ba11e8. PubMed: 19779307.
View Article • PubMed/NCBI • Google Scholar

27. Goodman DD, Zhou Y, Margot NA, McColl DJ, Zhong L et al. (2011) Low level of the K103N HIV-1 above a threshold is associated with virological failure in treatment-naive individuals undergoing efavirenz-containing therapy. AIDS 25: 325-333. doi:10.1097/QAD.0b013e3283427dcb. PubMed: 21157296.
View Article • PubMed/NCBI • Google Scholar

28. Metzner KJ, Giulieri SG, Knoepfel SA, Rauch P, Burgisser P et al. (2009) Minority quasispecies of drug-resistant HIV-1 that lead to early therapy failure in treatment-naive and -adherent patients. Clin Infect Dis 48: 239-247. doi:10.1086/595703. PubMed: 19086910.
View Article • PubMed/NCBI • Google Scholar

29. Metzner KJ, Rauch P, Braun P, Knechten H, Ehret R et al. (2011) Prevalence of key resistance mutations K65R, K103N, and M184V as minority HIV-1 variants in chronically HIV-1 infected, treatment-naive patients. J Clin Virol 50: 156-161. doi:10.1016/j.jcv.2010.10.001. PubMed: 21056001.
View Article • PubMed/NCBI • Google Scholar

30. Paredes R, Lalama CM, Ribaudo HJ, Schackman BR, Shikuma C et al. (2010) Pre-existing minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure. J Infect Dis 201: 662-671. PubMed: 20102271.
View Article • PubMed/NCBI • Google Scholar

31. Simen BB, Simons JF, Hullsiek KH, Novak RM, Macarthur RD et al. (2009) Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretrovira treatment-naive patients significantly impact treatment outcomes. J Infect Dis 199: 693-701. doi:10.1086/596736. PubMed: 19210162.
View Article • PubMed/NCBI • Google Scholar

32. Li JZ, Paredes R, Ribaudo HJ, Svarovskaia ES, Metzner KJ et al. (2011) Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. JAMA 305: 1327-1335. doi:10.1001/jama.2011.375. PubMed: 21467286.
View Article • PubMed/NCBI • Google Scholar

33. Wheeler WH, Ziebell RA, Zabina H, Pieniazek D, Prejean J et al. (2010) Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. AIDS 24: 1203-1212. doi:10.1097/QAD.0b013e3283388742. PubMed: 20395786.
View Article • PubMed/NCBI • Google Scholar

34. Wittkop L, Günthard HF, de Wolf F, Dunn D, Cozzi-Lepri A et al. (2011) Effect of transmitted drug resistance on virological and immunological response to initial combination antiretroviral therapy for HIV (EuroCoord-CHAIN joint project): a European multicohort study. Lancet Infect Dis 11: 363-371. doi:10.1016/S1473-3099(11)70032-9. PubMed: 21354861.
View Article • PubMed/NCBI • Google Scholar

35. Wainberg MA, Turner D (2004) Resistance issues with new nucleoside/nucleotide backbone options. J Acquir Immune Defic Syndr 37 Suppl 1: S36-S43. doi:10.1097/01.qai.0000137005.63376.6e. PubMed: 15319668.
View Article • PubMed/NCBI • Google Scholar

36. Borroto-Esoda K, Parkin N, Miller MD (2007) A comparison of the phenotypic susceptibility profiles of emtricitabine and lamivudine. Antivir Chem Chemother 18 297-300. PubMed: 18046962.
View Article • PubMed/NCBI • Google Scholar

37. Ly JK, Margot NA, MacArthur HL, Hung M, Miller MD et al. (2007) The balance between NRTI discrimination and excision drives the susceptibility of HIV-1 RT mutants K65R, M184V and K65r+M184V. Antivir Chem Chemother 18: 307-316. PubMed: 18320935.
View Article • PubMed/NCBI • Google Scholar

38. Cong ME, Heneine W, García-Lerma JG (2007) The fitness cost of mutations associated with human immunodeficiency virus type 1 drug resistance is modulated by mutational interactions. J Virol 81: 3037-3041. doi:10.1128/JVI.02712-06. PubMed: 17192300.
View Article • PubMed/NCBI • Google Scholar

39. Martinez-Picado J, Savara AV, Shi L, Sutton L, D'Aquila RT (2000) Fitness of human immunodeficiency virus type 1 protease inhibitor-selected single mutants. Virology 275: 318-322. doi:10.1006/viro.2000.0527. PubMed: 10998332.
View Article • PubMed/NCBI • Google Scholar

40. Martinez-Picado J, Savara AV, Sutton L, D'Aquila RT (1999) Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1.

J Virol 73: 3744-3752. PubMed: 10196268.
View Article • PubMed/NCBI • Google Scholar

41. van Maarseveen NM, Wensing AM, de Jong D, Taconis M, Borleffs JC et al. (2007) Persistence of HIV-1 variants with multiple protease inhibitor (PI)-resistance mutations in the absence of PI therapy can be explained by compensatory fixation. J Infect Dis 195: 399-409. doi:10.1086/510533. PubMed: 17205479.
View Article • PubMed/NCBI • Google Scholar

42. Avidor B, Girshengorn S, Matus N, Talio H, Achsanov S et al. (2013) Evaluation of a benchtop HIV ultradeep pyrosequencing drug resistance assay in the clinical laboratory. J Clin Microbiol 51: 880-886. doi:10.1128/JCM.02652-12. PubMed: 23284027.
View Article • PubMed/NCBI • Google Scholar

43. Messiaen P, Verhofstede C, Vandenbroucke I, Dinakis S, Van Eygen V et al. (2012) Ultra-deep sequencing of HIV-1 reverse transcriptase before start of an NNRTI-based regimen in treatment-naive patients. Virology 426: 7-11. doi:10.1016/j.virol.2012.01.002. PubMed: 22305619.
View Article • PubMed/NCBI • Google Scholar

44. Delobel P, Saliou A, Nicot F, Dubois M, Trancart S et al. (2011) Minor HIV-1 variants with the K103N resistance mutation during intermittent efavirenz-containing antiretroviral therapy and virological failure. PLOS ONE 6: e21655. doi:10.1371/journal.pone.0021655. PubMed: 21738752.
View Article • PubMed/NCBI • Google Scholar

45. Dudley DM, Chin EN, Bimber BN, Sanabani SS, Tarosso LF et al. (2012) Low-cost ultra-wide genotyping using Roche/454 pyrosequencing for surveillance of HIV drug resistance. PLOS ONE 7: e36494. doi:10.1371/journal.pone.0036494. PubMed: 22574170.
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ORIGINAL

Development and application of a simple LC-MS method for the determination of plasma rilpivirine (TMC-278) concentrations

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Abstract : Rilpivirine (TMC-278) is a second-generation non-nucleoside reverse transcriptase inhibitor that is high potent against both wild-type and drug-resistant HIV-1 strains. Therefore, rilpivirine is expected to treat therapy-experienced patients who failed to use current drugs due to the emergence of drug-resistant HIV mutants. The quantification of rilpivirine in human plasma is important to support clinical studies and determine pharmacokinetic parameters of rilpivirine in HIV-1 infected patients. Consequently, simple and easy system to determine plasma rilpivirine concentrations has been required. In this study, we developed a conventional LC-MS method to quantify plasma rilpivirine. Subsequently the method was validated by estimating the precision and accuracy for inter- and intraday analysis in the concentration range of 18-715 ng/ml. The calibration curve was linear in this range. Average accuracy ranged from 100.0 to 100.6%. Relative standard deviations of both inter- and intraday assays were less than 3.3%. Recovery of rilpivirine was more than 82.0%. These results demonstrate that our LC-MS method provides a conventional, accurate and precise way to determine rilpivirine in human plasma. This method can be used in routine clinical application for HIV-1 infected patients, and permits management of drug interactions and toxicity for rilpivirine. J. Med. Invest. 60 : 35-40, February, 2013

Keywords : *rilpivirine, LC-MS, HIV, therapeutic drug monitoring*

INTRODUCTION

The clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection has been advanced by the success of highly active antiretroviral therapy. The latest treatment guidelines recommend regimen including efavirenz, a first-generation non-nucleoside reverse transcriptase inhibitor (NNRTI), as one of the standard first-line regimen (1). However, efavirenz use is limited by low genetic barrier to resistance and central nervous system toxity (2, 3). Therefore, new antiretroviral drugs, which have long-term efficacy and good tolerability, are required to continue effective therapy for the treatment of HIV-1.

Received for publication August 20, 2012 ; accepted September 19, 2012.

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Rilpivirine (TMC-278) is a second-generation NNRTI that is high potent against both wild-type and drug-resistant HIV-1 strains (4). Consequently, rilpivirine is expected to treat therapy-experienced patients who failed to use current drugs due to the emergence of drug-resistant HIV mutants. In addition, rilpivirine shows a favourable safety profile (5-7). The recommended dose of rilpivirine is 25 mg (one tablet) once daily in combination with other antiretroviral agents. No dose adjustment is required in patients with moderate hepatic or renal impairment. However, rilpivirine is primarily metabolized by cytochrome P450 (CYP)3A. Therefore, co-administration of rilpivirine and CYP3A inducer may result in decreased plasma concentrations of rilpivirine, loss of virologic response, and possible resistance to rilpivirine. To avoid these risks, therapeutic drug monitoring of rilpivirine is essential.

Else *et al.* (8) recently determined plasma rilpivirine concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS). However, more simple and easy system to determine plasma rilpivirine concentrations has been required. Now we have a routine system, by which antiretroviral drug plasma concentrations are easily determined by HPLC (9). According to our preliminary HPLC application, the sensitivity of LC-MS method must at least be essential for quantification of plasma rilpivirine. In this study, we intended to develop a conventional method for determining plasma rilpivirine concentrations by LC-MS.

MATERIALS AND METHODS

Chemicals and Reagents

Rilpivirine was supplied by Janssen Pharmaceutica (Turnhoutseweg, Beerse, Belgium) and the internal standard (IS), 6,7-Dimethyl-2,3-di (2-pyridyl)-quinoxaline, was purchased from Sigma-Aldrich (St Louis, MO, USA). Methanol, *n*-hexane, ethyl acetate, and acetonitrile (Kanto Chemical, Tokyo, Japan) were HPLC grade. Ammonium acetate, EDTA and acetic acid were purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q® system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvents were of analytical grade.

Equipment

A Waters Alliance 2695 HPLC and a Micromass ZQ-2000 MS (Waters Assoc., Milford, MA, USA), controlled with MassLynx version 4.0 software, were used for detection. The analytical column was a SunFire C_{18} column (3.5 μm, 2.1×50 mm, Waters), protected by a SunFire C_{18} Guard Column.

Chromatographic and mass spectrometric conditions

The mobile phase was a mixture of 0.1 mM EDTA in 0.1% acetic acid (A), 100% acetonitrile (B) and 100% methanol (C). An isocratic mobile phase consisting of A-B-C (65 : 15 : 20) was used during the first 2 min of the run, followed by a linear gradient elution consisting of A-B-C (30 : 50 : 20) for the next 8 min. The final conditions were maintained for the final 5 min. The system was then reequilibrated for an additional 25 min using the initial conditions. The flow rate of the mobile phase was 0.2 ml/min, the column temperature was 40°C, and the amount of injected sample was 5 μl.

The mass spectrometer was operated in positive ion electrospray mode. The capillary sprayer voltage was 3.5 kV and the sample cone voltage was 30 V for both rilpivirine and the internal standard. The source temperature was 120°C and the desolvation temperature was 350°C. The desolvation and cone gas flow-rates were set to 600 and 50 L/h, respectively. The acquisition mass range is *m/z* 200-800 at 0.5 s per scan with a 0.1 s interscan delay. All mass spectra are acquired in centroid mode.

Quantitative analysis, carried out in selected-ion recording (SIR) mode, detected rilpivirine at *m/z* 367, and the IS, at *m/z* 313, all in the form of ions. The quantitation calculations were performed using analytical software, MassLynx version 4.0 (Waters).

Standard Solutions

Stock solutions of rilpivirine and the IS were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 143.0 μg/ml of rilpivirine and 588.0 μg/ml of the IS. These stock solutions were stored at -80°C and thawed on the day of analysis. The stock solution was diluted in drug-free plasma to yield rilpivirine concentrations of 18, 72, 143, 358 and 715 ng/ml.

Sample Preparation

Two milliliters of ethyl acetatate/*n*-hexane (50 : 50, v/v) containing the IS (177.5 ng/ml) and 1.0 ml of 0.2 M ammonium acetate were added to a 500 μl plasma sample prepared from peripheral blood anticoagulated with heparin. The mixture was vortexed for 5 min and then centrifuged at 3,500 g

for 5 min. The upper layer was separated and evaporated dry. The dried material was then dissolved in 50 μl of a mobile phase solution. Lastly, 5 μl of the upper solution was injected into the LC-MS system. The institutional review board of National Hospital Organization Nagoya Medical Center approved this study and each subject provided written informed consent.

Validation

Inter- and intraday precision values using this method were estimated by assaying control plasma containing five different concentrations of rilpivirine five times on the same day and on three separate days to obtain the relative standard deviation (RSD). The measured value was calculated as the peak area ratio of rilpivirine to the internal standard. The extraction recovery was determined by comparing the peak areas obtained from the extracted samples in plasma with those of direct injected standards, at the same concentrations. The mean recoveries were determined in triplicate. Accuracy was determined as the percentage of the nominal concentration.

RESULTS

LC-MS Chromatograms

Figures 1A and B show selected-ion recording chromatograms obtained from a spiked plasma sample containing 143.0 ng/ml of rilpivirine and 177.5 ng/ml of the IS. Under the described chromatographic conditions, retention times were 5.3 min for rilpivirine and 10.0 min for IS. Figures 1C and D show chromatograms obtained from a blank plasma sample. Assays performed on drug-free human plasma succeeded to show no interfering peaks during the interested intervals of the retention times. Figure 1D is the expanded figure of the baseline part of Fig. 1B. These peaks did not affect the quantification of the IS. Figures 1E and F show chromatograms of a plasma sample from an HIV-1-infected patient treated with rilpivirine. There were no interfering peaks affecting quantification of rilpivirine in this chromatogram. Anticoagulants of heparin and EDTA did not hinder the selected-ion recording chromatograms for rilpivirine and the IS.

Validation : Linearity, Precision, Accuracy and Recovery

Calibration curves of rilpivirine appeared linear in the concentration range of 18 to 715 ng/ml with a correlation of 0.995.

Precision, accuracy, and recovery of our LC-MS method are shown in Table 1. The selected concentration of rilpivirine covers the expected plasma concentrations found in the patients. The RSDs calculated for rilpivirine in the inter- and intraday

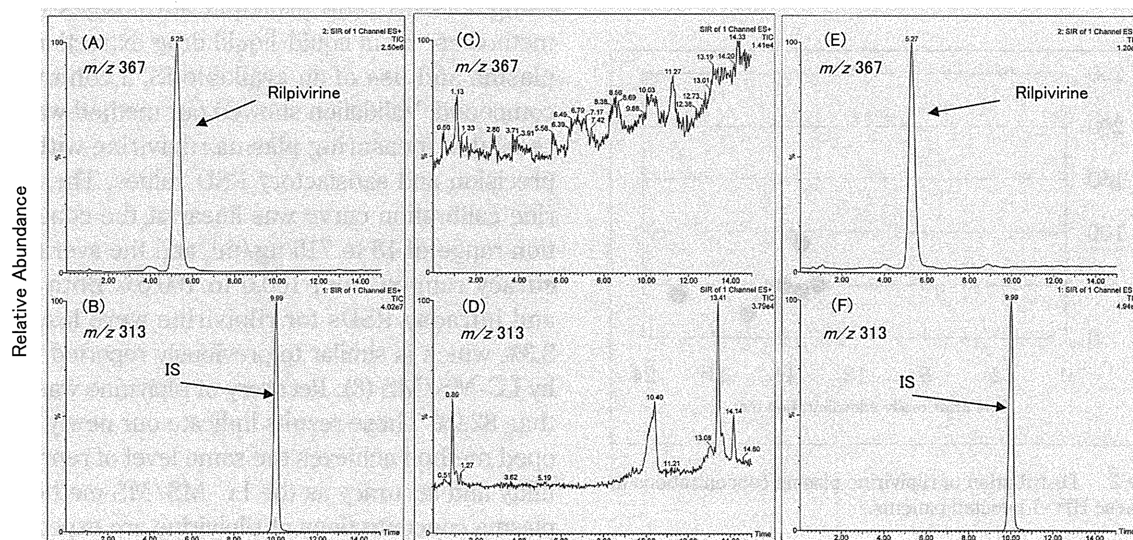

Figure 1. Selected-ion recording chromatograms for rilpivirine and the internal standard.
(A) and (B) were obtained from a spiked plasma containing 143 ng/ml of rilpivirine and 178 ng/ml of the internal standard (IS). (C) and (D) were obtained from a blank plasma sample. (E) and (F) were obtained from a plasma sample from an HIV-1-infected patient on rilpivirine at 16 h after orally administration. (A), (C) and (E) were monitored with *m/z* 367. (B), (D) and (F) were monitored with *m/z* 313. (C) and (D) are the expanded figures of the baselines in (A) and (B), respectively.

Table 1. Intraday and interday precision and accuracy for rilpivirine

	Intraday	(n=5)	Interday	(n=15)		
Expected (ng/ml)	Measured (ng/ml)	RSD (%)	Measured (ng/ml)	RSD (%)	Accuracy (%)	Recovery (%)
18	18.1±0.2	1.0	18.0±0.4	2.4	100.3±1.0	85.1±1.3
72	72.3±1.4	1.9	72.0±2.3	3.3	100.4±1.9	82.0±3.7
143	143.7±3.0	2.1	143.9±3.1	2.2	100.5±2.1	87.6±0.6
358	360.2±2.9	0.8	357.8±5.2	1.5	100.6±0.8	88.3±7.2
715	715.0±8.6	1.2	716.3±6.1	0.8	100.0±1.2	84.0±8.6

RSD, relative standard deviation
Means±SD

assays ranged from 0.8 to 3.3%, which are similar to values reported by LC-MS/MS method (8). Accuracies ranged from 100.0 to 100.6%. Recoveries from plasma ranged from 82.0 to 88.3%. These results indicate that this method achieves a high degree of reproducibility and accuracy.

Clinical application

Figure 2 shows the distribution of plasma rilpivirine concentrations in 6 Japanese HIV-1 infected patients. Rilpivirine plasma concentrations were measured at trough level (14-22 h after orally administration). Mean rilpivirine plasma concentration was 49±22 ng/ml (n=6, range : 23-90 ng/ml). Rilpivirine has been just approved at May 2012 in Japan. This result is the first rilpivirine concentration data for Japanese HIV-1 infected patients. These rilpivirine concentrations were similar to values reported by foreign healthy volunteers (12).

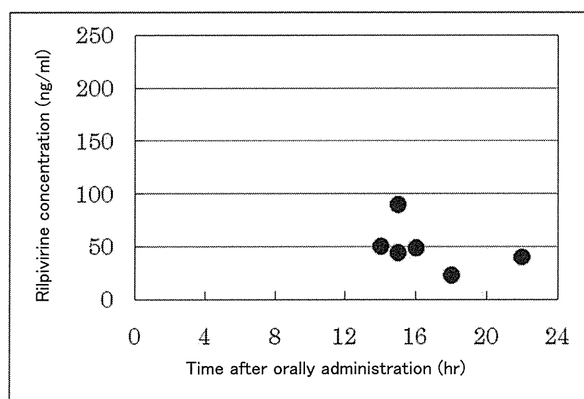

Figure 2. Distribution of rilpivirine plasma concentrations in 6 Japanese HIV-1 infected patients.

DISCUSSION

In NNRTI-based regimens, efavirenz is recommended as an initial combination regimen for antiretroviral-naïve patients, because no regime has proven superior to efavirenz-based regimens with respect to virologic responses. However, efavirenz-based regimens are associated with rash and central nervous system adverse effects (1-3). Clinical trials of rilpivirine (TMC-278) have showed the same efficacy compared with efavirenz, with a slightly increased incidence of virological failures, but a more favourable safety and tolerability profile (10, 11). Therefore, rilpivirine can be an alternative NNRTI-based regimen for antiretroviral therapy-naive patients infected with HIV-1.

Rilpivirine is a substrate of CYP3A4 and its pharmacokinetics is likely to be modulated by inhibitors and inducers of these enzymes. To manage these drug interactions and ensure optimal drug efficacy, monitoring plasma rilpivirine concentrations is essential. For this purpose, we developed a method for determining plasma rilpivirine concentrations using LC-MS. The principal advantages of our method are rapid liquid-liquid drug extraction from plasma and use of an available IS, a commercial compound. Validation showed our method was successful in measuring plasma rilpivirine with high precision and satisfactory RSD values. The rilpivirine calibration curve was linear at the concentration range of 18 to 715 ng/ml, and the average accuracy ranged from 100.0 to 100.6%. Both inter- and intraday RSDs for rilpivirine were less than 3.3%, which is similar to previously reported values by LC-MS/MS (8). Recovery of rilpivirine was more than 82.0%. These results indicate our newly developed method achieves the same level of reproducibility and accuracy as the LC-MS/MS method. As plasma concentrations of rilpivirine are expected in the 67 to 204 ng/ml range when rilpivirine is administered at single dose of 25 mg for healthy volunteers (12), our method successfully covers this region with good precision and accuracy. In clinical practice, mean rilpivirine plasma concentration

at trough was 49 ng/ml. This level compared favourably with trough concentrations of about 50-80 ng/ml seen in ECHO and THRIVE trials (10, 11, 13).

In conclusion, our LC-MS method provides a conventional, accurate and precise way to determine rilpivirine in human plasma. This method can be used in routine clinical application for HIV-1 infected patients, and permits management of drug interactions and toxicity for rilpivirine.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for Clinical Research from the National Hospital Organization to MT.

REFERENCES

1. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. October 14, 2011 ; 41-47. Available at http : //www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf Accessed July 5, 2012.
2. Ngo-Giang-Huong N, Jourdain G, Amzal B, Sang-A-Gad P, Lertkoonalak R, Eiamsirikit N, Tansuphasawasdikul S, Buranawanitchakorn Y, Yutthakasemsunt N, Mekviwattanawong S, Mclntosh K, Lallemant M : Resistance patterns selected by nevirapine vs. efavirenz in HIV-infected patients failing first-line antiretroviral treatment : a bayesian analysis. PLos One 6 : 1-12, 2011
3. Cavalcante GI, Capistrano VL, Cavalcante FS, Vasconcelos SM, Macedo DS, Sousa FC, Woods DJ, Fonteles MM : Implications of efavirenz for neuropsychiatry : a review. Int J Neurosci 120 : 739-745, 2010
4. Azijn H, Tirry I, Vingerhoets J, de Bethune MP, Kraus G, Boven K, Jochmans D, Van Craenenbroeck E, Picchio G, Rimsky LT : TMC278, a next-generation nonnucleoside reverse transcriptase inhibitor (NNRTI), active against wild-type and NNRTI-resistant HIV-1. Antimicrob Agents Chemother 54 : 718-727, 2010
5. Ford N, Lee J, Andrieux-Meyer I, Calmy A : Safety, efficacy, and pharmacokinetics of rilpivirine : systematic review with an emphasis on resource-limited settings. HIV AIDS (Auckl) 3 : 35-44, 2011
6. Wilkin A, Pozniak AL, Morales-Ramirez J, Lupo SH, Santoscoy M, Grinsztejn B, Ruxrungtham K, Rimsky LT, Vanveggel S : Long-term efficacy, safety, and tolerability of rilpivirine (RPV, TMC278) in HIV type1-infected antiretroviral-naïve patients : week 192 results from a phase IIb randomized trial. AIDS Res Hum Retroviruses 28 : 437-446, 2011
7. AIDSinfo Drug Database : Emtricitabine/Rilpivirine/Tenofovir disoproxil fumarate. Available at http : //www.aidsinfo.nih.gov/drugs/441/complera/patient/ Accessed July 5, 2012.
8. Else L, Watson V, Tjia J, Hughes A, Siccardi M, Khoo S, Back D : Validation of a rapid and sensitive high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assay for the simultaneous determination of existing and new antiretroviral compounds. J Chromatogr B Analyt Technol Biomed Life Sci 878 : 1455-1465, 2010
9. Takahashi M, Yoshida M, Oki T, Okumura N, Suzuki T, Kaneda T : Conventional HPLC method used for simultaneous determination of the seven HIV protease inhibitors and nonnucleoside reverse transcription inhibitor efavirenz in human plasma. Biol Pharm Bull 28 : 1286-1290, 2005
10. Cohen CJ, Andrade-Villanueva J, Clotet B, Fourie J, Johnson MA, Ruxrungtham K, Wu H, Zorrilla C, Crauwels H, Rimsky LT, Vanveggel S, Boven K ; THRIVE study group : Rilpivirine versus efavirenz with two background nucleoside or nucleotide reverse transcriptase inhibitors in treatment-naïve adults infected with HIV-1 (THRIVE) : a phase 3, randomized, non-inferiority trial. Lancet 378 : 229-237, 2011
11. Molina JM, Cahn P, Grinsztejn B, Lazzarin A, Mills A, Saag M, Supparatpinyo K, Walmsley S, Crauwels H, Rimsky LT, Vanveggel S, Boven K ; ECHO study group : Rilpivirine versus efavirenz with tenofovir and emtricitabine in treatment-naïve adults infected with HIV-1 (ECHO) : a phase 3 randomized double-blind active-controlled trial. Lancet 378 : 238-246, 2011
12. Rilpivirine PK Fact Sheet. Produced by www.hiv-druginterctions.org (October 2011). Available at http//www.hiv-druginteractions.org/

data/FactSheetImages/FactSheet_DrugID_511.pdf Accessed July 5, 2012.

13. Cohen CJ, Molina JM, Cahn P, Clotet B, Fourie J, Grinsztejn B, Wu H, Johnson MA, Saag M, Supparatpinyo K, Crauwels H, Lefebvre E, Rimsky LT, Vanveggel S, Williams P, Boven K ; ECHO Study Group ; THRIVE Study Group : Efficacy and safety of rilpivirine (TMC278) versus efavirenz at 48 weeks un treatment-naïve HIV-1-infected patients : pooled results from the phase 3 double-blind randomized ECHO and THRIVE Trials. J Acquir Immune Defic Syndr 60 : 33-42, 2012

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HIV-1 Drug-Resistance Surveillance among Treatment-Experienced and -Naïve Patients after the Implementation of Antiretroviral Therapy in Ghana

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Published: August 19, 2013 • DOI: 10.1371/journal.pone.0071972

Abstract

Background

Limited HIV-1 drug-resistance surveillance has been carried out in Ghana since the implementation of antiretroviral therapy (ART). This study sought to provide data on the profile of HIV-1 drug resistance in ART-experienced and newly diagnosed individuals in Ghana.

Methods

Samples were collected from 101 HIV-1-infected patients (32 ART-experienced cases with virological failure and 69 newly diagnosed ART-naïve cases, including 11 children), in Koforidua, Eastern region of Ghana, from February 2009 to January 2010. The *pol* gene sequences were analyzed by in-house HIV-1 drug-resistance testing.

Results

The most prevalent HIV-1 subtype was CRF02_AG (66.3%, 67/101) followed by unique recombinant forms (25.7%, 26/101). Among 31 ART-experienced adults, 22 (71.0%) possessed at least one drug-resistance mutation, and 14 (45.2%) had two-class-resistance to nucleoside and non-nucleoside reverse-transcriptase inhibitors used in their first ART regimen. Importantly, the number of accumulated mutations clearly correlated with the duration of ART. The most prevalent mutation was lamivudine-resistance M184V (n = 12, 38.7%) followed by efavirenz/nevirapine-resistance K103N (n = 9, 29.0%), and zidovudine/stavudine-resistance T215Y/F (n = 6, 19.4%). Within the viral protease, the major nelfinavir-resistance mutation L90M was found in one case. No transmitted HIV-1 drug-resistance mutation was found in 59 ART-naïve adults, but K103N and G190S mutations were observed in one ART-naïve child.

Conclusions

Despite expanding accessibility to ART in Eastern Ghana, the prevalence of transmitted HIV-1 drug resistance presently appears to be low. As ART provision with limited options is scaled up nationwide in Ghana, careful monitoring of transmitted HIV-1 drug resistance is necessary.

Figures

Citation: Nii-Trebi NI, Ibe S, Barnor JS, Ishikawa K, Brandful JAM, et al. (2013) HIV-1 Drug-Resistance Surveillance among Treatment-Experienced and -Naïve Patients after the Implementation of Antiretroviral Therapy in Ghana. PLoS ONE 8(8): e71972. doi:10.1371/journal.pone.0071972

Editor: Jason D. Barbour, University of Hawaii Manoa, United States of America

Received: April 7, 2013; **Accepted:** July 7, 2013; **Published:** August 19, 2013

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Funding: This study was supported by a grant of the Japan Initiative for Global Research Network on Infectious Diseases from the Ministry of Education, Culture, Sports, Science and Technology of Japan to the University of Ghana; the Tokyo Medical and Dental University; a Grant-in-Aid for AIDS Research from the Ministry of Health, Labour and Welfare of Japan (H22-AIDS-004); and a grant from the HIV Research Trust, United Kingdom. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

The number of people worldwide living with HIV/AIDS in 2010, according to the latest report from the United Nations Programme on HIV/AIDS, was estimated to be 34.0 million [1]. Although the highest prevalence of HIV/AIDS remains in sub-Saharan Africa, current massive and rapid scaling up of antiretroviral therapy (ART) has resulted in the decline of the epidemic in this region [1]. Indeed, HIV prevalence in Ghana gradually declined from a peak of 3.6% in 2003 to 2.1% in 2011 due to the National AIDS Control Programme implementing a strategy for achieving universal access to ART. The program has been continuously expanding since 2003, and the coverage of ART in 2011 was estimated to be 26.6% (59,007/221,884) and 63.6% (8,057/12,661) for total HIV-infected individuals and for HIV-positive pregnant women, respectively [2].

The first-line regimen of ART recommended in Ghana is the combination of two nucleoside reverse-transcriptase inhibitors (NRTIs) and a non-nucleoside reverse-transcriptase inhibitor (NNRTI) [3]. Specifically, the two NRTIs selected are lamivudine (3TC) and either zidovudine (AZT) or stavudine (d4T), then either nevirapine (NVP) or efavirenz (EFV) as the NNRTI [3]. For the second-line regimen in Ghana, two protease inhibitors (PIs) are available, nelfinavir (NFV) or lopinavir/ritonavir (LPV/r), either of which is recommended to use with two NRTIs, abacavir (ABC) and either tenofovir (TDF) or didanosine (ddI) [3].

Drug-resistant HIV variants selected during ART have the potential to be transmitted to others. Indeed, drug-resistant HIV has been widely described in ART-naïve individuals. For example, a recent systematic review revealed that the overall prevalence of drug-resistant HIV-1 transmission reached 12.9% in North America, 10.9% in Europe, 6.3% in Latin America, 4.7% in Africa, and 4.2% in Asia [4]. Thus, the higher prevalence of drug-resistant HIV-1 transmission has been reported in

higher ART coverage areas, mostly in developed countries. It is important to note that, along with ART scale-up, the prevalence of transmitted HIV-1 drug-resistance increased from 2.8% before 2001 to 5.3% after 2003 in African countries [4]. As the transmission of drug-resistant HIV may seriously affect the efficacy of first-line ART, surveillance to monitor the prevalence of transmitted HIV drug-resistance has become an important issue in African countries. The prevalence of transmitted HIV-1 drug resistance in Ghana was reported in two studies. One was conducted in 2003 in the Greater-Accra Region of Ghana [5], and the other one was conducted between 2002 and 2004 in Accra and two sites of the Eastern region, Agomanya and Atua [6]. Both studies reported no case of drug-resistant HIV-1 transmission [5], [6]. As at December 2009, the national response had established programs for the provision of ART in hospitals and health centers in several districts in the ten regions of Ghana [7]. However, since ART was expanded in Ghana, the situation of transmitted HIV-1 drug-resistance has not been reported.

To clarify the prevalence, pattern, and spectrum of HIV-1 drug resistance in the era of scaled up ART in Ghana, particularly in ART-experienced patients and transmission to new individuals, we surveyed HIV-1 drug resistance among ART-experienced and -naïve patients enrolled between 2009 and 2010 in Koforidua, the capital of the Eastern region, Ghana. Concomitantly, we analyzed HIV-1 subtypes in detail to further understand the epidemiology of HIV-1 infections in Ghana.

Methods

Patients

HIV-infected patients who visited the Koforidua Regional Hospital (KRH) from February 2009 to January 2010 were enrolled in the study. KRH is the main HIV/AIDS clinic in the capital of the Eastern region of Ghana. This hospital is responsible for HIV prevention and intervention programs in the area and provides free ART with care and support to HIV-infected patients. The Institutional Review Board of the Noguchi Memorial Institute for Medical Research granted ethical approval for this study. All patients or their caregivers gave written consent to participate in the study.

$CD4^+$ T-cell Count and Plasma HIV-1 Viral Load Monitoring

For an indication of immune status, $CD4^+$ T-cells were measured using a FACSCount flow cytometer (Becton Dickinson, San Jose, California, USA). Plasma HIV-1 viral loads (pVLs) were quantified using an in-house real-time reverse-transcription and polymerase chain reaction (RT-PCR) assay as previously reported [8]. ART-experienced patients with pVL>150 copies/mL were considered as virological failures.

HIV-1 Drug-resistance Genotyping

HIV-1 drug-resistance genotyping was performed as previously reported with some modifications [9]. In brief, viral RNA was extracted from 200 µL of plasma samples using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). RT-PCR was performed with QIAGEN one-step RT-PCR kit (Qiagen), and nested PCR was subsequently performed using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, USA). Specific primers known as DRPRO5, DRPRO2L, DRPRO1M, and DRPRO6 were used for the protease (PR) region (424 bp, positions 2,168 to 2,591 in the reference HXB2 sequence), and DRRT1L, DRRT4L, DRRT7L, and DRRT6L primers for the reverse transcriptase (RT) region (838 bp, positions 2,510 to 3,347) [9]. Details of the primers used in the study are shown in Table 1. Nucleotide sequencing was performed using ABI 3730 auto-sequencer followed by editing with SeqScape software v2.5 (Applied Biosystems). HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. In addition, transmitted HIV-1 drug-resistance mutations were defined using the mutation list proposed by Bennett et al. [11].

Target region	Amplicon		Primers			
	Size (bp)	Position*	Reaction	Direction	Name	Nucleotide sequence (5′ to 3′)
Protease	424	2,168 to 2,591	RT-PCR	Forward	DRPRO5	[illegible]
				Reverse	DRPRO2L	[illegible]
			Nested PCR	Forward	DRPRO1M	[illegible]
				Reverse	DRPRO6	[illegible]
Reverse transcriptase	838	2,510 to 3,347	RT-PCR	Forward	DRRT1L	[illegible]
				Reverse	DRRT4L	[illegible]
			Nested PCR	Forward	DRRT7L	[illegible]
				Reverse	DRRT6L	[illegible]

bp, base pairs; PCR, polymerase chain reaction; and RT-PCR, reverse transcription and polymerase chain reaction.
*Amplicon positions in the reference HIV-1 HXB2 sequence are represented.
doi:10.1371/journal.pone.0071972.t001

Table 1. List of primers used in HIV-1 genotypic drug-resistance testing.
doi:10.1371/journal.pone.0071972.t001

HIV-1 Subtyping

HIV-1 subtyping was performed using the *pol* gene sequences (1,095 bp, positions 2253 to 3347). Phylogenetic tree was constructed with the references of subtypes A-D, F-H, J, K, and all circulating recombinant forms (CRFs) 01 to 51, except 30, 41, and 50, obtained from the HIV Sequence Database at the Los Alamos National Laboratory. In addition, HIV-1 sub-subtype A3 (DDI579, DDJ360, and DDJ369) and A4 (97CD_KCC2, 97CD_KTB13, and 02CD_KTB035) isolates were added to the phylogenetic tree analysis, as these sub-subtypes have been reported as circulating in several African countries [12], [13]. Multiple sequences were aligned using the MUSCLE program, and genetic distances were calculated based on the maximum composite likelihood model using MEGA software v5.05 [14]. Phylogenetic trees were constructed using the neighbor-joining method with 1,000 bootstrap replicates. In similarity plotting and boot-scanning analyses, nine HIV-1 subtypes, A-D, F-H, J, and K, and three CRFs, CRF02_AG, CRF06_cpx, and CRF09_cpx, were used as references. Similarity plotting and boot-scanning were performed using SimPlot software v3.5.1 with window and step sizes of 250 and 20 nucleotides, respectively [15]. One HIV-1 isolate identified with an unknown mosaic pattern both in similarity plotting and boot-scanning analyses was considered as a unique recombinant form (URF).

Statistical Analysis

The Fisher's exact test and the Mann-Whitney U-test were used in SYSTAT software v10.2 (SYSTAT Software, Chicago, USA) for analysis of statistical significance between categorical variables and quantitative valuables, respectively. All tests were two-sided and the level of significance was set at $P<0.05$.

Accession Numbers

Nucleotide sequences have been registered as #AB751399 to AB751499 in the DNA databank of Japan.

Results

CRF02_AG is the Predominant HIV-1 Strain in Koforidua, Ghana

During the study period, 101 HIV-1-infected patients were enrolled in this study. As shown in Table 2, 90 cases were adults (≧15 years old), including 59 newly diagnosed ART-naïve cases and 31 ART-experienced cases. The remaining 11 cases were children (<15 years old), among which were 10 newly diagnosed ART-

naïve cases while one child was ART-experienced (Table 3). To understand the molecular epidemiology of HIV-1 infections in Ghana, we analyzed the *pol* gene sequences in detail through the construction of phylogenetic trees, similarity plotting, and boot-scanning analyses. Among the 101 cases, 75 (74.3%) were identified as HIV-1 subtypes and CRFs (Fig. 1A); 67 were CRF02_AG (66.3%), 4 were sub-subtype A3 (4.0%), 2 were CRF06_cpx (2.0%), and 2 were CRF09_cpx (2.0%). Thus, our analyses clearly showed the predominance of HIV-1 CRF02_AG in Koforidua.

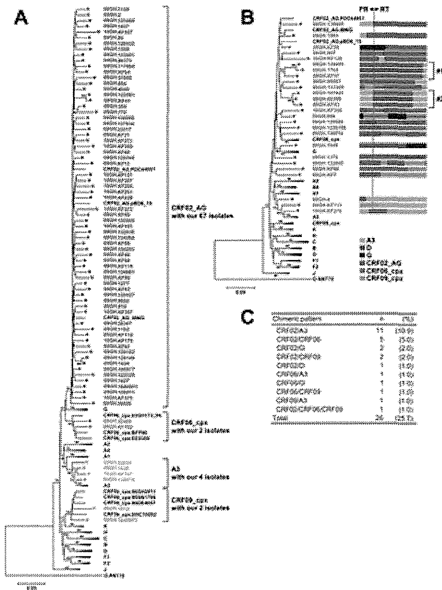


Figure 1. Molecular epidemiology of HIV-1 infections in Koforidua, Ghana.
HIV-1 subtypes of 101 isolates were determined through the construction of phylogenetic trees, similarity plotting, and boot-scanning analyses. (A) Phylogenetic tree containing our 75 isolates classified into known subtypes and CRFs. (B) Phylogenetic tree containing our 26 URF isolates identified with unknown mosaic patterns of the *pol* gene. Two clusters of URF isolates are represented by #1 and #2. (C) Summary on the chimeric patterns of 26 URF isolates. The trees were constructed by the neighbor-joining method. Bootstrap values were calculated from 1,000 analyses, and values greater than 70% are shown at tree nodes. Our isolates are represented by colored circles, and subtype reference isolates are represented by their subtype and name. Scale bar represents nucleotide substitutions per site. HIV-1 group O isolate, ANT70, was used as the outgroup. CRF, circulating recombinant form; PR, protease; RT, reverse transcriptase; and URF, unique recombinant form.
doi:10.1371/journal.pone.0071972.g001

Characteristic		ART-experienced, n = 31	ART-naïve, n = 59	P
Age, years	Median (IQR)	[illegible]	[illegible]	[illegible]
Sex (%)	Female	18 (58.1)	38 (64.4)	[illegible]
	Male	13 (41.9)	21 (35.6)	
Risk factor for HIV infection (%)	Heterosexual contact	31 (100.0)	57 (96.6)	[illegible]
	Transfusion	0 (0.0)	2 (3.4)	
HIV serology (%)[a]	HIV-1 positive	29 (93.5)	59 (100.0)	[illegible]
	HIV-1 and -2 positive	2 (6.5)	0 (0.0)	
CD4+ T-cell count, cells/μl	Median (IQR)	[illegible]	[illegible]	[illegible]
HIV-1 viral load, log_{10} copies/ml	Median (IQR)	[illegible]	[illegible]	[illegible]
HIV-1 genotype (%)	CRF02_AG	18 (58.1)	42 (71.2)	[illegible]
	A3	2 (6.5)	2 (3.4)	
	CRF06_cpx	1 (3.2)	1 (1.7)	
	CRF09_cpx	1 (3.2)	1 (1.7)	
	URF	9 (29.0)	13 (22.0)	
ART regimen (%)				–
First line	d4T+3TC+EFV	10 (32.3)	–	
	AZT+3TC+NVP	10 (32.3)	–	
	AZT+3TC+EFV	7 (22.6)	–	
	d4T+3TC+NVP	3 (9.7)	–	
Second line	AZT+3TC+NFV	1 (3.2)	–	
Duration of ART, months	Median (IQR)	[illegible]	–	–
Adherence (%)[b]	Good	17 (54.8)	–	–
	Satisfactory	8 (25.8)	–	
	Poor	6 (19.4)	–	

ART, antiretroviral therapy; AZT, zidovudine; CRF, circulating recombinant form; d4T, stavudine; EFV, efavirenz; IQR, interquartile range; NFV, nelfinavir; NVP, nevirapine; 3TC, lamivudine; and URF, unique recombinant form.
[a]HIV serology was determined using New Lav Blot I and II (BioRad Laboratories, Marnes-la-Coquette, France).
[b]Good, 100% pills taken; Satisfactory, ≧95% but < 100% pills taken; Poor, < 95% pills taken.
doi:10.1371/journal.pone.0071972.t002

Table 2. Demographic and clinical characteristics of ART-experienced and -naïve HIV-1-infected patients ≧15 years old (*n* = 90).
doi:10.1371/journal.pone.0071972.t002

Characteristic		Value
Age, years	Median (IQR)	5.0 (1.5–8.0)
Sex (%)	Female	6 (54.5)
	Male	5 (45.5)
CD4+ T-cell count, cells/μl	Median (IQR)	747 (474–1152)
HIV-1 viral load, log_{10} copies/ml	Median (IQR)	4.3 (3.4–4.8)
HIV-1 genotype (%)	CRF02_AG	7 (63.6)
	URF	4 (36.4)
ART (%)	Naïve	10 (90.9)
	d4T+3TC+EFV[b]	1 (9.1)

ART, antiretroviral therapy; CRF, circulating recombinant form; d4T, stavudine; EFV, efavirenz; IQR, interquartile range; 3TC, lamivudine; and URF, unique recombinant form.
[a]All were HIV-1 seropositive alone, and their risk factor for infection was mother-to-child transmission.
[b]Only one case had been on treatment for 9.6 months.
doi:10.1371/journal.pone.0071972.t003

Table 3. Demographic and clinical characteristics of HIV-1-infected patients <15 years old (*n* = 11)[a].
doi:10.1371/journal.pone.0071972.t003

Interestingly, the remaining 26 cases (25.7%) were identified as HIV-1 URFs (Fig. 1B). The most prevalent chimeric pattern was CRF02/A3 (n = 11, 10.9%), followed by CRF02/CRF06 (n = 5, 5.0%), CRF02/G (n = 2, 2.0%), CRF02/CRF09 (n = 2, 2.0%), and 6 other patterns (Fig. 1C). Of note, two interesting clusters were found in the phylogenetic tree of URF isolates (Fig. 1B). Cluster #1 with three isolates, 09GH.120495, 09GH.1765, and 09GH.KF47, shared the same mosaic *pol* gene comprising a large PR and RT fragment of CRF02 and a short RT fragment of A3 (cluster #1, Fig. 1B). Cluster #2 with the other three isolates, 09GH.107421, 09GH.60390, and 09GH.KF43, shared the same mosaic *pol* gene comprising the PR fragment of A3 and two RT fragments of CRF02 and A3 (cluster #2, Fig. 1B). Our data suggest that the two URF clusters are candidates for a new CRF spreading in this area of Ghana.

HIV-1 Drug-resistance Mutations are Highly Frequent among ART-experienced Cases with Virological Failure Status

Demographic and clinical characteristics of 31 ART-experienced adult cases are shown in Table 2. All patients except one (96.8%, 30/31) were treated with the first-line ART regimen of 2 NRTIs+NNRTI, and the remaining one (3.2%) with the second-line ART regimen of 2 NRTIs+PI. Their median duration of ART was 16.1 months (IQR, 6.8–30.3 months), and most cases maintained their adherence at a "good" or "satisfactory" level (80.6%, 25/31).

Among these ART-experienced adult cases, 22 cases (71.0%) possessed one or more HIV-1 drug-resistance mutations (Table 4). The most prevalent drug-resistance pattern was 2-class resistance to NRTI and NNRTI (n = 13, 41.9%), followed by 1-class resistance to NNRTI (n = 8, 25.8%). Of note, 3-class resistance was identified in one case (3.2%) treated with the second-line regimen AZT+3TC+NFV. This case possessed HIV-1 RT mutations M41L, V90I, A98G, M184V and T215Y, and the major NFV-resistance mutation L90M in PR. As shown in Table 4, the most prevalent drug-resistance mutation among the 31 cases was M184V (n = 12, 38.7%), followed by K103N (n = 9, 29.0%), and T215Y/F (n = 6, 19.4%). No drug-resistance mutation was detected in the remaining 9 cases (29.0%, Table 4), suggesting that acquisitions of drug resistance was not the primary cause of their virological failure. The cases with and without resistance did not differ significantly in their demographic characteristics.

Mutation	ART-experienced, n = 31 (%)	ART-naïve, n = 59 (%)
Any	22 (71.0)	6 (10.2)
NNRTI resistance	8 (25.8)	6 (10.2)
NRTI and NNRTI resistance	13 (41.9)	0 (0.0)
NRTI, NNRTI, and PI resistance	1 (3.2)	0 (0.0)
Transmitted drug resistance	Not applicable	[illegible]
None	9 (29.0)	53 (89.8)
NRTI-resistance mutation	14 (45.2)	0 (0.0)
A62V	1 (3.2)	0 (0.0)
M184V	12 (38.7)	0 (0.0)
TAMs	8 (25.8)	0 (0.0)
M41L	4 (12.9)	0 (0.0)
D67N	3 (9.7)	0 (0.0)
K70R	4 (12.9)	0 (0.0)
L210W	1 (3.2)	0 (0.0)
T215Y	[illegible]	0 (0.0)
T215F	1 (3.2)	0 (0.0)
K219Q	1 (3.2)	0 (0.0)
K219E	1 (3.2)	0 (0.0)
NNRTI-resistance mutation	22 (71.0)	6 (10.2)
V90I	4 (12.9)	4 (6.8)
A98G	[illegible]	0 (0.0)
K103N	9 (29.0)	0 (0.0)
V106I	[illegible]	1 (1.7)
V106A	1 (3.2)	0 (0.0)
V108I	1 (3.2)	0 (0.0)
E138A	1 (3.2)	2 (3.4)
Y181C	3 (9.7)	0 (0.0)
Y188L	2 (6.5)	0 (0.0)
G190A	2 (6.5)	0 (0.0)
P225H	3 (9.7)	0 (0.0)
M230L	1 (3.2)	0 (0.0)
PI-resistance major mutation	1 (3.2)	0 (0.0)
L90M	1 (3.2)	0 (0.0)

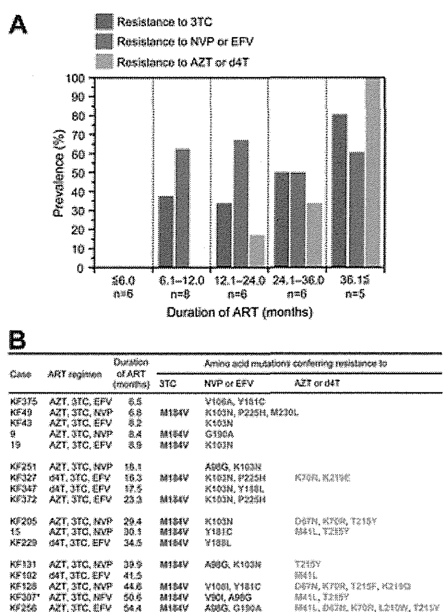
ART, antiretroviral therapy; NNRTI, non-nucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; and TAMs, thymidine analog-associated mutations.

[a]HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. For ART-naïve patients, transmitted drug resistance was defined according to the latest definition of the WHO drug-resistance surveillance [11].

doi:10.1371/journal.pone.0071972.t004

Table 4. Frequency of HIV-1 drug-resistance mutations in ART-experienced and -naïve adult patients (≧15 years old) (n = 90)[a].
doi:10.1371/journal.pone.0071972.t004

Furthermore, we analyzed the chronological order of acquiring drug resistance to 3TC, NVP, EFV, AZT, and d4T. As shown in Fig. 2A, no mutation was found in any patients, even with viremia, who had received ART for ≦6.0 months (0%, 0/6). However, M184V mutation was detected in 37.5% (3/8) of patients with 6.1–12.0 months of ART, and the prevalence increased to 80.0% (4/5) at ≧36.1 months of ART (red bars in Fig. 2A and 2B). In the case of NVP and EFV resistance, K103N, V106A, V108I, Y181C/L, G190A, P225H, and M230L mutations were detected in more than half of patients after 6.0 months of ART (blue bars in Fig. 2A and 2B). Importantly, the prevalence and accumulation of thymidine analog-associated mutations (TAMs) appeared to be higher with longer duration of ART; 16.7% (1/6) at 12.1–24.0 months to 100% (5/5) at ≧36.1 months of ART (green bars in Fig. 2A and 2B).



Case	ART regimen	Duration of ART (months)	Amino acid mutations conferring resistance to		
			3TC	NVP or EFV	AZT or d4T
KF375	AZT, 3TC, EFV	6.5		V106A, Y181C	
KF49	AZT, 3TC, NVP	6.8	M184V	K103N, P225H, M230L	
KF43	AZT, 3TC, EFV	8.2		K103N	
9	AZT, 3TC, NVP	8.4	M184V	G190A	
19	AZT, 3TC, EFV	8.9	M184V	K103N	
KF251	AZT, 3TC, NVP	16.1		A98G, K103N	
KF327	d4T, 3TC, EFV	16.3	M184V	K103N, P225H	K70R, K219E
KF347	d4T, 3TC, EFV	17.5		K103N, Y188L	
KF372	AZT, 3TC, EFV	23.3	M184V	K103N, P225H	
KF205	AZT, 3TC, NVP	29.4	M184V	K103N	D67N, K70R, T215Y
15	AZT, 3TC, NVP	30.1	M184V	Y181C	M41L, T215Y
KF229	d4T, 3TC, EFV	34.5	M184V	Y188L	
KF131	AZT, 3TC, NVP	39.9	M184V	A98G, K103N	T215Y
KF102	d4T, 3TC, EFV	41.5			M41L
KF128	AZT, 3TC, NVP	44.6	M184V	V108I, Y181C	D67N, K70R, T215F, K219Q
KF307*	AZT, 3TC, NFV	50.6	M184V	V90I, A98G	M41L, T215Y
KF256	AZT, 3TC, EFV	54.4	M184V	A98G, G190A	M41L, D67N, K70R, L210W, T215Y

Figure 2. Prevalence of 3TC-, NVP-, EFV-, AZT-, and d4T-resistance mutations by duration of ART in 31 HIV-1-infected patients ≧15 years old.
(A) Bar graph and (B) details of 17 patients identified with 3TC-, NVP-, EFV-, AZT-, and d4T-resistance mutations. HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. Amino acid mutations responsible for drug resistance are shown in

bold and color coded with bar graph in A. *Major NFV-resistance mutation L90M was found in the protease in the case of KF307. ART, antiretroviral therapy; AZT, zidovudine; d4T, stavudine; EFV, efavirenz; NFV, nelfinavir; NVP, nevirapine; and 3TC, lamivudine.
doi:10.1371/journal.pone.0071972.g002

Low HIV-1 Drug-resistance Transmission in ART-naïve Cases

The general demographics of the 59 adult ART-naïve cases were similar to those of the treated cases, however pVL was significantly higher in the naïve cases (P = 0.006) (Table 2). Among the ART-naïve cases, no transmitted HIV-1 drug-resistance mutation was found (Table 4). However, polymorphisms at NNRTI-resistance mutation loci, V90I, E138A, and V106I, were found in 6 cases (10.2% in Table 4). Our data indicated that drug-resistant HIV-1 transmission events are still low in Koforidua, Ghana.

Eleven children infected with HIV-1 through mother-to-child transmission were also analyzed in our study (Table 3). Their median age was 5.0 years (IQR, 1.5–8.0 years), and 10 of these cases were ART naïve. The remaining case had been treated with d4T+3TC+EFV for 9.6 months but had become viremic. In this case, both 3TC-resistance (M184V) and EFV-resistance (V108I and G190S) mutations were detected (Table 5). Importantly, among the 10 ART-naïve children, a 1.5-year-old case had K103N and G190S NNRTI-resistance mutations (Table 5), suggesting the importance of HIV-1 drug-resistance testing in infants.

ART	*n*	Amino acid mutations associated with	
		NNRTI resistance	NRTI resistance
Naïve	5	–	–
	2	V90I, V106I	–
	2	V90I	–
	1	**K103N, G190S**	–
d4T+3TC+EFV	1	K101E, V106I, V108I, G190S	M184V

ART, antiretroviral therapy; d4T, stavudine; EFV, efavirenz; NNRTI, non-nucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; and 3TC, lamivudine.
[a]HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. For ART-naïve patients, transmitted drug-resistance (shown in bold and underlined) was detected according to the latest definition of the WHO drug-resistance surveillance [11].
doi:10.1371/journal.pone.0071972.t005

Table 5. HIV-1 drug-resistance mutations in patients <15 years old (*n* = 11)[a].
doi:10.1371/journal.pone.0071972.t005

Discussion

Our results present a profile of the circulating subtypes and prevalence of drug resistance for HIV-1 infections in Koforidua, Ghana. The data clearly demonstrate the predominance of HIV-1 CRF02_AG (66.3%, n = 67) in the region (Fig. 1A). Our results, combined with three previous reports on the domination of CRF02_AG in Ghana between 1994 and 2004 [5], [6], [16], indicate that CRF02_AG has stabilized and maintained its predominance in the region for nearly 12 years. However, our study identified 26 isolates (25.7%) as URFs (Fig. 1B), indicating that active viral recombinations are ongoing in Ghana. Interestingly, a similar prevalence (25.1%) of HIV-1 URFs was reported from other cities in Ghana, Accra, Agomanya, and Atua [6]. Taken together, these data thus highlight the importance of HIV-1 URFs in understanding the dynamics of the HIV-1 epidemic in Ghana.

Regarding the situation of HIV-1 drug resistance in Ghana, most of the 31 patients on treatment with virological failure (n = 22, 71.0%) had HIV-1 drug-resistance mutations, suggesting that drug-resistant HIV-1 is the major risk factor for virological failure. Furthermore, nearly half of the cases (45.2%, 14/31) had both NRTI- and NNRTI-resistance mutations (Table 4), a pattern that is consistent with that observed in a recent systematic review on treatment-failure cases in sub-Saharan Africa [17], where M184V/I, K103N, and T215Y/F mutations predominate.

Regarding the timing of drug-resistance acquisition, our data demonstrated that 3TC-, NVP-, and EFV-resistance mutations were selected earlier (6.1–12.0 months) than AZT- and d4T-resistance mutations (12.1–24.0 months). Importantly, the prevalence of TAMs increased from 16.7% (1/6) at 12.1–24.0 months to 100% (5/5) at ≧36.1 months. As the accumulation of TAMs confers cross-resistance not only to the first-line NRTIs (AZT, d4T, and 3TC), but also to the second-line NRTIs (ABC, TDF, and ddI) to some extent [18], their accumulation should be avoided by conducting drug-resistance testing earlier and appropriately switching the regimen, once virological failure is suspected.

As no transmitted HIV-1 drug-resistance mutation was found among the 59 newly diagnosed treatment-naïve adult cases, the transmission of drug-resistant HIV-1 appeared to be a rare event in Koforidua, Ghana. Comparing our data with that from other African countries with a similar background, roll-out time of ART, and coverage rate of ART (26.6% in Ghana) [2], the low prevalence of transmitted HIV-1 drug resistance is not surprising and understandable. However, we cannot exclude the possibility of low levels of transmitted HIV-1 drug resistance in our 59 ART-naïve adult cases. The results of our study are limited by using direct sequencing, which may not have been sensitive enough to detect minority drug-resistant variants hidden among the wild-type strains. Indeed, several studies have reported 2- to 3-fold higher prevalence of drug-resistance transmission with ultra-deep sequencing than with direct sequencing [19], [20], which can detect 1% minority populations [21]. Furthermore, as ultra-deep sequencing can better detect the presence of dual or multiple infections of HIV-1 subtypes compared with direct sequencing [22], [23], using such new technology may identify subtypes of 26 URFs.

Finally, an eventual increase of transmitted drug-resistance cases is anticipated in Ghana as well. Thus, access to HIV-1 genotypic drug-resistance testing should ideally be expanded along with the scale-up of ART programs. In addition, vertical transmission of drug-resistant HIV-1 was found in one of 10 newly-diagnosed treatment-naïve children, suggesting that expanded access to HIV-1 genotypic drug-resistance testing is also needed for programs to prevent mother-to-child transmission in Ghana.

Acknowledgments

We thank the participating patients for their understanding and contributions to our study. We appreciate Mr. George Danquah Damptey, Mr. Samuel Nii-Azumah Morton, Mr. Richard Ansong, and Dr. Agyin Mensah for their help and support. We also thank Ms. Claire Baldwin for her help in preparing the manuscript. We thank the National AIDS Control Programme (NACP) and the Ghana AIDS Commission (GAC) for their support.

Author Contributions

Conceived and designed the experiments: NIN SI JSB KI JAMB SBO SY WKA WS. Performed the experiments: NIN SI JSB KI JAMB. Wrote the paper: NIN SI WS.

Organized the study team: KI SY WKA WS. Enrolled patients into the study: SBO. Prepared a clinical database: NIN JSB KI SBO. Revised the manuscript critically JSB KI JAMB SBO SY WKA.

References

1. UNAIDS (2011) UNAIDS world AIDS day report 2011. Available: http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2011/JC2216_WorldAIDSday_report_2011_en.pdf. Accessed 29 October 2012.

2. Ghana AIDS Commission (2012) Ghana country AIDS progress report. Available: http://www.unaids.org/en/dataanalysis/kn owyourresponse/countryprogressreports/2012countries/ce_GH_Narrative_Report[1].pdf. Accessed 29 October 2012.

3. National HIV/AIDS/STI Control Programme (2008) Guidelines for antiretroviral therapy in Ghana. Available: http://www.who.int/hiv/pub/guidelines/gh ana_art.pdf. Accessed 29 October 2012.

4. Frentz D, Boucher CAB, van de Vijver DAMC (2012) Temporal changes in the epidemiology of transmission of drug-resistant HIV-1 across the world. AIDS Rev 14: 17–27. doi: 10.1107/s0021889802016709
View Article • PubMed/NCBI • Google Scholar

5. Sagoe KWC, Dwidar M, Lartey M, Boamah I, Agyei AA, et al. (2007) Variability of the human immunodeficiency virus type 1 polymerase gene from treatment naive patients in Accra, Ghana. J Clin Virol 40: 163–167. doi: 10.1016/j.jcv.2007.07.016
View Article • PubMed/NCBI • Google Scholar

6. Delgado E, Ampofo WK, Sierra M, Torpey K, Pérez-Álvarez L, et al. (2008) High prevalence of unique recombinant forms of HIV-1 in Ghana: molecular epidemiology from an antiretroviral resistance study. J Acquir Immune Defic Syndr 48: 599–606. doi: 10.1097/QAI.0b013e3181806c0e
View Article • PubMed/NCBI • Google Scholar

7. Ampofo WK (2009) Current status of HIV/AIDS treatment, care and support services in Ghana. Ghana Med J 43: 142–143.
View Article • PubMed/NCBI • Google Scholar

8. Barnor J, Brandful J, Ampofo W, Yamamoto N, Bonney K, et al.. (2011) Establishment of in-house quantitative real-time RT-PCR assay for monitoring HIV-1 viral loads and efficacy evaluation of ART in Ghanaian AIDS patients in an urban setting. Abstract CDB346. 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention; 17–20 July 2011; Rome, Italy. Available: http://www.iasociety.org/Default.aspx?pageId=11&abstractId=200743770. Accessed 29 October 2012.

9. Chiba-Mizutani T, Miura H, Matsuda M, Matsuda Z, Yokomaku Y, et al. (2007) Use of new T-cell-based cell lines expressing two luciferase reporters for accurately evaluating susceptibility to anti-human immunodeficiency virus type 1 drugs. J Clin Microbiol 45: 477–487. doi: 10.1128/JCM.01708-06
View Article • PubMed/NCBI • Google Scholar

10. Johnson VA, Calvez V, Gunthard HF, Paredes R, Pillay D, et al. (2011) 2011 update of the drug resistance mutations in HIV-1. Top Antivir Med 19: 156–164.
View Article • PubMed/NCBI • Google Scholar

11. Bennett DE, Camacho RJ, Otelea D, Kuritzkes DR, Fleury H, et al. (2009) Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PLoS One 4: e4724. doi: 10.1371/journal.pone.0004724
View Article • PubMed/NCBI • Google Scholar

12. Meloni ST, Kim B, Sankalé J-L, Hamel DJ, Tovanabutra S, et al. (2004) Distinct human immunodeficiency virus type 1 subtype A virus circulating in West Africa: sub-subtype A3. J Virol 78: 12438–12445. doi: 10.1128/JVI.78.22.12438-12445.2004
View Article • PubMed/NCBI • Google Scholar

13. Vidal N, Mulanga C, Bazepeo SE, Lepira F, Delaporte E, et al. (2006) Identification and molecular characterization of subsubtype A4 in Central Africa. AIDS Res Hum Retroviruses 22: 182–187. doi: 10.1089/aid.2006.22.182
View Article • PubMed/NCBI • Google Scholar

14. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739. doi: 10.1093/molbev/msr121
View Article • PubMed/NCBI • Google Scholar

15. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, et al. (1999) Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73: 152–160.
View Article • PubMed/NCBI • Google Scholar

16. Fischetti L, Opare-Sem O, Candotti D, Sarkodie F, Lee H, et al. (2004) Molecular epidemiology of HIV in Ghana: dominance of CRF02_AG. J Med Virol 73: 158–166. doi: 10.1002/jmv.20070
View Article • PubMed/NCBI • Google Scholar

17. Barth RE, van der Loeff MFS, Schuurman R, Hoepelman AIM, Wensing AMJ (2010) Virological follow-up of adult patients in antiretroviral treatment programmes in sub-Saharan Africa: a systematic review. Lancet Infect Dis 10: 155–166. doi: 10.1016/S1473-3099(09)70328-7
View Article • PubMed/NCBI • Google Scholar

18. Whitcomb JM, Parkin NT, Chappey C, Hellmann NS, Petropoulos CJ (2003) Broad nucleoside reverse-transcriptase inhibitor cross-resistance in human immunodeficiency virus type 1 clinical isolates. J Infect Dis 188: 992–1000. doi: 10.1086/378281
View Article • PubMed/NCBI • Google Scholar

19. Simen BB, Simons JF, Hullsiek KH, Novak RM, MacArthur RD, et al. (2009) Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. J Infect Dis 199: 693–701. doi: 10.1086/596736
View Article • PubMed/NCBI • Google Scholar

20. Lataillade M, Chiarella J, Yang R, Schnittman S, Wirtz V, et al. (2010) Prevalence and clinical significance of HIV drug resistance mutations by ultra-deep sequencing in antiretroviral-naïve subjects in the CASTLE study. PLoS One 5: e10952. doi: 10.1371/journal.pone.0010952
View Article • PubMed/NCBI • Google Scholar

21. Wang C, Mitsuya Y, Gharizadeh B, Ronaghi M, Shafer RW (2007) Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1

drug resistance. Genome Res 17: 1195–1201. doi: 10.1101/gr.6468307
View Article • PubMed/NCBI • Google Scholar

22. Pacold M, Smith D, Little S, Cheng PM, Jordan P, et al. (2010) Comparison of methods to detect HIV dual infection. AIDS Res Hum Retroviruses 26: 1291–1298. doi: 10.1089/aid.2010.0042
View Article • PubMed/NCBI • Google Scholar

23. Redd AD, Collinson-Streng A, Martens C, Ricklefs S, Mullis CE, et al. (2011) Identification of HIV superinfection in seroconcordant couples in Rakai, Uganda, by use of next-generation deep sequencing. J Clin Microbiol 49: 2859–2867. doi: 10.1128/JCM.00804-11
View Article • PubMed/NCBI • Google Scholar

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Prevalence of Transmitted HIV Drug Resistance in Iran between 2010 and 2011

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Published: April 23, 2013 • DOI: 10.1371/journal.pone.0061864

Abstract

Objective

Drug-resistant (DR) HIV emerges during combined antiretroviral treatment (cART), creating concern about widespread transmission of DR-HIV as cART is expanded in resource-limited countries. The aim of this study was to determine the predominant HIV-1 subtypes and prevalence of transmitted DR mutations among antiretroviral-naïve patients in Iran.

Design

To monitor transmission of DR HIV, a threshold surveillance based on the world health organization (WHO) guidelines was implemented in Iran.

Methods

For this HIVDR threshold surveillance study, blood samples were collected from 50 antiretroviral-naïve HIV-1-infected patients. Antiretroviral-resistant mutations were determined by sequencing HIV-1 protease, reverse transcriptase and integrase regions. The HIV-1 subtype was determined by sequencing the p17 and C2-V5 regions of the *gag* and *env* genes, respectively.

Results

Phylogenetic analyses of the sequenced regions revealed that 45 (95.7%) of 47 samples that were successfully obtained were CRF35_AD. The remaining two cases were subtype B (2.1%) and CRF01_AE (2.1%). Consistent results were obtained also from Env and Gag sequences. Regarding prevalence of transmitted DR viruses, two cases were found to harbor reverse transcriptase-inhibitor-resistant mutations (4.3%). In addition, although not in the WHO list for surveillance of transmitted mutations, 13 minor protease-inhibitor-resistant mutations listed in the International AIDS Society-USA panel of drug resistance mutations were found. No DR mutations were detected in the integrase region.

Conclusions

Our study clarified that CRF35_AD is the major subtype among HIV-1-infected patients in Iran. According to the WHO categorization method of HIVDR threshold survey, the prevalence of transmitted drug resistant HIV in Iran was estimated as moderate (5–15%).

Figures

Citation: Jahanbakhsh F, Hattori J, Matsuda M, Ibe S, Monavari S-HR, et al. (2013) Prevalence of Transmitted HIV Drug Resistance in Iran between 2010 and 2011. PLoS ONE 8(4): e61864. doi:10.1371/journal.pone.0061864

Editor: Luis Menéndez-Arias, Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Spain

Received: December 24, 2012; **Accepted:** March 14, 2013; **Published:** April 23, 2013

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Funding: This study was supported by grants from the World Health Organization (APW/09/00252), Iranian Ministry of Health and Medical Education, and a Grant-in-Aid for AIDS research from the Ministry of Health,Labour and Welfare of Japan (H22-AIDS-004). The stipendiary of Fatemeh Jahanbakhsh's Ph.D. sabbatical leave in Japan, at Nagoya Medical Center was funded by Ministry of Health and Medical Education of Iran. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Selection and acquisition of HIV-1 drug resistance is inevitably associated with combined antiretroviral treatment (cART) of HIV-1-infected patients. Thus, the emergence of drug-resistant (DR) HIV is a major concern as a potential consequence of scaling up cART [1]. Indeed, expansion of cART might be jeopardized by widespread transmission of DR-HIV [2], particularly in countries where antiretroviral options are limited.

The first HIV-infected case in Iran was a hemophilic patient identified in 1986, and the first cases of HIV transmission through drug injections were reported in 1989 [3]. As of March 2012, 24290 HIV-1-infected individuals have been reported in Iran [4]. The transmission routes were attributed to injected drugs (69.6%), sexual contact (10.5%), transfusion of blood products (1.0%) and mother-to-child transmission (0.1%) [4]. Iran is experiencing a concentrated HIV-1 epidemic among injecting drug users (IDUs) [5], [6].

In Iran, cART was introduced in 1997 when the antiretrovirals zidovudine, lamivudine, and indinavir became available as a part of the country's healthcare system, making it possible to scale up cART in Tehran and other cities such as Kermanshah and Shiraz [7]. Subsequently, indinavir was replaced with nelfinavir from the list

of Iranian generic drugs. Later, stavudine, nevirapine, and didanosine in 2005, efavirenz, lopinavir/ritonavir, tenofovir, and abacavir in 2006, atazanavir in 2011 became available for HIV/AIDS patients [3], [7]. cART is in line with Iran's guidelines on clinical care for HIV/AIDS patients, which state the 14 possible three-drug combinations from the antiretrovirals mentioned above [3]. cART is supplied to HIV/AIDS patients for free at counseling and behavioral centers (triangular clinics) [7].

In addition to receiving cART, all HIV/AIDS patients in Iran are monitored for $CD4^+$ T cell counts periodically by the government at no cost for patients. On the other hand, the Iranian guidelines do not include measurement of viral load. Similarly, drug-resistance monitoring is available on a limited basis, and it is not tested on every Iranian patient for drug resistance. Therefore, to address concerns about the emergence and transmission of DR-HIV in Iran, we conducted a threshold survey among drug-naïve individuals by following recommendations of the World Health Organization (WHO) drug-resistance network. In this study, we report the estimated prevalence of DR-HIV transmission in Iran.

Methods

Eligibility Criteria

Samples were collected according to the HIV Drug Resistance Threshold Survey (HIVDR-TS) recommended by the WHO for the surveillance of DR-HIV in resource-limited countries [8]. Individuals were recruited from all counseling and behavioral centers in Tehran from January 2010 through February 2011 if they met these eligibility criteria: under age 25 years at HIV diagnosis and no previous pregnancy for females [8]. However, after these 9 months, only 15 newly diagnosed cases were recruited. Thus, after consultation with WHO experts, to reduce sample collection period, the inclusion criteria were expanded to less than 30 years of age, $CD4^+$ T cell counts >500/µl, without previous pregnancies for females and no previous exposure to antiretroviral drugs. In addition to Tehran, samples were collected from two other areas in Iran, Kermanshah in the west and Shiraz in the south of Iran, where antiretroviral therapy was started at the same time as Tehran.

Sample

Newly diagnosed, antiretroviral-naïve HIV-1 patients (n = 50) were enrolled in this study. They visited counseling and behavioral centers in Tehran (n = 30), Kermanshah (n = 10), or Shiraz (n = 10) between January 2010 and February 2011. After patients signed informed consent, 10 ml of their peripheral blood was collected into EDTA-containing vacutainer tubes. Plasma samples were obtained by centrifugation and aliquots were stored at -70°C until use. If possible, each patient was asked to complete a questionnaire regarding patient's basic information, including age, sex, risk behavior, marital status, and status of hepatitis B virus (HBV) or hepatitis C virus (HCV) co-infection.

Ethics Statement

This study was approved by the Ethics Committees in Medical Sciences Research at the Tehran University of Medical Sciences and Ministry of Health and Medical Education.

Determination of Drug-Resistance Mutations by Genotyping

We used the drug resistance genotypic testing protocol described previously by Sugiura, et al. with some modification [9]. Briefly, viral RNA was extracted from 140 µl of plasma by the QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Nucleotide sequences of whole HIV-1 protease (PR, HXB2 position 2253–2549), the N-terminal portion of reverse transcriptase (RT, 2550–3269), and full-length integrase (INT, 4230–5093) were amplified using region-specific primer pairs by reverse transcription (RT)-polymerase chain reaction (PCR) using SuperScript III one-step RT-PCR system with platinum Taq high-fidelity kit (Life Technologies Corp., Tokyo, Japan), and amplified further in nested PCR using LA Taq (Takara Bio Inc., Shiga, Japan). After gel electrophoresis, the amplified PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan). Sequencing reaction was performed using the BigDye terminator cycle sequencing kit v3.1 (Applied Biosystems, Tokyo, Japan). Nucleotide sequences were determined on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The sequenced data were aligned against HXB2 and amino acid mutations were detected by SeqScape 2.5 software (Applied Biosystems). Presence of transmitted HIV-1 drug-resistance mutations was determined using the WHO mutation list [10].

HIV-1 Subtype Determination

To determine the subtype of each sample, the nucleotide sequences including p17 of the *gag* gene (Gag, HXB2 position 708–1230) and the C2-V5 region of the *env* gene (Env, 6934–7651) were determined using the same protocol as for the drug-resistant genotypic test described above. The nucleotide sequences obtained for Gag and Env as well as PR, RT, and INT were aligned by ClustalW, and phylogenetic trees were constructed using neighbor-joining analysis with 1000 bootstraps as implemented in MEGA 5 [11].

Nucleotide Sequence Accession Numbers

The nucleotide sequences obtained in this study were deposited in the DNA Data Bank of Japan, and are available under the following accession numbers: AB716095–AB716141 for PR and RT, AB716142–AB716188 for INT, AB716189–AB716232 for Gag, and AB716233–AB716276 for Env. To be noted, one of the *env* sequences contained ambiguous regions. Thus, it was submitted in two parts: AB716271 and AB716272.

Results

The Major HIV-1 Transmission Route in Iranian Cases is Injecting Drug Use

Background information on the 50 newly diagnosed HIV-1-infected cases is summarized in Table 1. More than a half of study cases were male (64.0%) and in the 25–30 year-old age group (56.9%). Their average age was 26.0 years. Among 35 participants who completed the questionnaire, 51.4% were infected by drug injections. Together with 9 individuals who acquired HIV through heterosexual contact with IDU partners, IDU-related transmission accounts for 77.1%. Co-infection with hepatitis C virus and hepatitis B virus was identified among 46.7% and 7.1% of 30 and 28 tested participants, respectively.