

primer sequences were (5' → 3'): GP5 + M1, TTTRT-TACTGTTGTWACTACTAC, GP5 + M2, TGTWACTG-TTGTWACTACTAC, GP5 + M3, GTWACTGTTGTR-GACACCAC GP6 + M1, AATTGAAAWATAAACTGT-AAWTCATATTC, GP6 + M2, GAAACATAAAAYTGTAAATCAWATTC, and GP6 + M3, GAAAAATAAAAYTGC-AAATCAWACTC.

DNA quality was confirmed by detecting the beta-globin gene by PCR. Samples exhibiting a band of ~140 bp on agarose gels stained with ethidium bromide were defined as positive for HPV infection. HPV typing was determined by a slit blot hybridization method using type-specific FITC-labeled oligoprobes. The probe sequences were as follows (5' → 3'): low-risk HPV probe to detect HPV6, 11, 13, and 44, ACATGGCGCATG-TATTGTTTATA; HPV16, TCTGAAGTAGATATGGCAGC; HPV18, CCCAGGTACAGGAGACTG; HPV26, GG-ATGCAGATGCTGCAG; HPV30, CTTGAATTATATG-TGGATAACGTTTG; HPV31, TCACTGTTTGCAATT-GCAG; HPV33, ACTGTCACTAGTTACTTGTGT; HPV35, TCACTAGAAGACACAGCAGA; HPV39, ATGTA-GAAGGTATGGAAGACTC; HPV45, GACTTGGCAC-AGGATTTTG; HPV51, GGAAACCGCAGCAGTG; HPV52, GCTTTCCTTTTAACTCAGC; HPV53, ATGTA-GACATAGACTGTGTGG; HPV56, TCATATTTACTTA-CTGTTCTGTAGC; HPV58, CCTTCTTAGTTACTT-CAGTGC; HPV59, AGAAGAAGTAGTAGAAGCACAC; HPV66, GTTAATGTGCTTTTAGCTGCA; HPV67, GC-CTCTGATTTTCTCAGA; HPV68, AAATATTTGGT-ACAGTGATTCA; HPV70, CAGGTATGGCCGTTTC-G; and HPV82, TTGTGCAACAGATGGAGTA.

The slit blot hybridization method was similar to a dot blot hybridization. For the amplified PCR product (25 µl) of each sample, 5 µl were used to confirm HPV DNA amplification, and the remaining 20 µl were used for HPV typing. The amplified DNA was denatured by the addition of 200 µl of 0.4 M NaOH colored with blue ink and incubation at 95°C for 1 min, followed by cooling on ice. After denaturing, each sample was loaded onto a nylon membrane (Hydrobond N+; GE Healthcare, Tokyo, Japan) in one of 21 slit windows of a slit blot apparatus (Life Technologies, Rockville, MD) and aspirated under negative pressure. After aspiration, the membrane was lightly washed in 1× sodium salt citrate buffer (SSC) and semi-dried in air. The membrane was cut vertically between the slits, creating 21 strips for hybridization with different HPV probes. The membrane strips were hybridized with different HPV probes in plastic bags for 3 hr at 40°C in a hybridization oven. The membranes were washed in 1× SSC at room temperature for 20 min, and then in 1× SSC at 40°C for 20 min. A chemiluminescence detection system (CDP-Star; Amersham) was used to visualize the DNA hybridized with each HPV probe, and images were captured using a charge-coupled device camera.

The samples were classified into three risk groups based on HPV type: high-risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 67, 68, and 82), low-risk (types 6, 11, 13, and 44), and undetermined-risk (types 26, 30, 53, 66, and 70). Some samples contained an unknown type of

HPV, which was detected as an HPV band on agarose gels for PCR, but gave no signal in the slit blot hybridization.

Definition of Cervical Abnormalities

Pap smears were classified according to the 2001 Bethesda System [Solomon et al., 2002] as unsatisfactory, negative, atypical squamous cells of undetermined significance, atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesions, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, squamous cell carcinoma, adenocarcinoma in situ, or adenocarcinoma. The slides were initially screened by two cytotechnologists, and samples that were borderline abnormal (atypical squamous cells of undetermined significance/atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesions) and abnormal (low-grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, and cancer) were re-evaluated by a surgical pathologist. The final diagnosis of the cytological results was agreed upon by a cytotechnologist and a surgical pathologist. Cases showing abnormal cytology (n = 45), including atypical squamous cells of undetermined significance and atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesion, in the initial screening were histologically evaluated using biopsy specimens obtained by colposcopy. Discrepant results that showed a difference of more than two grades between cytology and histology were observed in four cases. The remaining abnormal cases were diagnosed by cytology alone, and thus only cytological diagnosis was used in the present study. Histologically confirmed cervical intraepithelial neoplasia grade 1 was counted as low-grade squamous intraepithelial lesion, and cervical intraepithelial neoplasia grade 2 or 3, as high-grade squamous intraepithelial lesion. One invasive squamous cell carcinoma was found in one HIV positive woman.

HIV Serological Testing, Lymphocyte Subset Counting, and Plasma HIV RNA Determination

Serological testing for HIV was performed using a kit (UniGold HIV-1/2, Trinity Biotech; HIV-1/2/0, Abbot Laboratories, Chicago, IL). HIV positive samples were confirmed with a particle agglutination test kit (KEMRI HIV PA kit; Kenya Medical Research Institute).

Lymphocyte subset counts were performed by standard flow cytometry, according to the method described by Maurer et al. [1990]. The details of the procedure were performed in accordance with the manufacturer's instructions (Tritest; Becton-Dickinson, Franklin Lakes, NJ). Briefly, 50 µl of whole blood with EDTA were incubated with three-color fluorochrome-labeled monoclonal antibodies. After incubation, flow cytometric analysis was performed on a FacsCalibur cytometer using an automatic acquisition and analysis program (Multiset; Becton-Dickinson). The CD4⁺ T cell count was used in the present study.

To quantify HIV-1 RNA, a NucliSens EasyQ assay (Biomerieux, Lyon, France) was used. This assay employs three methods: the Boom method for nucleic acid release and isolation, nucleic acid sequence-based amplification (NASBA) to amplify RNA, and real-time detection of amplicons using fluorescent molecular beacons. The measurement and interpretation were performed using an EasyQ analyzer. Kinetic analysis of the fluorescence signals revealed the respective amplification efficiency of the wild-type target and calibrator RNA, and thus the quantity of HIV-1 RNA in the original sample. The RNA level is presented as the estimated copy number of HIV RNA transcripts.

Statistical Analysis and Drawing Figure

The Chi-squared test or Fisher's test was used to compare the positive rate or prevalence between two groups. The number of CD4⁺ T lymphocytes and the HIV RNA copy number were compared between two groups using the Mann-Whitney test. The Chi-squared test for trends was used to demonstrate a change in positive rates according to increased age. Before the multivariate analysis using an unconditional logistic regression model, the forward and backward regression analyses were performed to select possible factors associated with HIV infection or abnormal cytology. The multivariate analysis was performed using JMP ver 5.1.1 (SAS Institute, Inc., Cary, NC), and the Chi-squared test for trends and other analyses, and drawing figure were conducted using Prism version 4 (GraphPad Software, San Diego, CA).

RESULTS

Prevalence of HIV and HPV Infections and Abnormal Cervical Cytology

The HIV positive rate was 32% (155/488) in the eligible women. The average ages were 31.2 years for all subjects, 33.9 years for HIV positive women, and

29.9 years for HIV negative women. HIV infection increased according to the age of the women ($P < 0.001$, Chi-squared test for trends; Fig. 1). Among HIV positive women, 23% were treated with antiretroviral drugs within 6 months, and the remaining 77% were not treated.

The overall prevalence of HPV infection in the uterine cervix was 27% (132/488) (Fig. 1). HPV was detected in 100% in HIV positive women aged 17–19 years, 58% of HIV positive women aged 20–24 years, and 43–56% of HIV positive women aged >25 years, while it was in 44% in HIV negative women aged 17–19 years, 25% of HIV negative women aged 20–24 years, and 10–15% of HIV negative women aged >25 years (Fig. 1). HPV infection decreased according to age in HIV negative ($P = 0.002$, Chi-squared test for trends), but no such tendency was seen in HIV positive women (Fig. 1).

Among HIV positive women, 49% (76/155) were positive for HPV, while 17% (56/333) of HIV negative women were HPV-positive (Fig. 2). Single- and multiple-type HPV infections were seen in 23% and 26% of HIV positive women, respectively, and in 12% and 4.5% of HIV negative women, respectively. The low- and high-risk HPV infection frequencies were 34% and 35%, respectively, in HIV positive women and 12% and 8.4%, respectively, in HIV negative women. The age-adjusted odds ratios for any HPV, single-type HPV, high-risk HPV, and multiple-type HPV infections in HIV positive women compared to HIV negative women were 2.8, 2.5, 8.6, and 11.0, respectively. In cytological tests, 47% of HIV positive women had an abnormal cervix (atypical squamous cells undetermined significance/atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesions, 20%; low-grade squamous intraepithelial lesion, 21%; and high-grade squamous intraepithelial lesion/cancer, 5.8%), whereas 14% of HIV negative women had an abnormal cervix (atypical squamous cells undetermined significance/atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesion, 6.6%; low-grade squamous intraepithelial lesion, 6.9%; and high-grade

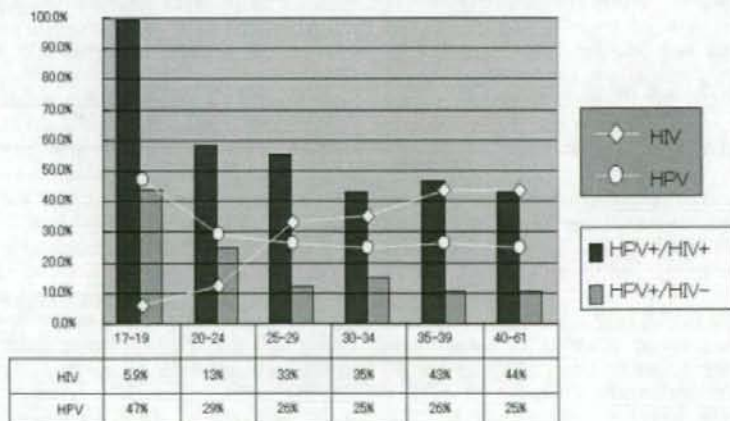


Fig. 1. Prevalence of HIV and HPV infections according to age. The lines indicate the prevalence of HIV and HPV infections, and the bars represent the HPV prevalence in HIV positive and HIV negative women.

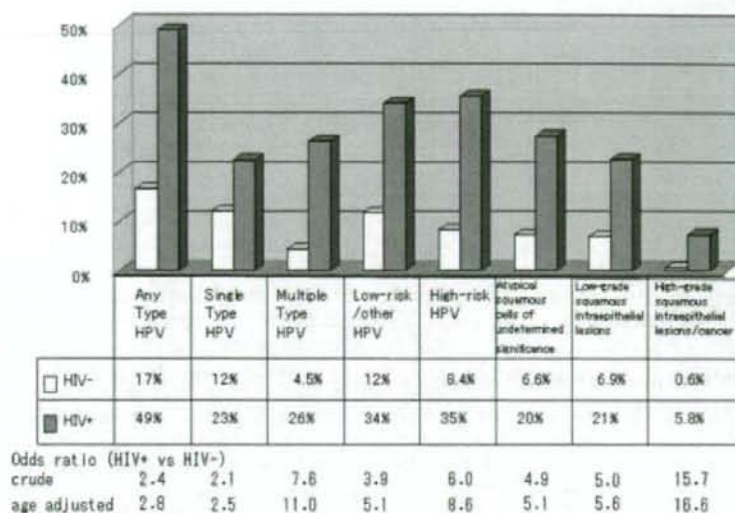


Fig. 2. HPV types and infection patterns, and cervical abnormalities in HIV negative/HIV positive women.

squamous intraepithelial lesion, 0.6%). The age-adjusted odds ratios for atypical squamous cells undetermined significance/atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesion, low-grade squamous intraepithelial lesion, and high-grade squamous intraepithelial lesion in HIV positive women compared to HIV negative women were 5.1, 5.6, and 16.6, respectively. These results suggest that HIV infection increases the susceptibility to HPV infection and increases the risk of cervical abnormalities.

Association Between HIV and Cervical HPV Infections and Abnormal Cervical Lesions

To determine the risk factors for HIV infection, the multivariate analysis using a logistic regression model was carried out. Forward and backward stepwise analyses showed that age >25 years, taking medication, age <20 years at the beginning of sexual activity, more than two life-time sex partners, a past history of condyloma or sexually transmitted diseases (STDs), cervical HPV infection, and cervical abnormalities were independently associated with HIV infection. In contrast, subjects who were visiting family planning clinics were at lower risk for HIV infection (Table I). History of education and present employment were not associated with the risk of HIV infection.

The same analysis to determine the risk of cervical abnormalities, including low-grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, and cancer (Table II), revealed that HPV infection was a factor, although past history of STDs or HIV infection were also associated with it in the univariate analysis. The latter factors were associated

with cervical abnormalities depending on HPV infection, because they became significant in the analysis after excluding the factor "HPV infection."

Relationships Between HIV Immunosuppression, HPV Infection, and Development of Cervical Lesions

The relationship between immunosuppression and HPV infection was evaluated. Among HIV positive women, those who were infected with HPV had a significantly lower number of CD4⁺ T leukocytes in the blood ($P < 0.0001$, Mann-Whitney test), and a significantly higher HIV RNA copy number (HIV load; $P < 0.05$, Mann-Whitney test) than those who were HPV-negative. Women with low-risk, high-risk, and multiple-type HPV infections had a significantly lower CD4⁺ T cell count and higher HIV RNA copy number (Fig. 3).

An analysis of cervical abnormalities showed that the CD4⁺ T cell count was significantly lower ($P = 0.021$) in women with high-grade squamous intraepithelial lesion than in those with a normal cervix, whereas the CD4⁺ T cell count did not differ significantly between women with low-grade squamous intraepithelial lesion and those with a normal cervix ($P = 0.094$; Fig. 4). The HIV RNA copy number was not significantly higher in women with either high- or low-grade squamous intraepithelial lesion compared to women with a normal cervix ($P > 0.1$; Fig. 4). Susceptibility to cervical HPV infection was related to immunosuppressive status, as represented by a low CD4⁺ T cell count and high HIV load. Development of abnormal cervical lesions was also related to immunosuppressive status, as represented by a low CD4⁺ T cell count.

TABLE I. Demographic Factors Associated With HIV Infection

Demographic factors	No. of cases	No. of subjects	%	Univariate analysis		Multivariate analysis		
				ORs	95% CI	ORs	95% CI	P-value
Age								
17-24 years	13	113	12	1		1		
25-61 years	142	375	38	3.29	1.94-5.58	5.89	2.60-14.7	<0.0001*
Taking medication								
No	70	391	18	1		1		
Yes	85	97	88	4.90	3.91-6.13	21.1	10.1-47.5	<0.0001*
Age at first sexual intercourse								
Older than 20 years	23	134	17	1		1		
10-19 years	132	354	37	2.17	1.46-3.23	2.29	1.12-4.81	0.025*
No. of lifetime sexual partners								
0-1	57	247	23	1		1		
More than 2	98	241	41	1.76	1.34-2.32	2.46	1.31-4.68	0.0053*
Present pregnancy								
No	143	403	35	1		1		
Yes	12	85	14	0.39	0.23-0.68	0.46	0.19-1.05	0.073
Visitor to family planning								
No	109	284	38	1		1		
Yes	46	204	23	0.59	0.44-0.79	0.23	0.11-0.45	<0.0001*
History of condyloma acuminata								
No	134	444	30	1		1		
Yes	21	44	48	1.58	1.13-2.22	3.29	1.18-9.14	0.022*
History of STD								
No	76	323	24	1		1		
Yes	79	165	48	2.04	1.58-2.62	2.16	1.12-3.82	0.0083*
Cytology								
Normal	82	368	22	1		1		
Abnormal including ASCUS	73	120	61	2.73	2.15-3.47	2.88	1.50-5.54	0.0014*
HPV infection								
Negative	79	357	22	1		1		
Positive (any types)	76	131	58	2.62	2.06-3.34	3.08	1.61-5.91	0.0007*

*Statistically significant.

TABLE II. Demographic Factors Associated With Cytological Abnormality

Demographic factors	Cervical abnormality			Univariate analysis		Multivariate analysis	
	No. of cases	No. of subjects	%	ORs	95% CI	ORs	95% CI
Age							
17-24 years	13	113	12	1		1	
25-61 years	54	375	14	1.25	0.71-2.21	0.88	0.38-2.03
No. of lifetime sexual partners							
0-5	62	465	13	1		1	
More than 6	5	23	22	1.63	0.73-3.67	0.66	0.18-2.42
Present pregnancy							
No	62	403	15	1		1	
Yes	5	85	6	0.38	0.16-0.92*	0.47	0.15-1.48
Visitor to family planning							
No	40	284	14	1		1	
Yes	27	204	13	0.94	0.60-1.48	1.29	0.64-2.57
History of STD							
No	32	323	10	1		1	
Yes	35	165	21	2.14	1.38-3.33*	1.83	0.94-3.53
HIV							
Negative	25	333	8	1		1	
Positive	42	155	27	3.61	2.29-5.70*	1.81	0.86-3.81
HPV infection							
Negative	79	357		1		1	
Positive (any types)	76	131		9.5	6.07-14.8*	16.1	8.21-31.5*

*Statistically significant.

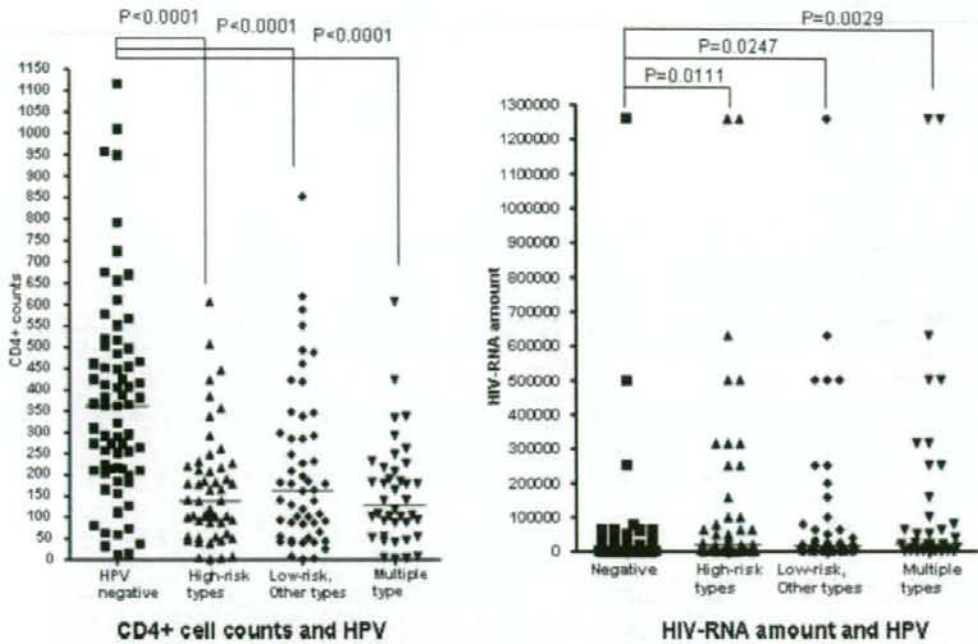


Fig. 3. CD4⁺ T cell counts and plasma HIV-RNA amount in HPV⁻/HPV⁺ women. Each dot represents the CD4⁺ T cell count and plasma RNA amount in each subject. Differences in CD4⁺ T cell counts between the groups were evaluated by the Mann-Whitney test.

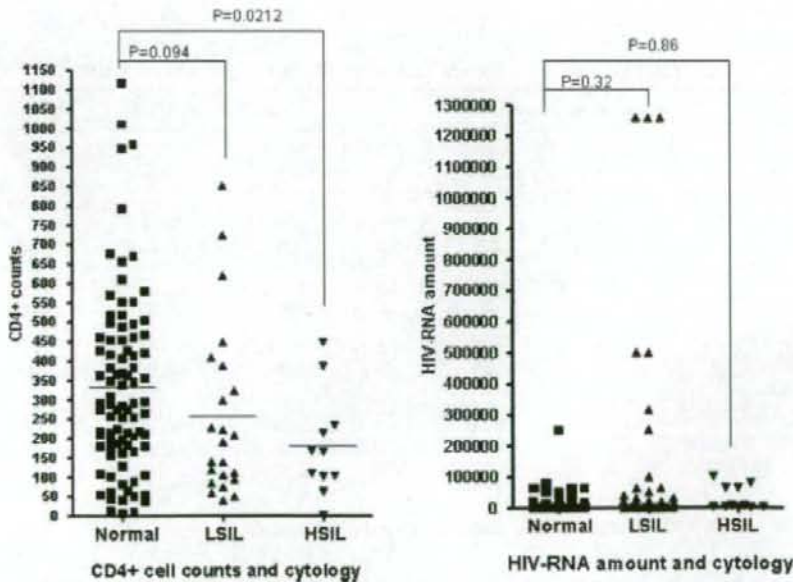


Fig. 4. CD4⁺ T cell count and plasma HIV-RNA amount in abnormal cytology. Each dot represents the CD4⁺ T cell count and plasma RNA amount in each subject. Differences in CD4⁺ T cell counts between the groups were evaluated by the Mann-Whitney test.

DISCUSSION

In the present study, 77% of HIV positive women received no treatment and 23% received treatment within 6 months, suggesting poor control of HIV infection in this population. The relationship between HIV and HPV infections and the development of cervical abnormalities was evaluated.

The prevalence of HIV in this study population was 32%. This is higher than the rate in the general population (12%) of Nairobi [Ministry of Health, Kenya, 2005], suggesting that the subjects may belong to a group at higher risk of HIV infection. This might have been expected. Because this health center has sponsored an AIDS prevention campaign, high-risk subjects would be more likely to have attended the center for voluntary counseling and HIV testing. Further, because the health center is located near a slum town in Nairobi, many of the subjects may have been disadvantaged economically. A multivariate analysis revealed that risk factors for HIV infection included older age, taking medication, <20 years old at sexual debut, having more than two life-time sex partners, a history of condyloma or STDs, cervical HPV infection, and cervical abnormalities, including atypical squamous cells of undetermined significance. Employment and education status were not significant factors. HIV infection cannot be cleared; thus, the number of HIV positive women is likely to increase with age. Sexual contact with a male partner appears to be the most important means of HIV transmission, and thus the number of sex partners and past history of condyloma or STDs were associated with the risk for infection. In the present study, 5% of the subjects had more than six sex partners, and 73% were 10–19 years old at sexual debut. In our previous study in Hokuriku, Japan [Sasagawa et al., 2005], 31% of the subjects had more than six life-time sex partners, and 67% had their sexual debut at 12–19 years of age. In the present study, the number of life-time sex partners was less than that in Japan, but the percentage of those <20 years old at sexual debut did not differ between the two study populations. Thus, the present subjects did not practice sexual behaviors as risky as those of the young Japanese women in the previous study. This suggests that HIV is transmitted readily to women from men in an area where HIV is highly prevalent. Cervical HPV infection and cervical abnormalities were independent risk factors for HIV infection in this study (Table I). Among HIV negative women, cervical HPV infection was more prevalent in younger women (Fig. 1), while the HIV positive rate in women increased with age (Fig. 1), suggesting that cervical HPV infection preceded HIV infection in some HIV positive women. HPV infection in the cervix may increase the risk of HIV transmission by sexual contact, although this hypothesis should be clarified in a future prospective study.

The prevalence of HPV was 49% in HIV positive women and 17% in HIV negative women in this study. This is similar to previous findings of HPV prevalence of 41% in HIV positive women and 14% in HIV nega-

tive women in Nairobi [Temmerman et al., 1999]. In Zimbabwe, the HPV prevalence was 54% in HIV positive women and 27.6% in HIV negative women [Baay et al., 2004]. Our multivariate analysis demonstrated that HIV infection was the most important risk factor for cervical HPV infection (data not shown). This relationship might be different in a population with lower prevalence of HIV infection. Many life-time sex partners, younger age at sexual debut, and history of STD are risk factors for HPV infection [Ho et al., 1998; Sasagawa et al., 2005]. The impact of sexual behavior on HPV infection may be attenuated by a high prevalence of HIV infection. High-risk and multiple-type HPV infections were significantly increased in HIV positive women in the present study, and this was similar to previous findings [Ahdieh et al., 2000]. It has been reported that immunosuppression induced by HIV not only allows cervical HPV infection but also leads to reactivation of HPV infection [Strickler et al., 2005]. Although it is not known whether either mechanism is responsible for an increased prevalence of HPV infection, it was found that a low CD4⁺ T cell count and high HIV load were strongly associated with cervical HPV infection and that a low CD4⁺ T cell count was associated with the development of cervical lesions. These findings were similar to those of previous studies [e.g., Clark et al., 1993]. The prevalence of oral, anal, and cervical HPV infection in HIV positive compared to HIV negative individuals increases with progressively lower CD4⁺ T counts, as does the incidence of high-grade intraepithelial neoplasia [Palefsky, 2006]. It is possible that attenuated HPV-specific immune responses induced by HIV infection allow for persistent HPV infection and premalignant lesions, thus providing sufficient time for the accumulation of genetic changes important for progression to cancer. However, late-stage cancer invasion is not influenced greatly by the immune status [Frisch et al., 2000]. In the present study, the CD4⁺ T cell count, rather than HIV load, was associated strongly with the presence of abnormal cervical cytology, in contrast to the findings of previous studies [e.g., Massad et al., 1999]. A low CD4⁺ T cell count may be associated more strongly with established immunosuppression rather than a high HIV RNA copy number (viral load).

Temmerman et al. [1999] reported a 3.6-fold increased risk for high-grade squamous intraepithelial lesions associated with HIV-1 in women who attended a family planning clinic in Kenya. We found low-grade squamous intraepithelial lesions in 6.9% and 21% of HIV negative and HIV positive women, respectively, and high-grade squamous intraepithelial lesions in 0.6% and 5.8%, respectively. One case of cervical cancer was observed in a HIV positive woman. The incidence of cervical intraepithelial neoplasia and cervical cancer is considered to be very high in Nairobi. The age-adjusted odds ratio for high-grade squamous intraepithelial lesions in HIV positive versus HIV negative women was 16.6, and there was a strong association between HIV infection and the presence of high-grade squamous intraepithelial

lesions. Some cervical cancer and high-grade squamous intraepithelial lesion cases may have been missed in the previous study in Nairobi, because in many cases, the conditions are asymptomatic and would not likely be detected without cervical cancer screening or an HPV DNA test.

The implementation of nationwide cervical cancer screening, and HIV screening, is required urgently for the promotion of health in women of reproductive age in Nairobi. The introduction of a HPV DNA test as a first screening for cervical cancer may be cost-effective in this area [Kuhn et al., 2000]. High-risk HPV infection and low CD4⁺ T cell count may be good markers for predicting women at high-risk of cervical cancer or precursor lesions.

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CASE REPORT

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HIV-2 amino acid substitutions in Gag and Env proteins occurring simultaneously with viral load upsurge in a drug-naïve patient

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Abstract It has been reported that the peptides of human immunodeficiency virus type 2 (HIV-2) most frequently recognized by cytotoxic T lymphocytes are firstly in Gag and secondly in Env proteins. In the present case study, we attempted to observe amino acid substitutions in Gag and Env proteins and related parameters possibly associated with an increase in HIV-2 load. A sudden, eightfold, increase in HIV-2 load occurred in a drug-naïve patient with human leukocyte antigen-B*5801 during the last phase of a longitudinal observation period from months 29 to 40. The genetic diversity of Gag and Env increased gradually prior to the HIV-2 load increase. The proportions of synonymous substitutions in both Gag and Env were greater than the proportions of nonsynonymous substitutions at every sampling point for 40 months, and the net charge of the V3-loop increased from months 29 to 40. Three amino acid substitutions (V286I in Gag, K303T and N337 K/R in Env) were observed from months 29 to 40. Only one amino acid substitution (V286I) was observed with an increase in HIV-2 load in the Gag region where the clustering of epitopes was reported. These results suggest that the sites encompassing these three substituted positions are candidates for HIV-2 epitopes, although further careful examinations will be required.

Key words HIV-2 · Viral load · Amino acid substitution · Gag protein · Env protein

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Introduction

In the design of vaccine candidates that could be broadly protective across HIV subtypes, we need to increase our knowledge of the immune mechanism required for the regulation of the highly divergent viruses, human immunodeficiency virus type 1 (HIV-1) and HIV-2, and to understand how the virus variability can be overcome. Reports have indicated several differences between the characteristics of HIV-1 and HIV-2. The majority of HIV-2-infected individuals with different human leukocyte antigen (HLA) molecules possess a dominant cytotoxic T-cell response able to recognize HIV-1 epitopes.¹ Also, HIV-2 has been characterized by amino acid sequences that are less frequently changed² at undetectable or stable low levels of viral load,³ although the HIV-2 proviral load is similar to that of HIV-1 at the same disease stage. Control by a specific host immune response could explain plasma viral suppression.⁴ However, the contribution of antigen-specific immune responses to the control of HIV-2 viremia has not yet been fully defined. Only recently, a study reported phenotype parameters such as the *in vivo* frequency of an HIV-2-specific T-cell response,⁵ where the most frequently recognized peptides were in Gag proteins (87.5% of patients) followed in frequency by Env proteins (51.6%). This study⁵ also demonstrated that Gag-specific interferon (IFN)- γ secretion dominated the immune response (66%), followed by Env-specific IFN- γ secretion (16.3%). Moreover, the most frequently targeted epitopes were clustered in a narrow part of the HIV-2 Gag (175–323) region. Thus, this study⁵ demonstrated the importance of Gag and Env, especially Gag (175–323) as the cytotoxic T-lymphocyte (CTL) target for the regulation of HIV-2.

It is expected that the importance of these peptides will be analyzed further in different populations through longitudinal studies, as well as through cross-sectional studies. Accordingly, in the present case study, we attempted to observe amino acid substitutions in Gag and Env, and related parameters, such as genetic diversity, possibly associated with a viral load increase.

Case report

The studied patient was a 35-year-old married man who had sexual contacts with multiple partners. He was referred to a clinic in Mumbai, India, and had his first medical examination there in August 1995, and then samples were collected periodically, with informed consent from him. His last visit was in January 1999 and he has been untraceable since then. His HIV-2 serological status was monitored until January 1999, using Serodia HIV 1/2 (Fujirebio, Tokyo, Japan). No antiretroviral drugs were given during the observation period. The patient's HLA type was A*0101/2402 and B*0705/5801 (or B*0706/5801). However, other immune status markers, such as the number of CD4-positive T lymphocytes, could not be evaluated.

Total DNA was extracted from cryopreserved peripheral blood mononuclear cells with the SMITEST EX-R&D kit (Genome Science Laboratories, Fukushima, Japan). Polymerase chain reactions (PCRs) for *gag* and *env* were performed as reported elsewhere.^{5,7} Three and two PCR products, respectively, for *gag* and *env* were cloned into a vector, using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), and sequenced as described previously.⁸

The distances of pairwise comparison of deduced Gag and Env amino acid sequences were calculated with the ClustalW analyzing system, using the Kimura two-parameter method, and the average of the distances was used as an evaluation measure of the genetic diversity of the strains.

The number of synonymous versus nonsynonymous base substitutions was calculated using the Synonymous Nonsynonymous Analysis Program,⁹ according to the instructions, after the sequences were provided with codons aligned by ClustalW.⁸

The net charge of the V3 loop of HIV-2 was calculated according to the formula:

$$\begin{aligned} & [((\text{Number of arginine and lysine residues}) \times 1) \\ & + ((\text{the number of glutamic acid and aspartic acid} \\ & \text{residues}) \times (-1))].^{10,11} \end{aligned}$$

A long terminal repeat (LTR) fragment of the HIV-2 provirus was amplified with OG01 and OG24 primers¹² and cloned into the pCR2.1-TOPO vector (Invitrogen) to construct an HIV-2 RNA transcription vector (pOG01/24). Fragments including a control sequence of HIV-2 RNA (382 bases, from +1 to the *Hind* III site) for an in-house real-time PCR were then transcribed using Riboprobe in vitro Transcription Systems (Promega, Madison, WI, USA). The concentration of RNA transcripts was calculated by the following equation:

$$\text{RNA (copies/ml)} = (\text{OD}_{260} \times \text{dilution rate} \times 40 \times 10^{-6}) \times (6.02 \times 10^{23}) / (1.3 \times 10^5)$$

$$1.3 \times 10^5: \text{Average molecular weight of the RNA (g/mol)} \\ 6.02 \times 10^{23}: \text{Avogadro constant (copies/mol)}$$

The patient's HIV-2 RNA was extracted from a plasma sample (100 μ l), using the SMITEST EX-R&D kit (Genome

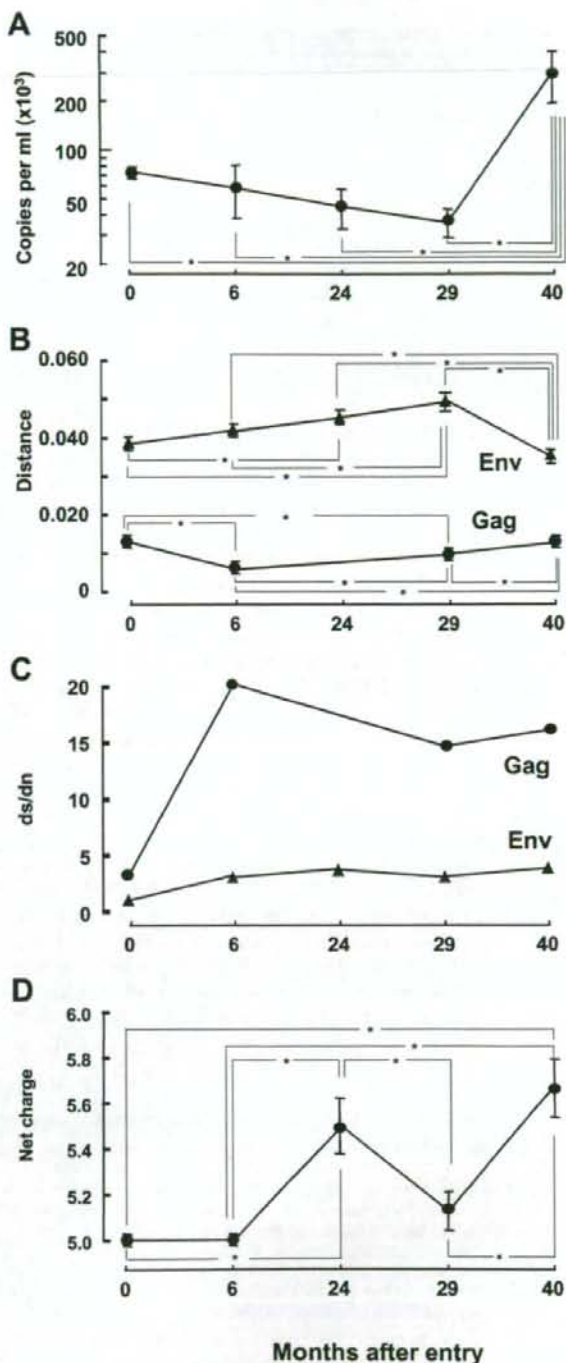
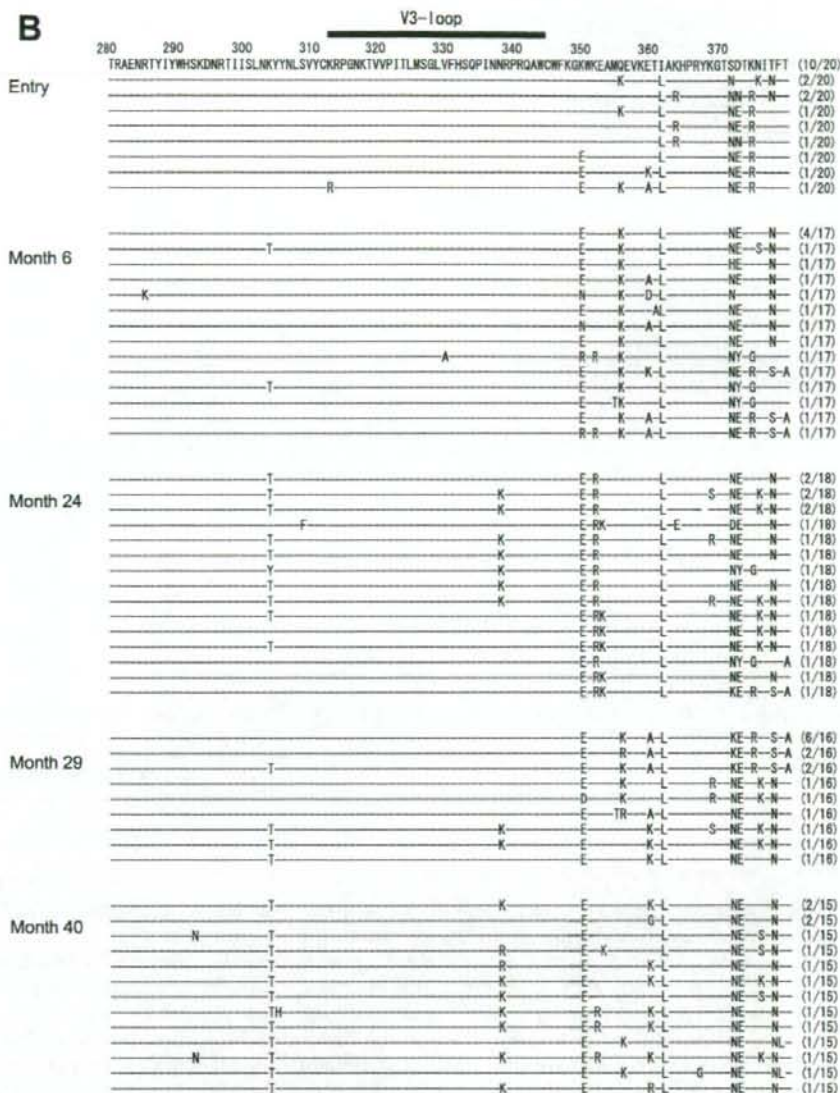


Fig. 1. A HIV-2 load; B the genetic diversity of strains; C the ratio of synonymous (*ds*) to nonsynonymous substitutions (*dn*); D, net charge of the V3-loop. The results are presented as means (A to D) with SE (A, B, and D). The HIV-2 load is shown on a logarithmic scale. **P* < 0.01.

Fig. 2. Continued



Discussion

The main findings of the present case study are the sudden increase in the HIV-2 load and the three amino acid substitutions found within Gag (V286I) and Env (K303T, N337 K/R). The studied Gag¹²⁶⁻³⁰⁶ (amino acid position, 126-306) mostly contained the region of Gag¹⁷⁵⁻³²³ where the most frequently targeted epitopes were found to be clustering in a large-scale cross-sectional study.⁵

The MHC class I allotype HLA-B*5801 had been identified as the restricting element for the HIV-2 Gag epitope (Gag¹⁸²⁻¹⁹⁰ of HIV-2_{ROD} TPYDINQML (TPY) and Gag²⁴¹⁻²⁵⁰ of HIV-2_{ROD} TSTVEEQIQW (TSTV)) and for the

equivalent epitopes in HIV-1 (Gag¹⁸⁰⁻¹⁸⁸ of HIV-1_{HXB2} TPQDLNMMML (TPQ) and Gag²⁴⁰⁻²⁴⁹ of HIV-1_{HXB2} TSTLQEQIQW (TSTL)).^{1,14,15} In addition, Gag²⁸⁶⁻³⁰⁴ of HIV-2_{ROD}, which is known as the major homology region (MHR), and is immuno-dominant, widely prevalent among different ethnic groups,¹⁶ highly conserved across all known retroviruses, and is also essential for viral assembly, maturation, and infectivity,¹⁷ has also been recognized as one of the important immune targets. These regions - TPY, TSTV, and MHR - are within the region of Gag¹⁷⁵⁻³²³ where the most frequently targeted epitopes cluster.⁵ Only one amino acid substitution (V286I in Gag) within the MHR was observed with the increase in HIV-2 load, and the substitution rate (35%) was not as high as expected. In the subject of our

case study, no other apparent substitution (including TPY and TSTV) was found in the tested region of Gag.¹²⁶⁻³⁰⁶

Two substituted positions in Env, K303T (87%) and N337 K/R (67%), could not be found in the reported HIV-2 epitopes, although a number of corresponding amino acid positions are located within the HIV-1 epitopes in the Los Alamos database.

These results suggest that the sites encompassing these three substituted positions (286 in Gag; 303 and 337 in Env) are candidates for HIV-2 epitopes, although further careful examinations will be required.

Acknowledgments The authors are grateful to Dr. S. Jujar for sample collection and other technological assistance. The Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research, 07044264, 10044253) supported this work.

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Molecular Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) and HIV-2 in Yaoundé, Cameroon: Evidence of Major Drug Resistance Mutations in Newly Diagnosed Patients Infected with Subtypes Other than Subtype B^V

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Prior to current studies on the emergence of drug resistance with the introduction of antiretroviral therapy (ART) in Cameroon, we performed genotypic analysis on samples from drug-naïve, human immunodeficiency virus (HIV)-infected individuals in this country. Of the 79 HIV type 1 (HIV-1) *pol* sequences analyzed from Cameroonian samples, 3 (3.8%) were identified as HIV-1 group O, 1 (1.2%) was identified as an HIV-2 intergroup B/A recombinant, and the remaining 75 (95.0%) were identified as HIV-1 group M. Group M isolates were further classified as subtypes A1 ($n = 4$), D ($n = 4$), F2 ($n = 6$), G ($n = 12$), H ($n = 2$), and K ($n = 1$) and as circulating recombinant forms CRF02_AG ($n = 41$), CRF11_cpx ($n = 1$), and CRF13_cpx ($n = 2$). Two *pol* sequences were identified as unique recombinant forms of CRF02_AG/F2 ($n = 2$). M46L ($n = 2$), a major resistance mutation associated with resistance to protease inhibitors, was observed in 2/75 (2.6%) group M samples. Single mutations associated with resistance to nucleoside reverse transcriptase inhibitors (T215V/F [$n = 3$]) and nonnucleoside reverse transcriptase inhibitors (V108I [$n = 1$], L100I [$n = 1$], and Y181C [$n = 2$]) were observed in 7 of 75 (9.3%) group M samples. None of the patients had any history of ART exposure. Population surveillance of transmitted HIV drug resistance is required and should be included to aid in the development of appropriate guidelines.

The current standard for antiretroviral drug therapy (ART) in developed countries is the combination of two nucleoside reverse transcriptase (RT) inhibitors (NRTIs) plus a non-nucleoside RT inhibitor (NNRTI) or a protease inhibitor (PI). Since the successful trials in the late 1990s, combination ART has benefited and continues to aid many human immunodeficiency virus type 1 (HIV-1)-infected patients in developed countries, and it is becoming increasingly available in resource-constrained countries (17, 20, 24, 29, 30).

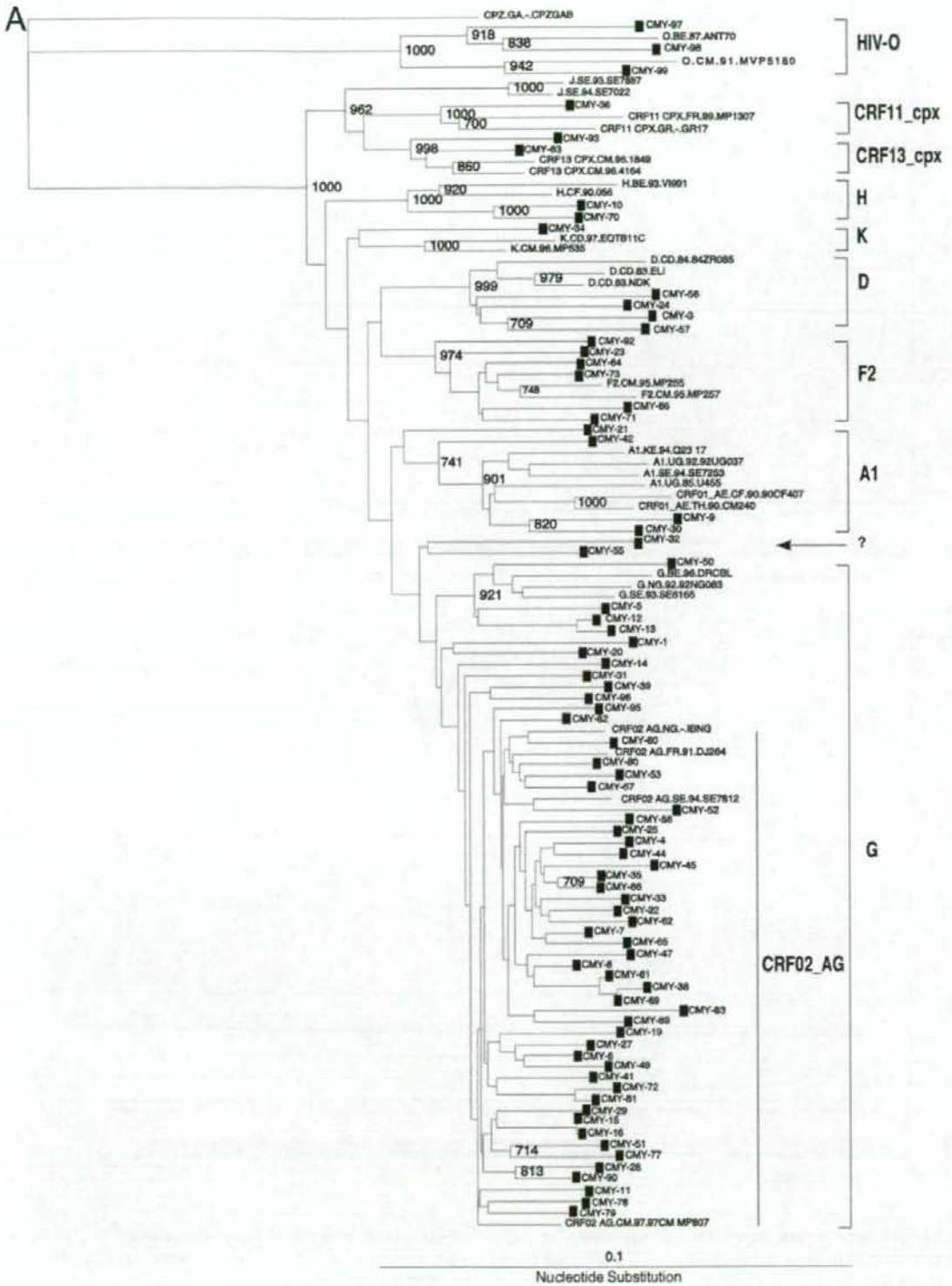
In countries with multiple antiretrovirals (ARVs) readily available, the prevalence of drug-resistant variants has ranged from 10 to 20% among drug-naïve patients (33), while in resource-constrained areas, resistance in the untreated HIV-infected population is rarely reported (23, 31). Recent interventions through such programs as the World Health Organization (WHO)'s 3 by 5 plan to treat 3 million people by the end of 2005 (33a) and the President's Emergency Plan for AIDS Relief have promoted significant access to ART in low- and middle-income countries. As of June 2005, about 500,000 people in sub-Saharan Africa were receiving ART, although the regional coverage rate was still 11% of the estimated number of patients with CD4 cell counts of ≤ 300 /ml (2% of all

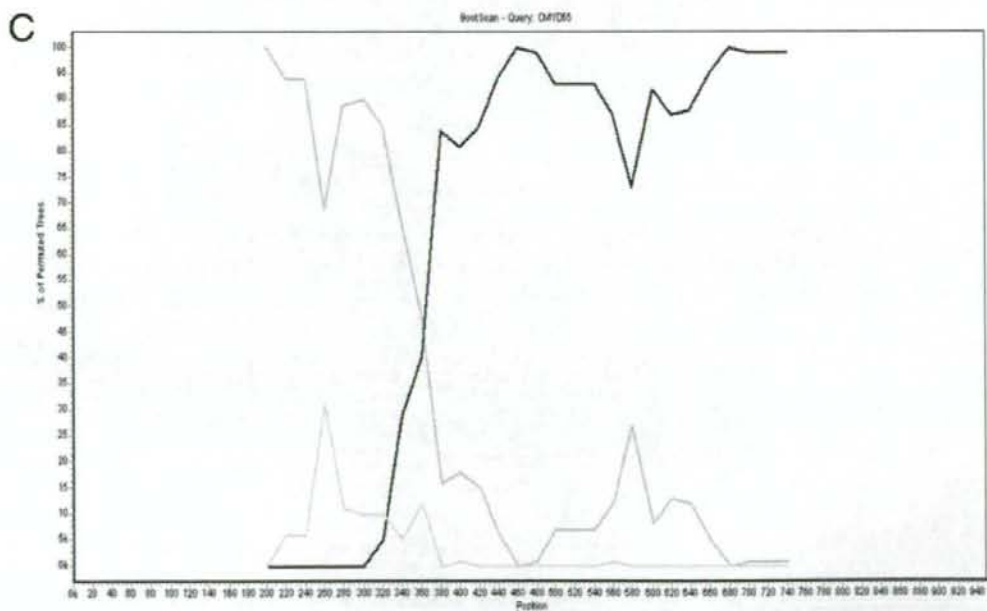
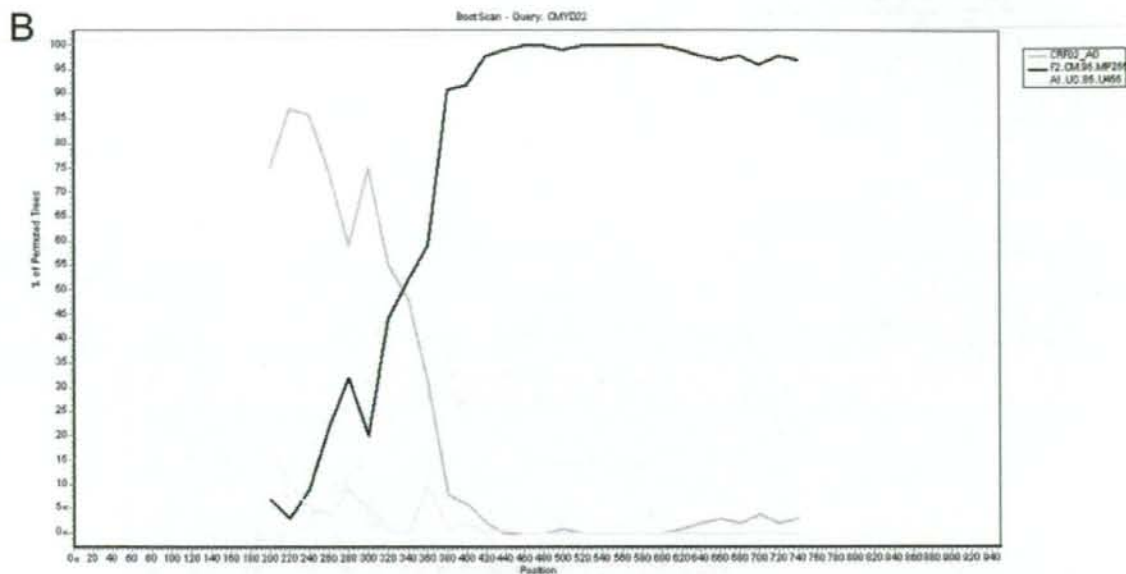
HIV-infected patients in this region) (33a). Developing countries, including Cameroon, are moving towards universal access to HIV prevention, care, and treatment for those in need and at high risk of infection. This has led to the widespread use of antiretroviral drugs through structured national ART scale-up plans. Because of the complexity and open-ended duration of HIV treatments and the need to begin programs to treat many patients quickly, fears have been raised that emergence of ARV resistance may become a serious public health concern and render anti-HIV drugs useless. To assist ART programs and to minimize the emergence and transmission of HIV drug resistance strains and their public health consequences, WHO has developed a minimum-resource strategy for the surveillance and monitoring of HIV drug resistance in resource-limited countries. In Kenya, for example, where ART has been provided for 12 to 17% of the estimated need, the prevalence of resistant strains among drug-naïve patients has recently risen from 1% (2002) to more than 5% (2003) (WHO, personal communication). In Botswana, where treatment is available to all patients with < 300 CD4 cells/ml, the prevalence of major mutations conferring PI resistance was estimated to be 4% among drug-naïve patients (4).

Unlike the case in southern and eastern African countries, where one or two HIV-1 subtypes dominate (22), all major groups and subtypes of HIV-1 cocirculate in Cameroon (1, 6, 14–19, 21, 22, 24, 28, 34–38). According to WHO/UNAIDS, as of the end of 2004, the prevalence of HIV-1 infection was estimated to be 4.8% overall and 9.8% for adults. To date,

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there have been several reports on the prevalence of ARV resistance mutations in the drug-naïve HIV-1-infected population of Cameroon (1, 6, 14–16, 19, 31). Baseline information on the frequency and types of ARV resistance mutations in

Cameroon will help to inform optimal ART and enable the government to monitor the success of the national AIDS treatment program.

ART in Cameroon is based on the WHO guidelines, i.e., the

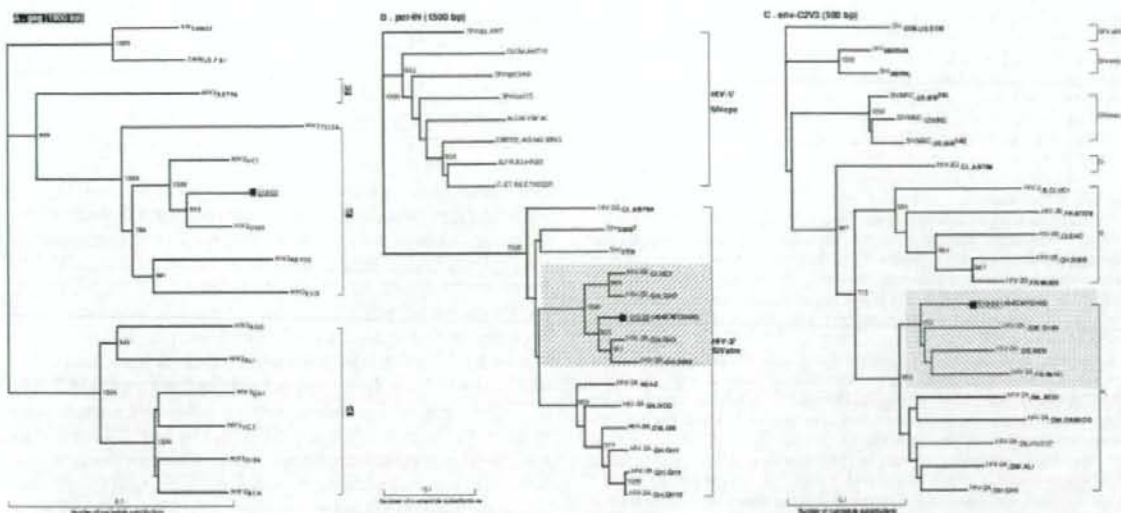


FIG. 2. Phylogenetic trees based on *gag-p17/p24* gene (1,900 bp) of HIV-2 (510-03/04CMYD-100) subtype B/A recombinant strain (A) and the *pol-IN* (1,500 bp) gene (B) and *env-C2V3* (500 bp) gene (C) from the Cameroonian HIV-2 strain. The bootstrap value at each node represents the number among 1,000 replicates that supported the branching order. Bootstrap values of >70% are shown. The brackets on the right represent the major HIV-2 subtypes. The newly analyzed sequence (510-03) is marked with a filled square.

combination of two NRTIs and one NNRTI. With the rapid introduction of ART and with limited health care infrastructure for care and monitoring, this country may face similar emergence rates of ARV resistance to those described for other developing countries (29, 30). With a higher prevalence of ARV resistance in the drug-naïve population (18, 32), resistance may emerge at an even higher rate.

In this study, we evaluated the prevalence of drug-resistant HIV-1 strains in treatment-naïve HIV-1-infected individuals in a resource-limited country where ART is being scaled up rapidly to determine whether standard first-line regimens will continue to be effective. Samples were obtained prior to the roll-out of significant ART programs in Yaoundé, the capital city of Cameroon. We examined the prevalence of ARV resistance mutations in 79 patient samples and found a low rate of major drug resistance mutations to RTIs and PIs.

MATERIALS AND METHODS

Study population. Blood specimens were drawn in 2004 from newly diagnosed HIV-1 patients attending a clinic in Yaoundé, Cameroon. All participants provided written informed consent and were likely to be recently infected. Sera found to be reactive for HIV by enzyme-linked immunosorbent assay confirmed with Western blotting were included in this study to explore the prevalence of intrinsic resistance to ARV drugs from treatment-naïve patients. This study received ethical clearance from the National Ethics Committee of Cameroon. Exclusion criteria included any previous form of ARV treatment, including that given to women for prevention of mother-to-child transmission.

PCR and sequencing. Peripheral blood mononuclear cells (PBMCs) from HIV-seroreactive blood donors were obtained by Ficoll-Hypaque density gradient centrifugation. Proviral DNA was extracted from uncultured PBMCs with a DNA extraction kit (Qiagen, Hilden, Germany). Nested PCR amplification was performed using AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, NJ). A segment of the PR-RT region of the *pol* gene was first PCR amplified using the universal external primers univ-PS1 (TTTTTTAGGGAAA ATTTGGCCTTC) and univ-RTA4 (CTGTATATCATTGACAGTCCAGCT), resulting in a 1.2-kbp product. Nested PCR was then performed with the uni-

FIG. 1. (A) Phylogenetic tree of HIV-1 PR-RT sequences from 78 HIV-1 group M and O isolates. "CMY" refers to PR-RT sequences from the cross-sectional analysis and indicates the country (Cameroon) and location (Yaoundé) of sample collection. The bootstrap value at each node represents the number among 1,000 bootstrap replicates that supported the branching order. Bootstrap resampling values of 70% or higher are shown. Brackets on the right represent the major group M subtypes. Newly derived sequences are marked with filled squares, and the novel unique recombinant form CRF02_AG/F2 is shown by an arrow. A 950-nt segment of the PR-RT coding region was used to construct this tree by the neighbor-joining method. PR-RT genetic subtypes A, D, F, G, H, and K and recombinants CRF02_AG, CRF11.cpx, and CRF13.cpx, as well as HIV-1 group O, are indicated. GenBank accession numbers for the reference sequences are as follows: A1.KE.93.Q23-17, AF004885; A1.UG.85.U455, M62320; A1.UG.92.92UG037, U51190; D.CD.83.ELL, K03454; D.CD.83.NDK, M27323; DCD.84.84ZR085, U88822; F2.CM.95.MP257, AJ249237; G.NG.92.92NG083, U88826; G.SE.93.SE6165, AF061642; G.BE.96.DRCBL, AF084936; H.BE.93.V1991, AF190127; J.SE.93SE7887, AF082394; J.SE.94.SE7022, AF082395; K.CM.96.MP535, AJ249239; K.CD.97.EQTB11C, AJ249234; O1.AE.TH.90.90CM240, U54771; O1.AE.CF.90.90CF4071, AF197341; O2.AG.NG.-IBNG, L39106; O2.AG.FR91.DJ264, AF063224; O2.AG.SE.94.SE7812, AF107770; O2.AG.CM.97.97CM.MP807, AJ251056; 11.CPX.CM.97.MP818, AJ291718; 13.CPX.CM.96.1849, AF460972; 13.CPX.CM.96.4164, AF460974; O.CM.-ANT70, L20587; O.CM.91.MVP5180, L20571; and CPZ.GA.-CPZGAB, X52154. (B and C) SimPlot analyses of unclassifiable Cameroonian PR-RT (approximately 1,000 nt) sequences 04CMY-32 (B) and 04CMY-55 (C), showing the recombination between subtype F2 and CRF02_AG (A). The bootscan analysis was performed against reference strains from clades A (strain A1.UG.85.U455), B (strain B.US.83.RF), D (strain D.CD.84.84ZR085), F1 (strain F1.FI.93.FIN9363), F2 (strain F2.CM.95.MP255), G (strain G.SE.93.SE6155), and O2_AG (strain AG.NG.-IBNG). (D) Segments derived from an IBNG-like strain and subtype F2 are shown.

versal primers univ-PS2 (5'-TCCTCAAACTCACTCTTTGGCAAC-3') and univ-RTA3 (5'-TTCATAACCCATCCAAAGAAATGG-3') to generate a fragment of 1.0 kbp. The PCR products were then purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced in the sense and antisense directions with a set of nested primers (25). All sequencing reactions were performed using an ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and an ABI 3730 DNA sequencer by Davis Sequencing, Inc. The chromatogram files were read using the Chromas 1.6 program (Helensvale, Australia). All sequences were edited with the BioEdit program.

Phylogenetic analysis and subtyping. Neighbor-joining phylogenetic trees including reference *pol* sequences were constructed using Clustal W and then drawn using Treeview PPC, version 1.6.6 (Institute of Biochemical and Life Sciences, Scotland, United Kingdom). Bootstrap resampling (1,000 data sets) of multiple alignments was performed to test the statistical robustness of the trees. Kimura-2 parameters were calculated with the DNADIST program in the PHYLIP package (13, 27).

Genotypic resistance analysis. Genotypic resistance was defined as the presence of one or more resistance-related mutations, as specified by the consensus mutation figures of the International AIDS Society—USA (11). The emergence of amino acid substitutions associated with resistance to RTIs and PIs has been characterized extensively, and these substitutions can be classified into major and accessory/minor (modifying) mutations. Major mutations lead to severalfold decreases in sensitivity to one or more ART drug. Accessory mutations may not result in a significant decrease in sensitivity but are associated with an increase in viral fitness (replication capacity) (9). Although resistance testing was performed retrospectively, for ethical reasons these results were fed back to the clinicians at the study site regarding the relative merits of change in therapy.

Nucleotide sequence accession numbers. The DNA sequences of HIV-1 *pol* PR-RT regions determined as part of this study were submitted to GenBank under the following accession numbers: DQ990400 to DQ990455.

RESULTS

HIV-1 subtype distribution. Seventy-nine HIV-infected samples from drug-naïve patients were obtained in 2004. A 1.0-kbp fragment encompassing amino acids 1 to 99 of PR and 1 to 234 of RT was PCR amplified and sequenced as described above. Sequences were then aligned and phylogenetic trees constructed to classify the different HIV sequences into groups, subtypes, and recombinant forms (Fig. 1A). Three sequences (3.8%; 95% confidence interval [CI], 1.6 to 5.9%) belonged to HIV-1 group O, and 75 sequences (94.9%; CI, 94.8 to 95.0%) were identified as HIV-1 group M. Group M isolates were further classified into the following six subtypes and three circulating recombinant forms (CRFs): subtypes A1 ($n = 4$), D ($n = 4$), F2 ($n = 6$), G ($n = 12$), H ($n = 2$), and K ($n = 1$) and CRF02_AG ($n = 41$), CRF11_cpx ($n = 1$), and CRF13_cpx ($n = 2$), with an intersubtype recombinant, CRF02_AG/F2 ($n = 2$). The two CRF02_AG/F2 isolates were identified using SimPlot for bootscanning analysis (Fig. 1B and C), with a 400-nucleotide (nt) rolling window and a significance threshold of 95% over the 1,000-bp PR-RT gene. Figure 1D shows the SimPlot output and a schematic representative plot and indicates that samples CMYD-32 and CMYD-55 have different breakpoints in the PR-RT gene, at 350 nt and 425 nt, respectively.

HIV-2 intersubtype B/A recombinant. One sample was seropositive for HIV infection but could not be PCR amplified by our set of primers. PCR amplification and subsequent DNA sequencing with a set of HIV-2-specific primers confirmed the identity of this isolate as not only HIV-2 but also the first documented case of an HIV-2 intersubtype B/A recombinant, based on *gag-p17/pol-IN/env-C2V3* sequence analyses, with three breakpoints in the *env-nef* gene (Fig. 2). The HIV-2 isolate (510-03) was subtype B based on *gag* and *pol* sequences,

while the *env-nef* region is an intersubtype recombinant of subtypes A and B, with three recombination breakpoints identified. Further data analyses are in progress (N. Ndambi and C. Brennan, unpublished data).

PI resistance-associated mutations. The amino acid sequence of each strain was compared to the subtype B consensus amino acid sequence, using the published HIV drug resistance algorithm from the International AIDS Society (10, 11) for mutations associated with resistance to PIs and RTIs. Based on subtype B sequences, drug resistance mutations in the protease region at positions 10, 13, 16, 20, 24, 30, 32, 33, 34, 36, 43, 46, 47, 48, 50, 53, 54, 58, 60, 62, 63, 64, 71, 73, 76, 77, 82, 84, 85, 88, 89, 90, and 93 (11), i.e., 33 mutations in total, have been shown to be associated with resistance to PIs.

Primary PI resistance-associated mutations were found in 2 of 75 cases (2.6%). These two patients harbored a CRF02_AG or CRF13_cpx HIV-1 isolate with an M46L amino acid substitution in the protease coding region. The M46L mutation in subtype B is associated with resistance to amprenavir, atazanavir (ATV), indinavir, and nelfinavir. The CRF02_AG-infected patient CMY-72 also contained a G48R mutation linked to the M46L mutation in the protease gene. The G48V mutation in subtype B is responsible for saquinavir, ritonavir, and ATV resistance (9). A V82I mutation was detected in the protease sequences of three patients, but the V82I mutation is a minor/accessory mutation and confers only minimal resistance to ATV and ritonavir (10). An alanine, threonine, phenylalanine, or serine at this position, however, is responsible for resistance to all PIs. Isoleucine at position 82 is also a naturally occurring polymorphism in subtype strains (9, 23) and was observed in 3 of 12 (25%; CI, 5.5 to 57.2%) of our G isolates. Minor or accessory PI resistance mutations were also found as wild-type sequences in Cameroonian isolates at the following positions, in order of decreasing frequency: M36I (74/75 isolates; 98.7%), K201M/R (67/75 isolates; 89.3%), L10V (5/75 isolates; 6.7%), L63P (4/75 isolates; 5.3%), and D60E (4/75 isolates; 5.3%).

RTI resistance-associated mutations. Based on subtype B consensus sequences, mutations leading to resistance to NRTIs and NNRTIs are well defined and differ between the two classes of inhibitors. The most common major RT mutations leading to NRTI resistance occur at positions 41, 62, 65, 67, 69, 70, 74, 75, 77, 115, 116, 151, 184, 210, 215, and 219 (16 in total), and major mutations leading to NNRTI resistance are known to occur at positions 100, 103, 106, 108, 181, 188, 190, 225, (11), and 236 (9 in total).

Of the 79 cases analyzed, 7 (9.3%) showed major mutations associated with resistance to RTIs (zidovudine [ZDV], nevirapine [NVP], delavirdine [DLV], and efavirenz [EFV]). A V108I mutation was found in a CRF02_AG-infected patient, a Y181C mutation was found in a CRF13_cpx-infected patient, and V118C and V179E mutations were found in subtype G isolates. The subtype B mutations V118C and V179E result in moderate NNRTI resistance, whereas Y181C and V108I mutations are responsible for high-level NNRTI resistance (DLV, EFV, and NVP resistance and EFV and NVP resistance, respectively). The L210W mutation in subtype B (ZDV resistance) and the Y181C mutation (in subtype B [NNRTI resistance]) are found as the wild-type sequences in most HIV-1 group O isolates, including the three group O samples from

TABLE 1. Overview of epidemiologic and genetic information for acutely HIV-1-infected subjects in central Cameroon

Patient no.	Age (yr)	Sex ^d	Genetic subtype ^a				Drug resistance-associated mutation(s) ^f			
			GenBank accession no.	Pol-PR	Pol-RT	Unique recombinant form	PR		RT	
							Primary	Secondary	Primary	Secondary
04CMYD1	50	F	DQ990377	G	G			K20I, M36I		
04CMYD3	21	M	DQ990378	D	D			M36I		
04CMYD4	29	F	DQ990379	CRF02_AG	CRF02_AG			K20R, M36I		
04CMYD5	28	F	DQ990380	G	G			K20I, M36I		
04CMYD6	25	F	DQ990381	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD7	45	M	DQ990382	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD8	27	F	DQ990383	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD9	23	F	DQ990384	A1	A1			M36I, D60E, V77I		
04CMYD10	33	M	DQ990385	H	H			K20R, M36I, D60E		
04CMYD11	23	F	DQ990386	CRF02_AG	CRF02_AG			L10I, K20I, M36I		
04CMYD12	21	F	DQ990387	G	G			K20I, M36I, (V82I)		
04CMYD13	23	F	DQ990388	G	G			K20I, M36I		
04CMYD14	40	F	DQ990389	G	G			K20I, M36I		
04CMYD15	34	M	DQ990390	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD16	25	F	DQ990391	CRF02_AG	CRF02_AG			K20I, M36I	V100I	
04CMYD19	29	F	DQ990392	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD20	33	F	DQ990393	G	G			K20I, M36I		
04CMYD21	47	M	DQ990394	A1	A1			M36I		
04CMYD22	43	M	DQ990395	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD23	54	M	DQ990396	F2	F2			M36I		
04CMYD24	28	M	DQ990397	D	D			K20I, M36I		
04CMYD25	14	M	DQ990398	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD27	35	F	DQ990455	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD28	56	M	DQ990399	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD29	45	F	DQ990400	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD30	26	M	DQ990401	A1	A1			M36I		
04CMYD31	46	M	DQ990402	G	G			K20I, M36I, (V82I)		
04CMYD32	30	F	DQ990403	CRF02_AG	F2	CRF02_AG)/F2 ^b		K20I, M36I, V77I		
04CMYD33	40	M	DQ990404	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD34	35	F	DQ990405	K	K			K20R, M36I		
04CMYD35	35	M	DQ990406	CRF02_AG	CRF02_AG			K20I, M36I	Y188C	
04CMYD36	31	F	DQ990407	CRF11_cpx	CRF11_cpx			D60E, V77I		
04CMYD38	49	M	DQ990408	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD39	34	F	DQ990409	G	G			K20I, M36I		
04CMYD41	32	M	DQ990410	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD42	43	F	DQ990411	A1	A1			K20I, M36I, L63P		
04CMYD44	33	M	DQ990412	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD45	36	F	DQ990413	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD47	29	F	DQ990414	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD49	24	M	DQ990415	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD50	35	M	DQ990416	G	G			K20I, M36I		
04CMYD51	28	F	DQ990417	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD52	26	M	DQ990418	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD53	50	M	DQ990419	CRF02_AG	CRF02_AG			K20I, M36I, L63P		
04CMYD55	20	M	DQ990420	CRF02_AG	F2	CRF02_AG)/F2 ^b		K20I, M36I		
04CMYD56	26	M	DQ990421	D	D			L10V, K20R, M36I		
04CMYD57	35	F	DQ990422	D	D			M36I		
04CMYD58	60	F	DQ990423	CRF02_AG	CRF02_AG			K20I, M36I	V108I	
04CMYD60	43	M	DQ990424	CRF02_AG	CRF02_AG			L10V, K20R, M36I		
04CMYD61	21	F	DQ990425	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD62	32	M	DQ990426	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD63	30	F	DQ990427	CRF13_cpx	CRF13_cpx			K20I, M36I, V77I		
04CMYD64	36	M	DQ990428	F2	F2			L10V, K20R, M36I		
04CMYD65	42	F	DQ990429	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD66	28	M	DQ990430	F2	F2			L10V, K20R, M36I		
04CMYD67	35	M	DQ990431	G	G			K20I, M36I, (V82I)		
04CMYD69	43	M	DQ990432	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD70	38	F	DQ990433	H	H			K20R, M36I, D60E		
04CMYD71	44	F	DQ990434	F2	F2			K20R, M36I, D60E		
04CMYD72	48	M	DQ990435	CRF02_AG	CRF02_AG			K20I, M36I	M46L	
04CMYD73	39	F	DQ990436	F2	F2			K20R, M36I		
04CMYD77	22	F	DQ990437	CRF02_AG	CRF02_AG			K20I, M36I	T215Y	
04CMYD78	36	F	DQ990438	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD79	33	F	DQ990439	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD80	55	M	DQ990440	CRF02_AG	CRF02_AG			K20I, M36I, L63P	T215F	
04CMYD81	33	F	DQ990441	CRF02_AG	CRF02_AG			K20I, M36I	T215Y	
04CMYD82	37	M	DQ990442	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD83	47	M	DQ990443	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD86	45	F	DQ990444	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD89	40	F	DQ990445	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD90	24	M	DQ990446	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD92	32	M	DQ990447	F2	F2			M36I, L63P		
04CMYD93	42	M	DQ990448	CRF13_cpx	CRF13_cpx			K20I, M36I	Y181C	
04CMYD95	31	M	DQ990449	CRF02_AG	CRF02_AG			K20I, M36I		
04CMYD96	37	F	DQ990450	G	G			K20I, M36I, L63P		
04CMYD97	23	M	DQ990451	HIV-1 group O	HIV-1 group O			M36L, I93L	Y181C, L210W	
04CMYD98	38	M	DQ990452	HIV-1 group O	HIV-1 group O			M36L, I93L	Y181C, L210W	
04CMYD99	25	F	DQ990453	HIV-1 group O	HIV-1 group O			M36L, I93L	Y181C, L210W	
04CMYD100	28	F	DQ990454	HIV-2 group A	HIV-2 group A	HIV2_B/A		NA	NA	

^a Typing of the *pol* gene (approximately 1,000 bp), encoding the Pol protease (Pol-PR) and Pol reverse transcriptase (Pol-RT) regions.

^b Possible recombination between subtype F and CRF_02 within the region.

^c Amino acid changes denote International AIDS Society (30) recognized mutations, while amino acid changes in parentheses stand for the presence of resistance mutations as minor mutations and subtype G naturally occurring polymorphisms. Primary drug resistance-associated mutations, shown in boldface type, lead to severalfold decreases in sensitivity to one or more ARTs. NA, not analyzed. All HIV group O samples contained Y181C as a natural occurring polymorphism. HIV-2B/A is a new recombinant strain, based on its *gag-p17/24* (1,500 bp), *pol-IN* (1,500 bp), and *env-C2F3* (500 bp) sequences.

^d F, female; M, male.

this cohort, i.e., CMYD-97, -98, and -99 (5, 19, 26). Possible accessory amino acid mutations R211K and G333E in subtype B isolates were also observed in the RT genes of viruses from 54 patients (Table 1).

Dual-class resistance-associated mutations. In one of the CRF13_cpx isolates (1.2%; CI, 0.93 to 1.46%), we identified primary amino acid sites associated with resistance to PIs (M46L mutation [resistance to amprenavir, indinavir, ATV, and nelfinavir]) and NRTIs (Y181C mutation [resistance to DLV, EFV, and NVP]). Further phenotypic resistance would be needed to confirm these genotypic analyses.

DISCUSSION

In the current study, we found 2.6% PI resistance and 9.3% major RTI resistance mutations in HIV-1-infected drug-naïve individuals in Yaoundé, Cameroon. Unlike the case in developed countries, where antiretroviral regimens containing PIs are readily available, the first line of ART in Cameroon is the combination of two NRTIs plus one NNRTI. Very few patients in Cameroon are currently being or have been treated with PIs (1, 16–18). Konings et al. (16) reported that only the minor mutations associated with PI resistance were detected among HIV-1-infected drug-naïve patients in Cameroon during the period of 2000 to 2002. Our study confirmed previous reports and describes a high frequency of minor mutations (isoleucine or valine at position 10 in CRF02_AG; K20I and M36I mutations), which were found in all sequences except one, i.e., the CMY-36 isolate classified as CRF11_cpx. Of greater concern is the appearance of the major PI resistance mutation M46L in two infected patients (one with CRF02_AG and another with CRF13_cpx). The identification of this amino acid mutation in the protease warrants a more thorough screen of CRF02_AG and CRF13_cpx protease sequences, which is currently under way.

Three major NRTI resistance mutations were observed as wild-type sequences in three CRF02_AG (T215Y/F) and one CRF13_cpx (Y118C) virus. The T215Y/F mutation confers resistance to ZDV in nearly all HIV-1 isolates, whereas Y118C is a mutation related to native versus nucleoside analog discrimination but confers only low-level resistance (10, 11, 30). Limited studies on ART drug resistance in Africa, especially for non-B subtypes in Europe, have shown a strong correlation between the presence of major mutations and phenotypic resistance, similar to the case for mutations seen in subtype B infections with similar treatment regimens (31, 33). However, studies have also documented some salient differences among patients infected with non-B subtypes. A study of single-dose NVP to prevent mother-to-child transmission of HIV-1, conducted in Uganda, showed that selection of genotypic mutations associated with resistance to NVP occurred more frequently in women infected with subtype D than in women infected with subtype A viruses (23, 24). In addition, there has been identification of new mutational patterns conferring high-level drug resistance, previously not characterized for subtype B isolates (3, 23, 25, 26). For example, the V106M mutation in subtype C, as opposed to the V106A mutation of subtype B, is generally selected and confers resistance to EFV (3, 4). In addition, a combination of three mutations (I135L, T139V, and V245T) found as “wild-type” sequences in a subtype D

HIV-1 isolate in Uganda conferred over 1,000-fold resistance to NVP and DLV and some cross-resistance to EFV (8). We are currently examining the phenotypic resistance of the PR-RT coding regions of Cameroonian HIV-1 isolates with or without any ARV resistance sequences. Although resistance testing was performed on PBMCs, this is a more sensitive method for detection of archived resistant mutants in persons lacking evidence of resistance by conventional assays.

This study provides the most recent data on molecular characterization of HIV-1 isolates in treatment-naïve individuals in Yaoundé, Cameroon. Overall, there is clear documentation of cocirculating HIV-1 group M and O strains as well as evidence for HIV-2 B/A recombinants, which are the subject of further investigation. At least six genetic subtypes (A, D, F2, G, H, and K) and three CRFs (CRF02_AG, CRF11_cpx, and CRF13_cpx) have been identified in HIV-1-infected patients in Yaoundé. Subtype CRF02_AG was responsible for 51.89% of the infections and was previously identified as predominant in west and west-central Africa (1, 6, 14–19, 21, 22, 24, 28). HIV-2 has been observed with a very low prevalence (0.06% of total HIV infections) in Douala but at a higher frequency in Yaoundé (0.2% to 1.2% of total HIV infections), based on independent epidemiological surveys (28, 36). A higher prevalence of HIV-2 infections was observed in commercial sex workers and tuberculosis patients, with no apparent link to other West African countries (36). However, the origin of the HIV-2 infection in our study was not available (7).

An obvious challenge in resource-limited settings such as Yaoundé, Cameroon, is maintaining a balance between rapid introduction of ART and continual surveillance of drug resistance to prevent treatment failures and to avoid a public health crisis. Expansion of molecular characterization on a nationwide basis would be useful to scientists developing prevention strategies based on vaccines and microbicides. Although there may be a cost factor involved, ART should be accompanied by testing for resistance before the choice of a particular ART regimen is made. This will reduce the selection pressure of resistance types, thus making first-line therapy more effective.

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