can be completed in a single step at a constant temperature without a programmed thermal cycler. LAMP provides highly efficient DNA amplification, up to 10^9–10^{10} times in 15–60 min, and the concentration of the LAMP product is much higher than that generated by conventional polymerase chain reaction (PCR). Conventional PCR is relatively time consuming (3–4 h) and much more complicated than RT-LAMP, requiring several amplification steps and the use of a high-precision thermal cycler. The RT-LAMP assay has been validated and applied to the rapid detection of a number of RNA viruses, such as rubella virus (Mori et al., 2006), Japanese encephali-

Table 1
HIV-1 genotype data for 57 infected individuals from eastern Cameroon and the results of HIV-1 RT-LAMP.

Sample ID	Genetic subtype[a]				LAMP	
	gag	*pol*	*env*-C2V3	gp41	Tt[b]	EP
01CM2213	CRF.01.AE	na[c]	CRF.01.AEA	na	19.2[d]	P[e]
01CF2214	G	U	U	na	25.8	P
01CM2215	CRF.02.AG	na	CRF.02.AG	na	28.7	P
01CM2216	A	na	A	na	21.2	P
01CM2217	CRF.11.cpx	na	CRF.11.cpx	na	26.5	P
01CM2218	CRF.11.cpx	CRF.11.cpx	nd	U	31.0	P
01CM2219	CRF.11.cpx	na	CRF.02.AG	na	No Tt	P
01CM2220	CRF.02.AG	na	A	na	29.2	P
01CM2222	CRF.02.AG	na	CRF.02.AG	na	29.2	P
01CM2223	CRF.01.AE	na	CRF.02.AG	na	26.2	P
01CM2224	CRF.02.AG	na	CRF.02.AG	na	28.8	P
01CM2225	B	na	A	na	24.3	P
01CM2226	CRF.02.AG	na	CRF.02.AG	na	26.4	P
01CM2227	CRF.02.AG	na	CRF.02.AG	na	27.2	P
01CM2228	CRF.02.AG	na	CRF.02.AG	na	30.9	P
01CM2229	CRF.11.cpx	na	CRF.11.cpx	na	27.0	P
01CM2230	A	na	A	na	22.7	P
01CM2231	CRF.02.AG	na	A	na	23.4	P
01CM2232	B	U	A	U	No Tt	P
01CM2234	CRF.11.cpx	na	CRF.02.AG	na	26.0	P
01CM2235	B	U	nd	U	21.9	P
01CM2236	CRF.02.AG	na	CRF.02.AG	na	25.2	P
01CM2237	F2	na	F2	na	25.1	P
01CM2238	CRF.13.cpx	na	CRF.01.AE	na	22.2	P
01CM2239	CRF.13.cpx	na	CRF.11.cpx	na	26.2	P
01CM2240	CRF.02.AG	na	CRF.13.cpx	na	29.6	P
01CM2241	CRF.01.AE	CRF.11cpx	nd	U	27.5	P
01CM2242	CRF.02.AG	na	CRF.02.AG	na	24.8	P
01CM2243	CRF.11.cpx	CRF.11cpx	nd	CRF.11.cpx	24.7	P
01CM2244	CRF.01.AE	na	CRF.11.cpx	na	23.1	P
01CM2246	B	na	CRF.01.AE	na	23.6	P
01CF2247	CRF.11.cpx	na	CRF.01.AE	na	24.1	P
01CM2248	CRF.01.AE	na	A	na	21.9	P
01CM2249	A	na	A	na	23.6	P
01CM2250	CRF.02.AG	CRF.02.AG	nd	U	30.5	P
01CM2252	CRF.02.AG	U	nd	U	28.6	P
01CM2253	CRF.01.AE	U	nd	A	21.7	P
01CM2256	CRF.01.AE	na	A	na	21.6	P
01CM2257	CRF.01.AE	na	A	na	21.9	P
01CM2260	CRF.13.cpx	U	A	CRF.13.cpx	23.7	P
01CM2262	B	na	CRF.02.AG	na	27.8	P
01CF2268	CRF.02.AG	CRF.02.AG	nd	CRF.02.AG	32.5	P
01CM2269	CRF.11.cpx	CRF.11.cpx	nd	CRF.11.cpx	26.7	P
01CM2270	CRF.02.AG	CRF.02.AG	nd	U	31.9	P
01CM2271	CRF.11.cpx	CRF.02.AG	nd	CRF.11.cpx	23.9	P
01CM2272	CRF.11.cpx	na	CRF.11.cpx	na	21.2	P
01CM2273	CRF.11.cpx	na	CRF.11.cpx	na	25.5	P
01CM2274	CRF.02.AG	na	CRF.02.AG	na	22.6	P
01CM2275	CRF.09.cpx	CRF.02.AG	nd	CRF.09.cpx	24.5	P
01CM2276	CRF.11.cpx	na	CRF.11.cpx	na	23.9	P
01CM2277	CRF.11.cpx	CRF.11.cpx	nd	CRF.11.cpx	21.4	P
01CM2278	B	na	CRF.02.AG	na	24.2	P
01CM2280	CRF.11.cpx	CRF.02.AG	nd	CRF.02.AG	29.8	P
01CM2281	CRF.02.AG	CRF.02.AG	nd	CRF.02.AG	23.4	P
01CM2284	CRF.11.cpx	CRF.11.cpx	nd	CRF.11.cpx	24.5	P
01CM2287	CRF.11.cpx	na	CRF.01.AE	na	33.2	P
02CM319	nd[f]	O[g]	nd	O	No Tt	N[h]

[a] Genotyping based on part of *gag*-p24 (460 bp), *env*-C2V3 (approximately 550 bp), *pol*-integrase, and *env*-gp41 (approximately 405 bp) regions.
[b] Threshold time by LA-200.
[c] Not available.
[d] Agarose gel electrophoresis.
[e] Positive.
[f] Not detected.
[g] Group O.
[h] Negative.

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tis virus (Toriniwa and Komiya, 2006), influenza virus (Ito et al., 2006), mumps virus (Okafuji et al., 2005), West Nile virus (Parida et al., 2004), severe acute respiratory syndrome corona virus (Hong et al., 2004; Poon et al., 2005), measles virus (Fujino et al., 2005), dengue virus (Parida et al., 2005), respiratory syncytial virus (Ushio et al., 2005), and HIV-1 (Curtis et al., 2008).

In the present study, another RT-LAMP assay was developed for the rapid detection of HIV-1 RNA. Its intended application is on-site confirmation of HIV diagnosis.

2. Materials and methods

2.1. Standard serum

WHO standard 97/656 (10^5 international units (IU) per vial, National Institute for Biological Standards and Control, Herts, UK) was used to determine the detection limit of the RT-LAMP assay (Davis et al., 2003; Holmes et al., 2001). The assay was carried out in octuplicate. The lowest concentration of genome copies with all octuplicate samples confirmed as positive was considered the detection limit.

2.2. Human plasma samples

Plasma samples were collected from 57 HIV-1-infected individuals in eastern Cameroon in 2001 (Ndembi et al., 2004) and 40 HIV-1-uninfected antenatal clinic attendees in western Cameroon in 2003. These samples were used to evaluate the sensitivity and specificity of HIV-1 RT-LAMP. In a previous study (Ndembi et al., 2004), phylogenetic analysis of genomic DNA samples from the 57 infected individuals revealed the presence of highly divergent strains of HIV-1 circulating in eastern Cameroon (Table 1). The 40 samples from uninfected individuals collected in 2003 were confirmed HIV-negative by HIV-Ab testing (AxSYM HIV1/2 and/or Determine HIV-1/2; Abbott Japan, Tokyo, Japan) and conventional PCR, as described previously (Ndembi et al., 2004).

2.3. RNA preparation

HIV RNA was extracted from plasma as follows: 200 μl of plasma was incubated with 400 μl of lysis buffer consisting of 10 mM Tris–HCl (pH 8.0), 68% (w/v) guanidine isothiocyanate, 3% (w/v) dithiothreitol, and 4 μl of co-precipitant (10 mg/ml amylopectin azure) at 25 °C for 10 min. HIV RNA was precipitated by adding 600 μl of isopropanol and centrifuging at 20,000 × g for 15 min. The RNA pellet was washed with 70% ethanol and resuspended in 10 μl of RNAse-free and DNAse-free water.

2.4. Primer design

A set of primers that recognizes eight distinct target sites in the HIV-1 *pol*-integrase gene, a well-conserved region of HIV-1 genome, was designed based on the HIV-1 genome sequence (GenBank accession number K02013) using a primer-designing software program for LAMP (Primer Explorer ver. 2.0; Net laboratory, Japan, http://venus.netlaboratory.com; Table 2). The set consisted of the six following primers: a forward inner primer (FIP), backward inner primer (BIP), two outer primers (F3 and B3), and two loop primers (loop F and loop B). Two additional inner primers comprise the combination of two functionally different primer parts: FIP consists of F1c (complementary to F1) and F2 and BIP consists of B1c (complementary to B1) and B2. The sequences of the two loop primers are complementary to the primers located between regions corresponding to F1 and F2 primer sequences.

2.5. RT-LAMP assay

The RT-LAMP reaction was carried out in 25 μl using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan) containing FIP (40 pmol), BIP (40 pmol), F3 (5 pmol), B3 (5 pmol), loop F (40 pmol), loop B (40 pmol), *Bst* DNA polymerase (16 U), AMV reverse transcriptase (2 U), and 5 μl of target RNA. The reaction mixture was incubated at 60 °C for 60 min in a Loopamp real-time turbidimeter (LA-200; Teramecs, Kyoto, Japan; Fig. 1A). A turbidity value of more than 0.1 was considered positive. The amplified products of RT-LAMP were resolved by 2% agarose gel electrophoresis (Agarose S; Wako Pure Chemical Industries, Ltd., Osaka, Japan); the gel was stained with ethidium bromide and visualized using an ultraviolet (UV) transilluminator (Fig. 1B). The turbidity of the amplified products was also ascertained by naked eye. The amplified products were inspected further under UV irradiation with or without adding ethidium bromide, an intercalating dye, when RT-LAMP assay was carried out in the presence of Fluorescent Detection Reagent (Eiken Chemical Co., Ltd., Tokyo, Japan; Fig. 1C).

3. Results

3.1. Development of the HIV-1 RT-LAMP assay

Using the primer sets targeting the HIV-1 *pol*-integrase gene (Table 2), a one-step RT-LAMP assay for the rapid detection of HIV-1 RNA was standardized. The success of amplification was assessed using a real-time turbidimeter (LA-200; Fig. 1A). Threshold time (Tt), the time required for the turbidity value to exceed 0.1, is shown in Table 1. Amplification was also detected by the presence of a ladder-like pattern on a 2% agarose gel. The ladder-like pattern results from a mixture of stem-loop DNAs of various stem lengths and cauliflower-like structures with multiple loops (formed by annealing between alternately inverted repeats of the target sequence in the same strand; Fig. 1B). Furthermore, amplification was detected by naked eye inspection of turbidity; visual detection was enhanced further by the addition of Fluorescent Detection Reagent and/or the intercalating dye under UV irradiation (Fig. 1C).

3.2. Sensitivity and specificity of the HIV-1 RT-LAMP assay

The sensitivity of the RT-LAMP assay for detecting HIV-1 RNA was determined using RNA from WHO standard HIV-1 97/656 (10^5 IU/vial) diluted to 6000, 600, 240, 120, 90, and 60 copies/ml. One IU was reported to be equivalent to 0.62 genome copies (Davis et al., 2003). The assay was carried out in octuplicate using viral RNA extracted from the equivalent of 100 μl of diluted serum. The reproducible 100% detection limit of the RT-LAMP assay was 120 copies/ml.

Of the 57 HIV-1-positive samples, 54 were positive for RT-LAMP in 19.2–33.2 min as assessed by turbidity using the LA-200 detec-

Table 2
Sequences of primers used for HIV-1 RT-LAMP.

Primer name	Sequence	Genome position[a]
F3	5′-GGTAAGAGATCAGGCTGAACATC-3′	4721–4743
F2	5′-AGACAGCAGTACAAATGGCA-3′	4747–4766
Loop F	5′-TTAAAATTGTGGATGAAT-3′	4786–4769
F1c	5′-CCCCAATCCCCCCTTTTCTT-3′	4806–4787
B1c	5′-AGTGCAGGGGAAAGAATAGTAGAC-3′	4812–4835
Loop B	5′-GCAACAGACATACAAACTAAAG-3′	4842–4863
B2	5′-CTGCTGTCCCTGTAATAAACCC-3′	4921–4900
B3	5′-GCTGGTCCTTTCCAAAGTGG-3′	4945–4926
FIP	F1c + F2	
BIP	B1c + B2	

[a] In HIV-1_{HXB2}.

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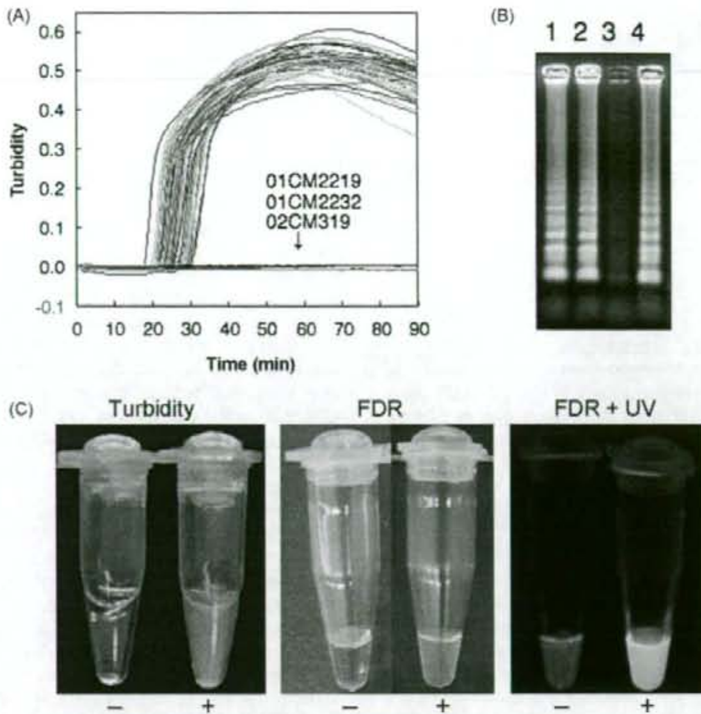

Fig. 1. Real-time detection of HIV-1 RT-LAMP products of 57 HIV-1-positive samples from Cameroon by turbidimeter (LA-200). (A) Agarose gel electrophoresis of HIV-1 RT-LAMP products that were undetectable by LA-200. A turbidity value of more than 0.1 was considered positive. Turbidity of three samples (01CM2219, 01CM2232, and 02CM319) was less than 0.1. (B and C) Representative pictures of HIV-1 RT-LAMP products with (B) and without (C) Fluorescent Detection Reagent. (B) Lane 1: 01CM2219; lane 2: 01CM2232; lane 3: 02CM319; and lane 4: 01CM2213 (positive control). (C) HIV-1 RT-LAMP positive (+) and negative (−). FDR: Fluorescent Detection Reagent; UV: ultraviolet irradiation.

tion system (Table 1 and Fig. 1A). HIV-1 RT-LAMP products of the two samples that were not detected by the real-time turbidimeter (01CM2219 and 01CM2232) could be detected by agarose gel electrophoresis (Fig. 1B) and by the naked eye after adding the intercalating dye under UV irradiation in the presence of Fluorescent Detection Reagent (data not shown). The remaining sample (02CM319) containing HIV-1 group-O RNA was RT-LAMP negative (Table 1 and Fig. 1B). Thus, all 56 samples that harbored HIV-1 group-M were positive by HIV-1 RT-LAMP assay.

Plasma specimens obtained from 40 pregnant women without HIV infection were also subjected to RT-LAMP and all were confirmed negative.

4. Discussion

An RT-LAMP assay was developed to detect HIV-1 RNA. This method was simple, rapid, and highly sensitive and specific for group-M HIV-1. Therefore, the HIV-1 RT-LAMP assay can be used as a rapid confirmatory test for HIV-1 group-M infection.

The HIV genome is usually detected by RT-PCR and PCR performed on plasma RNA and proviral DNA, respectively. These methods require at least 2–3 h despite the implementation of real-time PCR. In this study, the HIV-1 RT-LAMP assay was completed within 35 min, considerably faster than by RT-PCR or PCR. In addition, unlike RT-PCR and PCR, a simple apparatus such as a water bath can be used to maintain the constant incubation temperature at 60 °C.

The RT-LAMP reaction yields a white precipitate of magnesium pyrophosphate in the reaction mixture, indicating a positive result. This white precipitate is easily detected by the naked eye (Fig. 1C); thus, the results of the assay can be assessed without a turbidimeter. Although the amount of HIV-1 RT-LAMP products was monitored by a real-time turbidimeter (LA-200) in the current study, the results of visual inspection were consistent with those determined by turbidimeter (data not shown). According to the manufacturer's instructions for the Loopamp DNA amplification kit, visual detection can be enhanced by the addition of Fluorescent Detection Reagent to the reaction mixture. Interestingly, HIV-1 RT-LAMP products of the two samples that were undetectable by LA-200 (01CM2219 and 01CM2232) could be visualized by adding the intercalating dye under UV irradiation, when the assay was carried out in the presence of Fluorescent Detection Reagent. Thus, the HIV-1 RT-LAMP assay has the advantage of enabling the amplification of HIV-1 RNA and/or DNA in resource-limited settings in which sophisticated machines such as the thermal cycler and real-time turbidimeter are unavailable. In the two samples that were not detected by LA200, the production of magnesium pyrophosphate was prevented by unknown inhibitor(s). The cause and frequency of this phenomenon are under investigation.

RT-LAMP assay exhibits high specificity due to its use of multiple primers, including two loop primers, that recognize eight distinct regions of the target sequences. Previous studies in which RT-LAMP was used to detect various viral RNAs have documented the high specificity of RT-LAMP (Mori et al., 2006; Toriniwa and Komiya, 2006; Ito et al., 2006; Okafuji et al., 2005; Parida et al., 2004, 2005; Hong et al., 2004; Poon et al., 2005; Fujino et al., 2005; Ushio et al., 2005). Similarly, HIV-1 RT-LAMP analysis of 40 sero-negative

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and PCR-negative samples showed 100% specificity, making the RT-LAMP assay ideal for confirming diagnosis.

The 100% detection limit of the HIV-1 RT-LAMP assay was found to be 120 copies/ml (12 copies/100 μl/assay). This sensitivity is inferior to the quantification limit (50 copies/ml) of the UltraSensitive Assay of the COBAS AMPLICOR HIV-1 MONITOR test, v 1.5 (Roche), but superior to the detection limit of the Standard Assay in the kit (400 copies/ml), and typical RT-PCR assays. Furthermore, the sensitivity of the current HIV-1 RT-LAMP could be improved to reach or exceed that of the UltraSensitive Assay by using a larger initial plasma sample (more than 240 μl) for extracting viral RNA.

The HIV-1 RT-LAMP assay was evaluated using 57 HIV-1 strains belonging to nine different group-M subtypes/CRFs and one group O based on *gag* and *pol* sequences, respectively (Table 1): subtypes A ($n = 3$), B ($n = 6$), F2 ($n = 1$), G ($n = 1$), CRF_01AE ($n = 8$), CRF_02.AG ($n = 17$), CRF_09.cpx ($n = 1$), CRF_11.cpx ($n = 16$), CRF_13.cpx ($n = 3$), and group O ($n = 1$; Ndembi et al., 2004). This assay system identified all of the 56 group-M HIV-1 strains despite their diversity, but did not detect the group-O strain, indicating that the primers used in the current HIV-1 RT-LAMP assay were group-M specific. Thus, in order to detect not only all of the HIV-1 groups but also HIV type-2 strains as well, the design of universal primer set will be necessary.

Although the viral RNA extraction method used in this study is relatively easy and cheap as compared to conventional methods, it still requires knowledge and training not usually available in resource-limited settings. Therefore, it will be necessary to revise and simplify the extraction method in order to use this assay as a confirmatory test for HIV diagnosis in the field. Future evaluation of the direct use of plasma or serum after heating as a test material is warranted (Curtis et al., 2008).

In conclusion, a one-step RT-LAMP assay for detecting group-M HIV-1 has been developed. The RT-LAMP assay is simple, rapid, and highly sensitive and specific for group-M HIV-1; therefore, this assay can be used to confirm group-M HIV-1 diagnosis. Once the RNA extraction method is simplified, the group-M HIV-1 RT-LAMP assay will be ideal for use in resource-limited settings.

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High-Risk HPV Types in Lesions of the Uterine Cervix of Female Commercial Sex Workers in the Philippines

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In order to prevent cervical cancer, vaccines against human papilloma virus types 16 (HPV-16) and 18 (HPV-18) have been implemented worldwide. However, the HPV types that cause cancer can differ according to geographical area and ethnicity. In this new era of the HPV vaccine, it is important to elucidate the prevalent HPV types in each area. Therefore, the prevalence of HPV infection and cervical abnormalities among 369 female commercial sex workers in the Philippines were examined. HPV *L1* gene was amplified by polymerase chain reaction (PCR) using modified GP5+/6+ primers, and genotyping was performed by sequencing cloned PCR products. HPV DNA was detected in 211 (57.2%) women, among whom 46 HPV types were identified. HPV-52 was most common and multiple-type infection was observed in 44.5%. Among 56 women with abnormal cervical cytology (low- and high-grade squamous intraepithelial lesions and adenocarcinoma in situ), HPV-52 was most common (23.2%), followed by HPV-16 (19.6%), -58 (10.7%), and -67 (10.7%). Only 27% of these women were positive for HPV-16 and -18. Multivariate analysis revealed that HPV-16, -39, -52, -67, and -82 were significantly associated with abnormal cytology. Repeated analysis of HPV-52 single-positive samples using the original GP5+/6+ PCR primers produced negative results in 57% of cases, suggesting that the prevalence of HPV-52 infection may have been underestimated in previous studies, and the current vaccines may not be sufficient for preventing infection and the development of premalignant lesions of the cervix in women in the Philippines. ***J. Med. Virol. 81:545–551, 2009.*** © 2009 Wiley-Liss, Inc.

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INTRODUCTION

Human papillomavirus (HPV) is the most important risk factor for cervical cancer [Muñoz et al., 2003], which is the second most common malignancy and the third most common cause of cancer-related death in women [Parkin et al., 2005]. The incidence and mortality of cervical cancer are very high in women of reproductive age, especially in developing countries [Parkin et al., 2005; Frain et al., 2006]. Cervical cancer screening using cytological testing and HPV vaccination are paramount for preventing cervical cancer in young women.

More than 40 HPV types have been identified in the mucosal epithelia of the human genital tract; these are classified into high-risk and low-risk types according to their ability to cause cancer [Muñoz et al., 2003]. HPV type 16 (HPV-16) is the most common high-risk type and is detected in 50–60% of high-grade squamous

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intraepithelial lesions and invasive cervical cancers [Muñoz et al., 2003; Wheeler, 2007]. HPV-18 is identified in 10–20% of cancers [Muñoz et al., 2003; Wheeler, 2007]. Thus, HPV-16 and -18 are thought to be responsible for about 70% of cervical cancer cases in many countries [Bosch et al., 1995; Brown et al., 1999; Muñoz et al., 2003].

To reduce the incidence of cervical cancer, vaccines against HPV-16 and -18 have been developed and have been found 100% effective in preventing infection by these HPV types [Harper et al., 2006; Wheeler, 2007]. However, the distribution of common HPV types may vary depending on the geographic area and ethnicity of the population. Thus, the impact of these HPV vaccines on the prevention of infection and cancer may differ in different areas. In Japan, the prevalence of HPV-16 and -18 in cancers and high-grade squamous intraepithelial lesions is approximately 50% and 33%, respectively [Sasagawa et al., 2001]. HPV-52 is more common than HPV-18 in Japan, Taiwan, and eastern Africa [de Sanjosé et al., 2007]. Thus, in order to estimate the effectiveness of the current HPV vaccines for preventing cervical cancer, it is essential to determine the predominant cancer-causing HPV type in each area.

Degenerate and/or consensus primers for polymerase chain reaction (PCR) have been used to amplify a variety of HPV types from clinical specimens. The GP5+/6+ primers that target the HPV *L1* gene have been considered one of the best primer sets for HPV PCR and have been used in many epidemiological studies. However, it has been reported that some HPV types, such as HPV-52, may not be amplified by GP5+/6+ PCR as effectively as HPV-16 and -18 because of sequence mismatches between the target gene and the primers [Matsukura and Sugase, 2004]. Therefore, the GP5+/6+ primers have been modified to broaden the spectrum of detectable HPV types [Yamada et al., 2008].

In a case–control study of the cause of cervical cancer in the Philippines [Ngelangel et al., 1998], the most common HPV type in women with squamous cell carcinomas was HPV-16 (42.9%), followed by HPV-18 (25.3%) and -45 (13.5%). HPV-45 (17.2%) was the most common type found in cytology-normal women (controls), followed by HPV-16 (14.3%) and -18 (14.3%). However, the original GP5+/6+ PCR primers were used in this study. Little other information about the prevalence of HPV types in premalignant lesions of the cervix in the Philippines is available.

In the current study, the prevalence of HPV types and their associations with abnormal cervical cytology among female commercial sex workers in the Philippines were examined. HPV types were detected by PCR using modified GP5+/6+ primers. The goal was to ascertain whether the current HPV vaccine is sufficient for preventing infection and the development of premalignant lesions of the cervix in the Philippines. The prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections and their association with HPV infection were also investigated.

SUBJECTS AND METHODS

Subjects and Sample Collection

Three hundred seventy female commercial sex workers who were attending the Makati Social Hygiene Clinic or its mobile clinic at a night bar in Manila, Philippines, for a regular check-up in January or July 2006 were enrolled in this study. Written informed consent was obtained from all participants.

Specially trained technicians used cervical brushes to collect two cervical specimens from each participant (Honest Uterine Cervical Brushes Type S, Honest Medical, Tokyo, Japan). The first sample was smeared onto a microscope slide, fixed with alcohol solution (Rapid Fix, Muto, Tokyo, Japan), and stained according to the Pap test. The second sample was suspended in 1 ml of cell lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 2% SDS) and stored at −80°C for DNA extraction.

Classification of Cervical Cytology

Cervical cytology was diagnosed according to the Bethesda system [Solomon et al., 2002] and classified as normal (negative for intraepithelial lesion or malignancy), atypical glandular cells/atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, or adenocarcinoma in situ.

Detection and Typing of HPV DNA

DNA was extracted from cervical cells using a DNA extraction kit (SMI test; Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. The quality of the extracted DNA was evaluated by amplifying the glyceraldehyde-3-phosphate dehydrogenase gene (primers: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′) [Fujimori et al., 2002]. All but one of the samples, (n = 369 of 370) were confirmed as adequate for HPV, *C. trachomatis*, and *N. gonorrhoeae* testing.

HPV DNA detection was carried out using three pairs of modified GP5+/6+ primers: GP5+M1-2 (5′-TTTRTTACTGTTGTWGATACTAC-3′); GP5+M2-2 (5′-TGTWACTGTTGTWGATACCAC-3′); GP5+M3-2 (5′-GTWACTGTTGTRGACACCAC-3′); GP6+M1-2 (5′-AATTGAAAWATAAACTGTAAWTCATATTC-3′); GP6+M2-2 (5′-GAAACATAAAYTGTAAATCAWATTC-3′); and GP6+M3 (5′-GAAAATYTGCAAATCAWACTC-3′).

These primers were designed to amplify a 140-bp fragment of the HPV *L1* gene. Amplification was performed as follows: one cycle at 95°C for 10 min followed by 45 cycles at 95°C for 30 sec, 45°C for 30 sec, and 74°C for 30 sec, with a final extension at 74°C for 10 min. The presence of HPV DNA was confirmed by ethidium bromide staining of the PCR products following agarose gel electrophoresis. HPV DNA-negative samples were retested using the original GP5+/6+ primers [de Roda Husman et al., 1995; van den Brule et al., 2002]. The PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and

sequenced as described previously [Ndembi et al., 2003]. The similarity between *L1* sequences obtained by PCR and those of various HPV genotypes in the GenBank database was determined by BLAST analysis (http:www.ncbi.nlm.nih.gov/BLAST/). Ten clones from each sample were analyzed. HPV types were classified as high-risk (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, and -82), low-risk (HPV-6, -11, -40, -42, -43, -44, -54, -61, and -81), unknown risk (HPV-26, -30, -32, -34, -53, -55, -62, -66, -67, -70, -74, -83, -84, -86, -87, -90, -91, and -102), epidermodysplasia verruciformis (HPV-9 and -38), and unclassified types (JEB2 and unclassified), according to previous reports [Muñoz et al., 2003; Schiffman et al., 2005].

Detection of *C. trachomatis* and *N. gonorrhoeae*

C. trachomatis and *N. gonorrhoeae* were detected using the LAMP method as described elsewhere [Hong et al., 2004; Poon et al., 2005].

Statistical Analysis

Statistical analysis was performed using SPSS Version 15.0 J for Windows. Odds ratio (ORs) and 95% confidence intervals (CIs) were calculated as approximations of relative risks. Univariate analyses were performed to assess the association between HPV infection and demographic factors and between abnormal cervical cytology and HPV types. Any variables shown significant in univariate analysis were analyzed by a multivariate model. The level of statistical significance was set at $P < 0.05$.

Nucleotide Sequence Accession Numbers

GenBank accession numbers of the sequences reported in this study are EU911006–EU911930.

RESULTS

Risk Factors for Cervical HPV Infection

This study evaluated the presence of HPV, *C. trachomatis*, and *N. gonorrhoeae* DNA in cervical samples from 369 commercial sex workers (mean age ± SD: 24.5 ± 5.1 years; range: 18–40 years) working in Manila, Philippines. HPV DNA was detected in 198 of the 369 women by PCR using modified GP5+/6+ primers and in 13 of the remaining 171 women using the original GP5+/6+ primers. Ultimately, 211 (57.2%) women were positive for HPV DNA. *C. trachomatis* and *N. gonorrhoeae* DNA were detected in 84 (22.8%) and 24 (6.5%) women, respectively.

In order to determine the risk factors for cervical HPV infection, multivariate analysis using a logistic regression model was performed. Being 25 years of age and younger (OR: 2.5; 95% CI: 1.4–4.3) and having worked for at least 6 months to 2 years (OR: 3.3; 95% CI: 1.9–5.8) were significantly associated with HPV infection (Table I). Cervical *C. trachomatis* and *N. gonorrhoeae* infections,

TABLE I. Demographic Factors Associated With HPV Infection

Demographic factors	No. of subjects	No. of cases	%	Univariate analysis		Multivariate analysis		
				OR	95% CI	OR[a]	95% CI	*P*-value
Age (years)								
>25	127	59	47	1		1		
≤25	239	150	63	1.9	1.3–3.0	1.9	1.1–3.4	0.028
No answer	3	2	67					
Marital status								
Married	37	19	51	1		1		
Single	326	189	58	1.3	0.7–2.6	1.3	0.6–2.7	0.484
No answer	6	3	50					
Duration of sex work (months)								
≤6	120	58	48	1		1		
7–24	137	100	73	2.9	1.7–4.9	3.3	1.9–5.8	<0.0001
>24	97	45	46	0.9	0.5–1.6	1.5	0.8–2.8	0.212
No answer	15	8	53					
Age at first coitus (years)								
≥20	103	56	54	1		1		
<20	261	153	59	1.2	0.8–1.9	1.0	0.6–1.6	0.885
No answer	5	2	40					
C. trachomatis								
Negative	285	161	57	1		1		
Positive	84	50	60	1.1	0.7–1.9	1.2	0.7–2.1	0.507
N. gonorrhoeae								
Negative	345	195	57	1		1		
Positive	24	16	67	1.5	0.6–3.7	1.3	0.5–3.2	0.609

OR, odds ratio; CI, confidence interval.
[a]Adjusted for all other variables in the table.

marital status, and age at sexual debut were not significantly associated with HPV infection. Interestingly, having worked longer than 24 months did not increase the risk when compared with working at least 6 months (OR: 1.5; 95% CI: 0.8–2.8).

Profile of HPV Infection

Of the 211 women with HPV infection, 117 (55.5%) had a single-type infection and 94 (44.5%) had multiple-type infection. Among the infected women, 46 different HPV types were detected; HPV-52 (16.1%) was most prevalent, followed by HPV-66 (12.3%), -16 (11.8%), -45 (10.0%), and -67 (9.5%). One hundred eighty-two women (86.3%) were infected with high-risk types, 38 (18.0%) were infected with low-risk types, and 28 (13.3%) were infected with unknown-risk types. Among those with high-risk HPV infection, HPV-52 (18.7%) was the most common, followed by HPV-16 (13.7%) and -45 (11.5%). HPV-16 and -18 comprised only 20.3% of the high-risk HPV types (Fig. 1).

Risk Factors for Abnormal Cervical Cytology

Among the 369 women, 239 (64.8%) had normal cytology, 74 (20.1%) had atypical glandular cells/atypical squamous cells of undetermined significance, and the remaining 56 (15.2%) had abnormal cytology (low-grade squamous intraepithelial lesion, n = 42; high-grade squamous intraepithelial lesion, n = 12; adenocarcinoma *in situ*, n = 2). HPV DNA was detected in 91 (38.1%) of the 239 women with normal cytology, in 61 (82.4%) of the 74 women with atypical glandular cells/atypical squamous cells of undetermined significance, and in all (100%) of the 56 women with abnormal cytology. Stepwise regression analysis revealed that HPV infection was only the factor significantly associated with abnormal cytology ($P < 0.0001$; OR: 18; 95% CI: 7.6–52).

Twenty-five different HPV types were detected in the 56 women with abnormal cervical cytology. Of these types, HPV-52 was most prevalent (23.2%), followed by HPV-16 (19.6%), -58 (10.7%), -66 (10.7%), and -67 (10.7%). Multivariate analysis using a logistic regression model revealed that HPV-16, -39, -52, -67, and -82 were significantly associated with abnormal cytology (Table II).

PCR Using Modified Versus Original GP5+/6+ Primers

In order to evaluate the efficacy of the modified primers, HPV DNA samples from women with single-type infection according to PCR with modified GP5+/6+ primers were re-analyzed using the original GP5+/6+ primers (Table III). None (0%) of the seven women with HPV-16 infection, eight (57%) of 14 women with HPV-52 infection, and one (20%) of five women with HPV-67 infection tested negative for HPV DNA using the original GP5+/6+ PCR.

DISCUSSION

In this study, the prevalence of HPV infection among female commercial sex workers in the Philippines was 57.2%. The reported prevalences of HPV infection in this

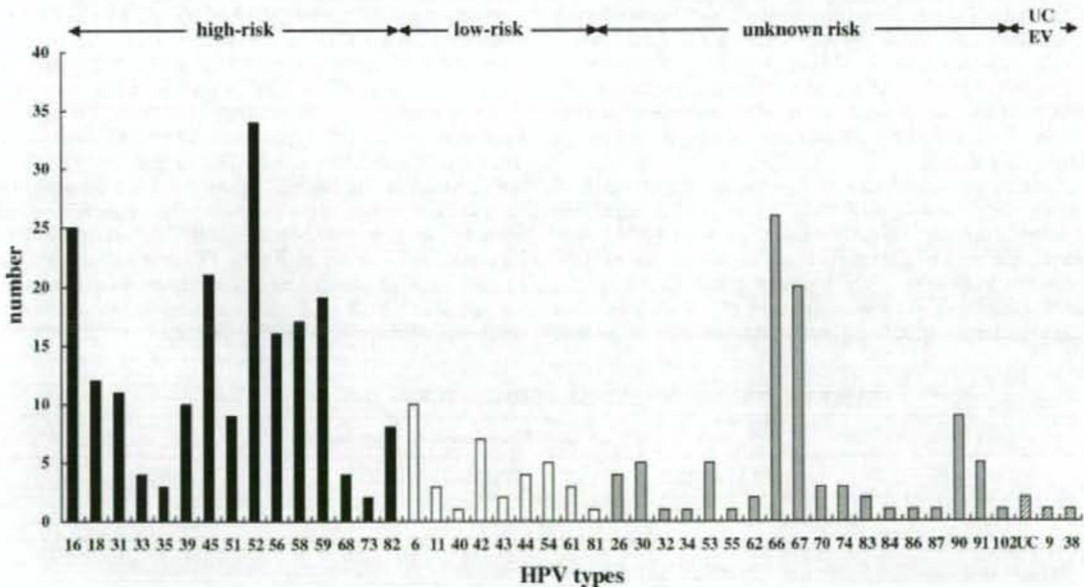

Fig. 1. The prevalence of HPV types among female commercial sex workers (n = 211) in the Philippines. ■, high-risk type; □, low-risk type; ▨, unknown-risk type; ▨, unclassified (UC) and epidermodysplasia verruciformis (EV) types.

TABLE II. HPV Types Associated With Abnormal Cervical Cytology*

HPV type	No. (%) of study participants positive for HPV DNA (n = 295)		Univariate analysis	Multivariate analysis	
	With abnormal cervical cytology (n = 56)	With normal cytology (n = 239)	OR (95% CI)	OR[a] (95% CI)	P-value
16	11 (19.6)	10 (4.2)	5.6 (2.2–14)	7.3 (2.7–20)	<0.0001
18	4 (7.1)	6 (2.5)	3.0 (0.8–11)		
26	1 (1.8)	2 (0.8)	2.2 (0.2–24)		
30	2 (3.6)	2 (0.8)	4.4 (0.6–32)		
31	3 (5.4)	5 (2.1)	2.6 (0.6–11)		
34	1 (1.8)	0 (0.0)	—		
35	1 (1.8)	1 (0.4)	4.3 (0.3–70)		
39	5 (8.9)	2 (0.8)	12 (2.2–62)	14 (2.4–83)	0.004
40	1 (1.8)	0 (0.0)	—		
43	1 (1.8)	1 (0.4)	4.3 (0.3–70)		
44	1 (1.8)	2 (0.8)	2.2 (0.2–24)		
45	4 (7.1)	11 (4.6)	1.6 (0.5–5.2)		
51	3 (5.4)	3 (1.3)	4.5 (0.9–23)		
52	13 (23.2)	9 (3.8)	7.7 (3.1–19)	10 (3.9–28)	<0.0001
53	1 (1.8)	4 (1.7)	1.1 (0.1–9.7)		
54	2 (3.6)	0 (0.0)	—		
56	5 (8.9)	9 (3.8)	2.5 (0.8–7.8)		
58	6 (10.7)	8 (3.3)	3.5 (1.2–10)	3.4 (0.96–12)	0.057
59	2 (3.6)	11 (4.6)	0.8 (0.2–4.0)		
66	6 (10.7)	11 (4.6)	2.5 (0.9–7.0)		
67	6 (10.7)	7 (2.9)	4.0 (1.3–12)	4.3 (1.2–15)	0.024
82	3 (5.4)	2 (0.8)	6.7 (1.1–41)	7.6 (1.0–55)	0.046
86	1 (1.8)	0 (0.0)	—		
90	1 (1.8)	6 (2.5)	0.7 (0.1–6.0)		
91	1 (1.8)	3 (1.3)	1.4 (0.1–14)		

OR, odds ratio; CI, confidence interval.
*Abnormal cervical cytology: low-grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, and adenocarcinoma in situ.
[a]Adjusted for HPV-16, -39, -52, -58, -67, and -82.

population in other countries are 28–39% in Spain [Cañadas et al., 2004; del Amo et al., 2005; Ortiz et al., 2006], 32% in Australia [Tideman et al., 2003], 47% in Korea [Choi et al., 2003], and 55% in Japan [Ishi et al., 2000]. Thus, the prevalence of HPV infection in this group of women in the Philippines is similar to that in Japan and Korea.

For the women in this group, being 25 years old or younger and having worked for a period of 6 months to 2 years were significantly associated with HPV infection, consistent with reports that the prevalence of HPV infection increases with decreasing age [Burk et al., 1996; Hassen et al., 2003; Matos et al., 2003; Baseman and Koutsky, 2005]. As expected, women who had worked longer than 6 months had a higher risk of being infected with HPV than did women who had worked <6 months. However, working longer than 2 years did not increase the risk of HPV infection, suggesting that these women may have acquired some immunity against common HPV types within 2 years of beginning this type of work. The role played by immunity against HPV in decreasing the risk of infection is supported by data from a recent report showing that age-dependent decrease in the prevalence of HPV is attenuated in HIV-infected women in Kenya [Yamada et al., 2008]. In the current study, no information was available for variables such as condom usage by sex partners, smoking, education, area of origin, and annual income,

TABLE III. Modified Versus Original GP5+/6+ PCR for Detecting HPV DNA

HPV infection	No. of positive samples					
	HPV-16 (n = 23)		HPV-52 (n = 34)		HPV-67 (n = 19)	
	Modified[a]	Original[b]	Modified	Original	Modified	Original
PCR positive	23	22	34	23	19	17
Multiple infection	16	15	20	17	14	13
Single infection	7	7	14	6	5	4

[a]Modified: PCR using modified GP5+/6+ primers.
[b]Original: PCR using original GP5+/6+ primers.

which could be factors associated with HPV infection. The absence of these data could limit the types of conclusions that can be drawn from this study.

In the current study, HPV genotyping was performed by sequencing cloned PCR products. In previous studies, direct sequencing or hybridization with HPV type-specific oligo-probes has been used for HPV genotyping. Although these methods are easier and quicker than the method used in this study, their results can sometimes be difficult to interpret [Qu et al., 1997; Coutlée et al., 2002; Perrons et al., 2002; Asato et al., 2004; Gheit et al., 2006]. The direct sequencing method rarely detects multiple-type HPV infection, whereas the hybridization method can detect only HPV types for which probes are available and cross-hybridization of type-specific probe with untargeted HPV types can occur in the dot-blot hybridization method. In contrast, sequencing of cloned PCR products can detect multiple-type HPV infection and identify distinct HPV types. In fact, 46 different HPV types and many cases of multiple-type HPV infections (44.5%) were identified in this study. Therefore, sequencing of cloned PCR products should be considered a preferred method for assessing HPV infection, especially multiple-type infections. However, unless a sufficiently large number of clones are analyzed, a number of types might not be detected, especially in cases of multiple infection with three or more types.

HPV-52 was found to be the most prevalent infecting HPV type in the Philippines; this is not the case in western countries. This difference could be due in part to differences in the methods for detecting HPV DNA. In this study, 57% of the women with single-type HPV-52 infection detected by the modified GP5+/6+ PCR were missed by the original GP5+/6+ PCR, which has been used in previous studies. In contrast, there was no significant difference in the detection of HPV-16 and -67 DNA between modified and original GP5+/6+ PCR, suggesting that the prevalence of HPV-52 may have been underestimated in previous studies.

In the current study, high-risk HPV types were detected in 86% of female commercial sex workers with HPV infection; however, HPV-16 and -18 were detected in only 20% of these women and the most prevalent HPV type was HPV-52. Although HPV-16 is known to be the most prevalent type worldwide [Muñoz et al., 2003; Wheeler, 2007], it has been reported that HPV-52 and -58 are also prevalent in Japan and South Taiwan [Asato et al., 2004; Inoue et al., 2006; Lin et al., 2006]. These results suggest that in addition to HPV-16, HPV-52 may be common in Asian countries in general.

HPV-16, -39, -52, -67, and -82 were found to be significantly associated with abnormal cytology in this study group. HPV-16 and -18 were detected in only 27% of the women with abnormal cervical cytology. Furthermore, HPV-18, -34, -45, and -59 were identified in cases of adenocarcinoma in situ. These results suggest that many high-risk types of HPV other than HPV-16 and -18 might play important roles in cervical carcinogenesis in the Philippines. This is the first study to examine the prevalence of HPV types and their association with abnormal cervical cytology in the Philippines.

The results from clinical trials of first generation vaccines in humans look promising. The data show that an HPV vaccine can prevent HPV infection and precancerous lesions in vaccinated women [Koutsky et al., 2002]. Most HPV vaccines target both HPV-16 and -18 and have been shown highly effective for preventing type-specific HPV infections [Harper et al., 2006; Wheeler, 2007]. However, in the current study, high-risk types of HPV other than HPV-16 and -18, such as HPV-39, -52, -67, and -82, were significantly associated with abnormal cervical cytology in women in the Philippines. In addition, it has been reported that in Japan not only HPV-16 and -18 but also HPV-31, -33, and -58 are significantly associated with cervical cancer (OR > 100) [Asato et al., 2004]. Therefore, the current vaccines might not be sufficient for preventing premalignant and malignant lesions of the cervix in women in Asia, although some cross protection of the vaccine has been reported [Harper et al., 2006]. Area-specific vaccines might be needed. Alternatively, the development of type-common HPV vaccines might be more ideal, in light of data showing that a type-common neutralization epitope exists in minor capsid protein *L2* and that a vaccine using the *L2*-epitope is expected to be effective in preventing infection by all high-risk types of HPV [Kondo et al., 2007].

In conclusion, this study determined that HPV-52 is the most prevalent infecting HPV type among female commercial sex workers in the Philippines and that several high-risk HPV types other than HPV-16 and -18 are significantly associated with abnormal cytology. Therefore, the current vaccines may not be sufficient to prevent HPV infection and the subsequent development of pre-malignant lesions of the cervix in women in the Philippines.

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Changes in the HIV Type 1 Envelope Gene from Non-Subtype B HIV Type 1-Infected Children in Kenya

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Abstract

A switch of coreceptor usage from CCR5 to CXCR4 occurs in about half of HIV-1-infected individuals in the natural course of infection. To investigate whether antiretroviral therapy (ART) enhances the coreceptor switch of HIV-1, we genotypically analyzed the env-V3 amino acid sequences from 81 HIV-1-infected children in Kenya whose plasma samples were obtained between 2000 and 2007. Of 41 children on ART, 35 had HIV-1 using CCR5 as a coreceptor at baseline. In 7 (20%) of them HIV-1 switched the coreceptor usage during the follow-up period. The mean duration of ART to the time of coreceptor switch was 2.6 years (range: 0.5–5.2). Of the remaining 40 children without ART, 32 had HIV-1 using CCR5 as a coreceptor at baseline and in 3 (9.4%) HIV-1 switched the coreceptor usage. The mean age of the children with HIV-1 coreceptor switch with and without ART was 7.3 and 9.7 years, respectively. The difference in the rate and age of coreceptor switch between treated and untreated children was not significant ($p = 0.38$ and 0.31, respectively). Of the HIV-1-infected children, 10 started ART by the age of 5 years (rapid progressors) and 23 did not need ART by the age of 10 years (slow progressors). The rate of coreceptor switch was strongly higher in rapid progressors (40%) than slow progressors (8.7%) ($p = 0.053$). These results suggest that switching of coreceptor usage from CCR5 to CXCR4 among HIV-1-infected children is not influenced by ART, but by factors responsible for rapid disease progression.

Introduction

Human immunodeficiency virus type 1 (HIV-1) coreceptor usage plays a critical role in the virus tropism. HIV-1 infection requires interactions between the viral envelope (env) glycoprotein (gp120) and cellular receptors, CD4 as a major receptor and CCR5 or CXCR4 as a coreceptor.[1] Based on the coreceptor usage, HIV-1 variants are classified as CCR5-tropic (R5 variants), CXCR4-tropic (X4 variants), and dual tropic (R5/X4 variants).[2] R5 variants are responsible for the establishment of HIV-1 infection and predominate in the early stage of HIV-1 infection.[3–5] X4 variants emerge later as disease develops.[6–9] A switch in HIV-1 coreceptor usage from CCR5 to CXCR4, which correlates with the subsequent accelerated decrease in $CD4^+$ T cell count and disease progression, occurs in the late stage of HIV-1 infection in about half of HIV-infected individuals.[10–14]

It has been reported that in HIV-1 subtype C-vertically infected children, R5 variants are predominantly transmitted and the virus may evolve to use CXCR4 as a coreceptor in older children,[15] and that X4 variants emerge later as disease develops in HIV-1 subtype B-infected children.[6–9] However, the late appearance of X4 variants with relation to disease progression is less clear in children than in adults.[16] Especially in HIV-1-infected infants disease progression was not necessarily associated with the switch in HIV-1 coreceptor usage.[17,18] In one study the coreceptor switch of HIV-1 was found in two of six rapid-progressor infants after vertical infection.[19] In another study, 14 of 15 infants with rapid disease progression harbored viruses that used CCR5 as a coreceptor, and only the remaining one had a virus that used both CCR5 and CXCR4.

In developed countries the clinical profile of HIV-1 infection in children shows a bimodal distribution, with approximately one-quarter developing severe symptoms and dying within the first 24–36 months of life (rapid progressors). Most children, however, develop AIDS more slowly, with some surviving beyond 5 years (slow progressors).[20] A similar bimodal disease pattern is seen among children living in

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developing countries,[21] although considerably less is known about the underlying virological and immunological factors, which may differ from those in developed countries.

Recently several longitudinal studies regarding the effect of highly active antiretroviral therapy (HAART) in the dynamics of evolution of HIV tropism in patients under HAART were carried out and reported a higher prevalence of X4 variants in those HIV-1-infected individuals exposed to HAART than in drug-naive individuals.[22–29] In addition, effective HAART was reported to enhance CCR5 to CXCR4 coreceptor switch.[30] However, the dynamics of viral tropism during the course of HIV-1 infection in persons exposed to antiretroviral therapy (ART) still remain unclear. Moreover, there are indeed limited reports regarding the effect of ART on HIV-1 coreceptor usage among children.

The aim of this study was to investigate the dynamics of coreceptor usage and whether ART enhanced the coreceptor switch among non-B subtype HIV-1-vertically infected children in Kenya.

Materials and Methods

Study population

As of August 2007, 95 HIV-1-infected children resided in a children's home in Nairobi. These children were born to HIV-1-infected mothers who either died of, or were too debilitated by HIV/AIDS and could not offer basic care to the children. All children were admitted into the home by their first birthday, where their HIV-1 status was confirmed serologically at 18 months of age. None of these children had a history of previous exposure to ARVs by the time of admission. Of the 95 children 81 were followed up at least three times during the period between 2000 and 2007, and blood samples were obtained from them every 6 months since the year 2000. Of the 81 children, 41 received ART consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) and one nonnucleoside reverse transcriptase inhibitor (NNRTI). The mean duration of ART varied among those children was 7.6 years with range of 1 to 15 years. The remaining 40 did not receive ART during the follow-up period.

This study was approved by the Kenya Medical Research Institute's National Ethical Review Committee on behalf of the Kenyan Government and conducted according to the national and international regulations governing the use of human subjects in biomedical research. The study was conducted within the continuing antiretroviral, medical, and healthcare programs of the institution without additional demand for blood samples solely for research purposes.

$CD4^+$ T cell counts and plasma viral loads

$CD4^+$ T cell counts of peripheral blood were determined using the FACSCOUNT (Becton-Dickinson, Beiersdorf, Germany). Plasma HIV-1 RNA loads were determined by the Amplicor HIV-1 Monitor kit version 1.5 (Roche Diagnostics, Alameda, CA) using the standard procedure (with detection limit of 400 copies/ml) according to the manufacturer's instructions.

Extraction and amplification of plasma HIV-1 viral RNA

HIV-1 RNA was extracted from 100 μl of plasma using SMITEST EX-R & D (Medical & Biological Co. Ltd., Fukushima, Japan) according to the manufacturer's instructions. A part of the HIV-1 group M *env* gene covering the C2V3 region (corresponding to 6975–7520 nt in HIV-1 HXB2) was amplified by both one-step RT-PCR and nested polymerase chain reaction (PCR) with primers M5 (5′-CCAATTCCCATAC ATTATTGTGCCCCAGCTGG-3′ and M10 (5′-CCAATTGT CCCTCATATCTCCTCCTCCAGG-3′) in the first round and M3 (5′-GTCAGCACAGTACAATGCACACATGG-3′) and M8 (5′-TCCTTGGATGGGAGGGGCATACATTGC-3′) in the second round,[31] according to the manufacturer's instructions. Amplification was done with one cycle of 95°C for 10 min and 35 cycles of 95°C for 30s, 55°C for 30 s, and 72°C for 1 min with a final extension of 72°C for 10 min. PCR amplification was confirmed by visualization with ethidium bromide staining of the gel.

Sequencing, cloning, and subtyping of the env-C2V3 region

The amplified PCR products were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and sequenced as described previously,[31] to take into account both the majority and minority virus populations.

The sample nucleotide sequences were aligned with HIV-1 subtype/circulating recombinant form (CRF) reference sequences from the Los Alamos database using CLUSTAL W (version 1.83), with minor manual adjustments. A phylogenetic tree was constructed by the neighbor-joining method, and its reliability was estimated by 1000 bootstrap replications. The profile of the tree was visualized with TreeViewPPC1.6.5.[31] To improve the accuracy of HIV-1 subtyping, we used the National Center for Biotechnology Information (NCBI) genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) and the REGA subtyping tool (http://dbpartners.stanford.edu/RegaSubtyping/), as needed.

Determination of the predicted coreceptor usage of HIV-1

The predicted coreceptor usage of HIV-1 was determined based on the *env*-V3 loop amino acid sequences. R5 and X4 variants were identified according to (1) the 11/25 amino acid rule [uncharged residues at position 11 of V3 (mostly serine/glycine), negatively charged residues at position 25 (mostly glutamic (E)/aspartic (D) acid), and a net charge of the V3 loop less than +5 have been reported to predict CCR5 chemokine receptor usage. Conversely, positively charged residues at position 11 or 25 (mostly arginine (R)/lysine (K)) and a net charge of the V3 loop equal and more than ±5 have been reported to predict CXCR4 chemokine receptor usage], and (ii) the net charge of the V3 region, which was calculated by subtracting the number of acidic amino acids (aspartate and glutamate) from the number of basic amino acids [lysine, histidine (H), and arginine]. A net charge of equal and less than +5 in the V3 region has been shown to predict CCR5 chemokine receptor usage (R5 variants), whereas a net charge of more than +5 has been shown to predict CXCR4-chemokine receptor usage (X4 variants).[32–36] In our study, a net charge of +5 was considered to predict an R5 phenotype, unless this was accompanied by the appearance of either an arginine or lysine amino acid residue at positions 11 or 25.

Determination of the rate of disease progression

The children were categorized into three groups, rapid progressors, slow progressors, and the others, based on the rate of disease progression. *Slow progressors* were the children over 10 years of age when they received ART, and were classified in stage N1 or A1 according to the Centers for Disease Control and Prevention (CDC) classification for children. Children over 10 years of age who did not need ART were also included in this category. *Rapid progressors* were the children who received ART within the first 5 years of their lives either because they had an onset of severe clinical manifestations (CDC category C) and/or profound immune suppression (CDC category 3).

Results

Predicted HIV-1 coreceptor usage

Of the 41 children on ART, 35 had HIV-1 that used CCR5 as a coreceptor at baseline (the first time point at which sample analysis was done). The mean age, viral load,and $CD4^+$ T cell count of these 35 children at baseline were 5.5 years old (range: 1–12), 5.2 $\log_{10}$ copies/ml (range: 3.9–6.1), and 537 cells/μl (range: 93–1760), respectively. In 7 (20%) of them the virus switched coreceptor usage from CCR5 to CXCR4, and in 28 the virus used CCR5 as a coreceptor during the follow-up period. The duration from the start of ART to the time of HIV-1 coreceptor switch varied considerably (mean: 2.6 years, range: 0.5–5.2 years) (Table 1). The remaining six children on ART had HIV-1 that used CXCR4 as a coreceptor from baseline to the end of the study.

Of the 40 children without ART, 32 had HIV-1 that used CCR5 as a coreceptor at baseline. The mean age, viral load, and $CD4^+$ T cell count of these 32 children at baseline were 8.0 years old (range: 3–19), 4.8 $\log_{10}$ copies/ml (range: 2.3–6.0), and 684 cells/μl (range: 70–1335), respectively. In three (9.4%) of them the virus switched the coreceptor usage, and in 29 the virus used CCR5 as a coreceptor during the follow-up period. The remaining eight children without ART had HIV-1 that used CXCR4 as a coreceptor from baseline to the end of the study. Although more of the treated children had HIV-1 that switched coreceptor usage from CCR5 to CXCR4, the difference in the rate of the coreceptor switch between treated (7/35, 20%) and untreated (3/32, 9.4%) children ($p = 0.38$) was not statistically significant (Table 1).

The seven children whose viruses switched their coreceptor usage started ART at younger ages than the 28 children whose viruses used CCR5 as a coreceptor from baseline to the end of the study (mean 5.3 and 7.6 years, respectively). The mean age of the children with HIV-1 coreceptor switch with and without ART was 7.3 and 9.7 years, respectively. The difference in the age of the coreceptor switch between treated and untreated children was not significant ($p = 0.31$).

Chronological changes of the env-V3 amino acid sequences from the serial study points for the 10 children whose HIV-1 showed the coreceptor switch with and without ART are shown in Fig. 1. No significant association was observed between the changes in coreceptor usage and plasma viral load in the children.

HIV-1 coreceptor switch with different rates of disease progression

Of the HIV-1-infected children who had the virus that used CCR5 as a coreceptor at recruitment, 10 started ART by the age of 5 years (rapid progressors) and 23 did not need ART by the age of 10 years (slow progressors). The rate of coreceptor usage was strongly higher in rapid progressors (4/10, 40%) than slow progressors (2/23, 8.7%), though the association was not statistically significant ($p = 0.053$) (Table 2).

HIV-1 subtypes

Phylogenetic analysis based on the *env*-C2V3 region revealed that all the 81 children were infected with non-B subtype HIV-1: subtypes A1 ($n = 65$), A2 ($n = 4$), D ($n = 9$), C ($n = 2$), and CRF_02AG ($n = 1$) (data not shown). No significant relationship between HIV-1 subtype/CRF and

Table 1. Characteristics of the Study Children at Baseline, ART Start, and Coreceptor Switch

	Coreceptor usage	*Baseline mean (range)*			*ART start mean (range)*			*Coreceptor switch mean (range)*			
		Viral load[a]	*$CD4^{+b}$*	*Age*[c]	*Viral load*[a]	*$CD4^{+b}$*	*Age*[c]	*Viral load*[a]	*$CD4^{+b}$*	*Age*[c]	*Duration of ART (years)*
ART	R5▶R5 ($n = 28$)	5.3 (3.9–6.1)	472 (6–1566)	5.7 (1–12)	5.3 (3.9–6.1)	447 (93–1340)	6.6 (1–12)				
	R5▶X4 ($n = 7$)[d]	4.8 (3.6–5.4)	479 (178–1760)	5.0 (1–8)	4.7 (3.6–5.4)	357 (147–1442)	4.7 (2.5–7)	4.8 (4.2–5.4)	677 (157–1439)	7.3 (3–12)	2.6 (0.5.–5.2)
	X4▶X4 ($n = 6$)[e]	3.5 (2.3–5.0)	590 (17–1620)	11.2 (7–18)							
No ART	R5▶R5 ($n = 29$)	4.8 (2.3–6.0)	697 (70–1637)	8.1 (2–19)							
	R5▶X4 ($n = 3$)	4.4 (3.3–5.2)	573 (338–700)	7.7 (5–11)				4.8 (4.6–5.1)	462 (411–550)	9.7 (7–12)	
	X4▶X4 ($n = 8$)	4.4 (2.9–5.3)	716 (345–1570)	8.6 (6–13)							

[a]Log (copies/ml).
[b]$CD4^+$T cell count (cells/μl).
[c]Years old; R5, CCR5; X4, CXCR4.
[d]One of the seven children had already received ART at baseline.
[e]All the six children in this group had received ART at baseline.

Child ID	Date of sample collection	V3 amino acid sequence (11 / 25)	Viral load (log/ml)	net charge	11/25 amino acid	predicted phenotype
36m*	aug,02	CTRPGNNTRESVRIGPGQAFYATKDVIGDIRQAHC	4.9	+3	S/D	R5
	apr,03	.I..S....................I.........	5.8	+3	S/D	R5
	feb,04	.........R.I.............IG........	5.6	+4	S/D	R5
	Oct,05	......K..R.I.....RV..T.NVIR........	5.1	+7	S/V	X4
38m*	mar,03	....ST...K.............GEIT........	4.6	+5	S/E	R5
	dec,03	....S....K.............GEIT........	4.8	+5	S/E	R5
	feb,04	....SSP..TR.A..R.......SAIT.T..K.Y.	4.6	+6	R/A	X4
	sep,05	....S.P..RR.A..........SAIX.T..T.Y.	4.9	+6	R/A	X4
51m*	apr,03	....N....KG.H.....S.FT.GNI.....K.Y.	5.3	+5	G/N	R5
	nov,04	....N....KG.H.....S.FT.GNI.....K.Y.	5.2	+5	G/N	R5
	apr,05	....N....KG.H.....SLFT.GNI.....K.Y.	5.5	+5	G/N	R5
	oct,05	....N....KG.H.....SLFT.GNI..N..K.Y.	5.4	+6	G/N	X4
69m*	mar,03	.I..N....QGIH.......WV.N....E....Y.	5.4	+4	G/D	R5
	may,04	.I..N....QGIH....R..WV..K.V.IK...Y.	5.1	+7	G/K	X4
85f*	feb,03	....N....K.I......T....G.IT........	4.5	+4	S/D	R5
	dec,03	....N....K.IH....RT....G.IT........	3.5	+5	S/D	R5
	apr,04	....N....K.IH....RT....G.IT........	4.9	+5	S/D	R5
	apr,05	....N....K.I......T....G.IT........	4.3	+5	S/D	R5
	sep,05	....N....K.IH....RT....G.I..N......	4.5	+6	S/D	X4
89f*	feb,04	..S.T...SRGIHM...RS....D.I..N......	5.2	+5	G/D	R5
	jul,04	..SNTSS.SRGIHM...RS....D.I..N......	5.0	+5	G/D	R5
	mar,05	..S.T...SRGIHM...RS....D.I..N......	5.5	+5	G/D	R5
	oct,05	..SRT...SRGIHM..LRS....DRI..N......	4.8	+6	G/R	X4
91f*	mar,05	....N....K.IHF.....L.T.DNI..N....Y.	4.9	+4	S/N	R5
	mar,06	....N....R.IH......L.T.NRI..N......	4.2	+6	S/R	X4
	aug,06	....N....KGIHF.....L.T.NRI....KK.Y.	4.0	+6	G/R	X4
21f**	jul,03	....S....K.IHL...R.....G.I.........	4.2	+5	S/D	R5
	dec,04	....N....K.IHL...R.....GRI..N......	4.7	+7	S/R	X4
	apr,06	....S....K.IHL.A.R.....GRI.........	4.2	+6	S/R	X4
49f**	aug,02	.S..S....K.............G.IV........	4.2	+5	S/D	R5
	jun,03	.S.......K.............G.IV........	4.5	+5	S/D	R5
	feb,04	.S.......K.........V...GAIV........	4.1	+5	S/A	R5
	mar,05	.S.......K..H..........GAIV....R...	4.6	+6	S/A	X4
72f**	Jul,02	.I.VN....Q.L..........MG.I..N..D...	5.2	+3	S/D	R5
	Jun,03	.I..Y.....GTHM...K.YFT...I.....D...	5.1	+4	G/D	R5
	feb,04	.I..N....Q..N.........MG.I.....D...	NT	+2	S/D	R5
	sep,04	....N....K.IHF.....L.TNNII..N..D...	5.1	+6	S/I	X4

FIG. 1. Changes in the HIV-1 V3 amino acid sequences during follow-up of the 10 children whose infected viruses switched from CCR5 to CXCR4 coreceptor usage. A net charge of less than and more than +5 in the V3 region was considered as CCR5-using (R5) and CXCR4-using (X4) variants, respectively, and a net charge of +5 was considered as R5 variants, unless this was accompanied by the appearance of either an arginine or lysine residue at position 11 or 25 of the V3 amino acid sequences. *, **Children whose HIV-1 showed a switch in coreceptor usage from CCR5 to CXCR4 with treatment (*) and without (**) treatment. NT, not tested.

TABLE 2. HIV-1 CORECEPTOR USAGE IN ASSOCIATION WITH THE RATE OF DISEASE PROGRESSION

	Change in coreceptor usage[a]	*Number of children (on ART)*	*Mean age (range) at ART start/recruitment*	*Mean age (range) at switch*	*Children with coreceptor switch*
Rapid progressor	R5→R5	6 (6)			
	R5→X4	4 (4)	3.0 (1–4)	4.8 (3–6)	40%[b]
Slow progressor	R5→R5	21 (6)			
	R5→X4	2 (0)	7.5 (6–9)	12.0 (10–14)	8.7%[b]

[a]R5, CCR5; X4; CXCR4.
[b]$p = 0.053$.

coreceptor usage was observed among the children (data not shown).

Discussion

In the current study we conducted a longitudinal study to investigate the evolution of the *env*-V3 region in terms of coreceptor usage among non-B subtype HIV-1-infected Kenyan children in relation to ART. These children were vertically infected with HIV-1 and have been virologically and immunologically followed up since the year 2000. Most studies on viral evolution and coreceptor usage from HIV-1-infected adults are often compromised by a lack of knowledge of the duration of infection. It makes this study particularly useful that the precise timing of HIV-1 infection is known in the children studied.

Recently it has been reported that the prevalence of X4 variants was higher in HIV-1-infected individuals exposed to ART than in drug-naive individuals,[22–25] and that effective HAART enhanced the coreceptor switch from CCR5 to CXCR4.[30] In our study, however, no significant difference in the rate of the coreceptor switch between the children with and without ART was observed ($p = 0.38$). In addition, the duration of time from the start of ART to the time of HIV-1 coreceptor switch varied considerably from 0.5 to 5.2 years (mean: 2.6 years) in our study, though it was expected to be synchronized if ART was directly associated with a switch in HIV-1 coreceptor usage. These results suggest that switching of coreceptor usage from CCR5 to CXCR4 among HIV-1-infected children is not directly influenced by ART.

We further analyzed the children who harbored HIV-1 who showed a switch in coreceptor usage according to the rate of disease progression. The rate of HIV-1 coreceptor switch was found to be strongly higher in the rapid progressors (40%, 4/10) than the slow progressors (8.7%, 2/23) ($p = 0.053$). The strong association between rapid disease progression and HIV-1 coreceptor switch in our study may suggest that those factors associated with rapid disease progression in children, such as high viral load at infection,[32] poor cell-mediated immune responses,[33] lack of neutralizing antibodies,[34] and the biological properties of the virus, would be more directly implicated. Studies using animal models also showed that reduced HIV-specific immunity may result in HIV-1 coreceptor switch.[35]

In the current study, the predicted coreceptor usages based on the env-V3 amino acid sequence according to previous reports[37,38] were not always correlated with those based on the amino acids residues at positions 11 and 25[39–41] (Fig. 1). Therefore, we mainly used the net charge of the env-V3 amino acid sequence to predict HIV-1 coreceptor usage, except when the net charge of the env-V3 amino acid sequence was +5, in which cases we used amino acid residues at positions 11 and 25 to predict the coreceptor usage.[39–41] Phenotypic assay for HIV-1 coreceptor usage might be needed to confirm our prediction of the coreceptor usage.

In conclusion, our data suggest that ART does not enhance the HIV-1 coreceptor switch from CCR5 to CXCR4. This switch in HIV-1 coreceptor usage was associated with rapid disease progression among non-B subtype HIV-1-infected children. We are currently carrying out more detailed analyses on the genetic host factors associated with delayed or rapid disease progression among HIV-1-infected children, hoping to focus more light on the possible factors that influence the HIV-1 coreceptor switch.

Sequence Data

GenBank accession numbers of the sequences reported in this study are EU602350 to EU603148 for env-C2V3.

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Disclosure Statement

No competing financial interests exist.

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Human Papillomavirus Infection and Cervical Abnormalities in Nairobi, Kenya, an Area With a High Prevalence of Human Immunodeficiency Virus Infection

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Human papillomavirus (HPV) infection and cervical abnormalities, and their association with human immunodeficiency virus (HIV) infection were studied in 488 women who visited a health center in Nairobi. PCR-based HPV and cervical cytology tests were carried out on all participants, and peripheral CD4+ T cells and plasma HIV RNA were quantitated in HIV positive women. HIV were positive in 32% (155/488) of the women; 77% of these were untreated, and the others had been treated with anti-retroviral drugs within 6 months. Cervical HPV infection was detected in 17% of HIV negative and 49% of HIV positive women. Low-grade squamous intraepithelial lesions were observed in 6.9% of HIV negative and 21% of HIV positive women, while high-grade squamous intraepithelial lesions and cancer were seen in 0.6% and 5.8%, respectively. Multivariate analysis revealed that HIV and HPV infections were associated with each other. Cervical lesions were significantly associated with high-risk HPVs and with HIV infection, depending on HPV infection. HPV infection increased in accordance with lower CD4+ T cell counts and higher HIV RNA levels, and high-grade lesions were strongly associated with high-risk HPV infection and low CD4+ T cell counts. Immunosuppression as a result of HIV infection appears to be important for malignant progression in the cervix. Nationwide prevention of HIV infection and cervical cancer screening are necessary for the health of women in this area. High-risk HPV infection and low CD4+ T cell counts are the risk factors for cervical cancer. ***J. Med. Virol. 80:847–855, 2008.*** © 2008 Wiley-Liss, Inc.

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KEY WORDS: HIV; HPV; cervical abnormality; HIV RNA; $CD4^+$ T cell count

INTRODUCTION

Uterine cervical cancer is the second most common cancer and the fifth most common cause of cancer mortality worldwide. Around 468,000 new cases of cervical cancer occur annually, and more than 233,000 women die from the disease worldwide. About 80% of such cases are in resource-poor developing countries [Parkin et al., 2001]. The highest incidence is observed in Latin America, the Caribbean, sub-Saharan Africa, and South and Southeast Asia [Parkin et al., 2001]. Cervical cancer remains a pervasive public health problem in developing countries. Implementation of nationwide cervical cancer screening has been successful in reducing the incidence and mortality of cervical cancer in many developed countries [Peto et al., 2004]. However, the high mortality rate from cervical cancer in women of child-bearing age may be one of the most important social problems in developing countries.

In sub-Saharan Africa, the annual incidence of cervical cancer is more than 93.9 per 100,000 women

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[Parkin et al., 2001]. Although the national incidence of invasive cervical cancer is unknown in Kenya, it has been estimated at 45 per 100,000 women [Gichangi et al., 2002]. In Kenya, 55% of cervical cancer cases are reported to be clinical stage 3 or more advanced, whereas this figure is only 25% in developed countries [Claeys et al., 2003]. Only 6% of women who present with invasive cervical cancer in Kenya have a history of screening [Gichangi et al., 2002]. The lack of a systematic cervical cancer screening program in Kenya appears to be a factor in the high incidence of progressive disease.

Human papillomavirus (HPV) has been identified as an important causative agent of cervical cancer. HPV DNA testing, rather than cytological testing, is recommended for cervical cancer screening in some resource-poor areas [Kuhn et al., 2000] and in those regions with high prevalence of the human immunodeficiency virus [HIV; Womack et al., 2000]. Cervical cancer is a major problem in areas of Africa with a high prevalence of HIV infection. Women with HIV infection are more likely to have a concurrent HPV infection [Temmerman et al., 1999; Womack et al., 2000], and HPV infection is associated with a greater risk for high-grade squamous intraepithelial lesions in HIV positive women than in HIV negative women. HIV-associated attenuation of HPV-specific immune responses may allow for persistent high-grade intraepithelial neoplasia, thus providing sufficient time for the accumulation of genetic changes important in progression to cancer [Palefsky, 2006]. Furthermore, compared to their non-infected counterparts, women infected with HIV and with invasive cervical cancer are more likely to present with advanced clinical disease [Maiman et al., 1997].

In Kenya, 11% of adult women were infected with HIV in 1996, and the incidence decreased to 8.7% in 2003. However, the incidence was higher in women living in large towns such as Nairobi, where it was 12% in 2003 [Ministry of Health, Kenya, 2005]. The high prevalence of HIV infection in Kenya may increase the incidence of cervical cancer and its precursor lesions, although Gichangi et al. [2002] have demonstrated that a two- to threefold increase in the prevalence of HIV in Kenya did not have a proportionate effect on the incidence of cervical cancer. Unlike cervical cancer, the incidence of Kaposi's sarcoma seems to mirror the incidence/prevalence of HIV, being increased significantly in HIV-infected individuals [Goedert et al., 1998]. It has been hypothesized that HIV-infected women die from HIV-related opportunistic infections before they develop invasive cervical cancer [Gichangi et al., 2002]. At the time of this report, the mean survival time for women with HIV infection in Kenya is 5 years, while typically more than 10 years elapse before the development of cervical cancer after HPV infection. Another possibility is that the diagnosis of subclinical cervical cancer may be missed in many women who die from opportunistic infections in AIDS, as many cases of cervical cancer are asymptomatic.

In the present study, a nested cross-sectional study was undertaken within an ongoing prospective study of HPV/HIV infection and cervical abnormality in Nairobi, Kenya, to establish their prevalence and any association between these conditions.

SUBJECTS AND METHODS

Study Design

This cross-sectional study was part of an ongoing cohort study conducted in Nairobi, Kenya, from November 2004 to August 2009. The subjects were sexually active women, aged 16–61 years old, who attended the Riruta Health Center in Nairobi from November 2004 to June 2005 for pregnancy, family planning, and gynecological concerns. The health center is located near a large slum town. After providing written informed consent, all of the women were invited to participate in this study. The recruitment criteria included women who were willing to undergo voluntary counseling and testing for HIV infection, a cervical Pap test, and an HPV DNA test. Ethics committee in Kenya Medical Research Institute (KEMRI) approved this study. More than 650 women volunteered, and 488 eligible women, including 83 pregnant women who were within 30 weeks of the gestation period, were evaluated in the present study. The remaining subjects were not eligible mainly because some information and data necessary for analysis were not available.

An educational talk about cervical cancer and its risk factors, screening methods, and management was presented to all of the women who visited the health center. The women who agreed to participate in this study were invited for a detailed explanation of the procedures involved. After an informed consent was signed, a structured questionnaire was administered, and a pelvic examination, Pap test, and HPV DNA test were performed in all participants. The questionnaire sought employment status, education, past and current sexually transmitted infection, and current pregnancy status. The first of two cervical scraped-cell samples was placed on a glass slide and fixed immediately in 95% ethanol for the Pap test, and the second was placed into a tube containing DNA extraction solution (10 mM Tris, pH 8.0, 1 mM EDTA, 2% SDS) for the HPV DNA test. After cytological screening, some abnormal and borderline cases ($n = 45$) were recalled and subjected to further examination by colposcopy and pathological diagnosis using punch biopsy specimens. HPV test samples were stored at −20°C. Blood samples were collected for screening of HIV. The samples were separated into serum and blood using a particle agglutination kit and were stored at −20°C.

HPV DNA Detection and Typing

DNA was extracted from cervical cell samples using a DNA extraction kit (SMI test). HPV L1 genes were amplified by PCR using modified GP5+ and GP6+ [de Roda Husman et al., 1995] multiprimers, designed to avoid mismatches between primer sequences and complement target HPV L1 genes. The modified GP