HIV-1 subtypes and the HIV epidemics in four cities in sub-Saharan Africa

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Objective: To describe the distribution of HIV-1 subtypes in two cities with high HIV prevalence (Kisumu, Kenya and Ndola, Zambia) and two with relatively low prevalence (Cotonou, Benin and Yaoundé, Cameroon), and to examine whether the differences in prevalence of HIV infection could be due to the predominance within the infected populations of subtypes with differing efficiency of heterosexual transmission.

Methods: For around 100 randomly selected HIV-positive sera from the general population and 60 from sex workers in each city, the HIV-1 subtype was determined in the *env* fragment. For between 19 and 52 of the sera from the general population and 20–32 sera from sex workers, the subtype was also determined in the *gag* fragment.

Results: Over 70% of infections in Cotonou, Yaoundé and Kisumu were with subtype A (by *env*). However, around one-half of subtype A infections in Cotonou and Yaoundé were found to be the circulating recombinant form CRF02_AG when the *gag* fragment was also examined. A large number of different HIV strains were found in Yaoundé, including some belonging to group O. Over 20% of infections in Kisumu and around 10% in Yaoundé were with isolated intersubtype recombinant forms. All but a few infections in Ndola were with subtype C and no recombinants were found.

Conclusions: The pattern of distribution of subtypes that we found does not suggest that differences in circulating subtypes play a major role in explaining the differences in prevalence of HIV-1 infection between the four cities. The emergence and spread of recombinants requires close surveillance to adapt testing strategies if needed, to inform vaccine development and to ascertain their role in the future spread of HIV.

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Introduction

HIV is characterized by a high degree of genetic variability. HIV-1, the primary focus of this paper, is divided into three genetic groups called major (M), outlier (O) and non-major–non-outlier (N) [1]. Group M is further divided into a number of genetic subtypes. Originally, 10 HIV-1 subtypes (labelled A–J) were identified based on the *env* sequence. In recent years, full-length genome sequencing has become more widespread and the number of HIV-1 viruses that have been fully sequenced has increased dramatically. Based on full-length genome sequencing, group M is currently divided into nine genetic subtypes, labelled A, B, C, D, F, G, H, J, and K. Recombinant lineages, which are spreading epidemically, are referred to as circulating recombinant forms (CRF). So far, four CRFs have been identified: CRF01_AE, CRF02_AG, CRF03_AB, and CRF04_cpx.

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In addition, numerous unique intersubtype recombinant forms (IRF) have been described [2].

The genetic variability of HIV has implications for testing, treatment and vaccine development. It has also been hypothesized that there are differences in transmission efficiency and pathogenesis between different subtypes or CRFs. For example, it has been suggested that CRF01_AE (formerly called subtype E) and subtype C may be more easily transmitted during heterosexual intercourse, and are a driving force behind the explosive epidemics in Thailand [3] and in Southern Africa [4], respectively. So far, however, there is no convincing consistent evidence (epidemiological or biological) that major differences in transmission efficiency exist between different subtypes [5].

For all of the presented reasons, surveillance of subtypes is important. We conducted a population-based study on the epidemiology of HIV-1 in two African cities with a high prevalence of HIV infection (Kisumu, Kenya and Ndola, Zambia) and two cities with a relatively low prevalence of HIV infection (Cotonou, Benin and Yaoundé, Cameroon), and studied the distribution of HIV-1 subtypes and recombinants in the general population and among sex workers [6]. We believe that the differences in prevalence between the four cities were due to differences in rate of spread of HIV rather than differences in time since the start of the epidemics. The overall objective of the study was to identify factors that could explain the differences in prevalence of HIV infection between the four cities. The main objective of the study on subtypes was to examine whether the differences in HIV prevalence between the four cities could be due to the predominance within the infected populations of subtypes with different transmission efficiencies for heterosexual intercourse. If the distribution of HIV-1 subtypes showed differences consistent with the high and low HIV prevalence rates in the four cities, it would support the hypothesis of differential transmission. The HIV-1 subtype data were also used to examine other aspects of the epidemiology of HIV-1 in each city.

Methods

In each city, we aimed to interview and examine a random sample of around 1000 men and 1000 women aged 15–49 from the general population, as well as a representative sample of 300 women engaging in sex work. Detailed questionnaires on sexual behaviour were administered to all consenting eligible respondents, and blood and urine samples were taken to ascertain HIV status and other sexually transmitted infections. We also collected a self-administered vaginal swab from each woman, which was inoculated in a culture medium for the detection of *Trichomonas vaginalis*. Details of the methods are described elsewhere in this supplement [6,7].

Blood was collected into ethylenediamine tetraacetic acid tubes and centrifuged within 24 h. The serum was divided into two aliquots. The first aliquot was tested locally for HIV-1 and syphilis. The second aliquot was frozen at –20°C and shipped on dry ice to the Institute of Tropical Medicine in Antwerp, where subtyping was carried out.

We aimed to carry out subtyping on 100 isolates from the general population and 60 from the sex workers for each city. The required number of HIV-positive sera was selected randomly from among the frozen samples for each city. RNA was extracted from the serum [8], followed by reverse transcription of the RNA and polymerase chain reaction. In Yaoundé, a V3-peptide enzyme-linked immunosorbent assay, which was developed by Behring (Marburg, Germany) for research purposes, was used to make a first differentiation between HIV-1 group M and group O viruses. Samples that were suspected of belonging to HIV-1 group O were further analysed by a specific group O/M polymerase chain reaction and *Pst*I restriction analysis [9] to make the distinction between group M and group O infections. HIV-1 group M subtyping was performed by the heteroduplex mobility assay (HMA) using a 700 base pair (V3–V5) fragment of the *env* gene [10]. Those virus strains that could not be unequivocally classified by HMA into one of the known HIV-1 subtypes were sequenced directly and phylogenetic analysis performed on them. When a sample could not be classified by HMA and by direct sequencing, the *env* fragment was cloned in a TA vector (Invitrogen, Leck, The Netherlands). An *env* HMA performed on the clones allowed the identification of a number of multiple infections.

A novel heteroduplex mobility assay was developed for the *gag* fragment [11]. Of the samples that had been successfully subtyped using *env*, the *gag* HMA was applied to between 19 and 51 randomly selected sera from the general population and between 20 and 32 randomly selected sera from the sex workers in each city. The results obtained by *gag* HMA were confirmed by sequencing and phylogenetic analysis of the *gag* fragment. Application of the *gag* HMA in combination with the *env* HMA provided a minimal estimate of the prevalence of recombinant strains.

The emphasis of the statistical analysis was on describing how the distribution of the different subtypes varied between the cities and between different population groups. The χ^2 test or Fisher's exact test (if the assumptions for the χ^2 were not valid) were used to compare distributions. All analysis was carried out using Stata 6 (Stata Corp., Texas, USA, 1999).

Results

Table 1 presents details of sample sizes obtained for the various components of the study. All available HIV-

Table 1. Serum samples tested for HIV and HIV-1 subtypes, for the general population and sex workers in four African cities

Country	Interview	HIV test	HIV-positive	Samples selected for subtyping	Positive PCR	*env* subtype unclassified	*env* subtype classified	Subtype based on *gag* and *env*
General population								
Cotonou (Benin)	2116	1943	66 (3.4%)	63[a]	58 (92%)	1	57	19
Yaoundé (Cameroon)	2089	1913	110 (5.8%)	110	104 (95%)	0	104	44
Kisumu (Kenya)	1889	1519	392 (25.8%) (+ 4 indeterminate)	118	103 (87%)	3	100	52
Ndola (Zambia)	1730	1536	435 (28.3%) (+ 2 indeterminate)	120	114 (95%)	0	114	40
Sex workers								
Cotonou (Benin)	433	275	158 (57.5%)	60	57 (95%)	0	57	20
Yaoundé (Cameroon)	328	340	110 (32.3%) (+ 4 indeterminate)	67	59 (88%)	0	59	21
Kisumu (Kenya)	300	296	221 (74.7%)	60	56 (93%)	2	54	32
Ndola (Zambia)	332	324	219 (67.6%) (+ 5 indeterminate)	61	57 (93%)	0	57	22

[a] Out of the 66 samples that tested positive in Cotonou, 63 were available for testing in Antwerp, including 60 serum samples and three dried blood spots. Polymerase chain reaction (PCR) was positive for 58 out of the 60 sera, while all three dried blood spots were negative on PCR.

Table 2. Distribution of subtypes in the four cities (based on the *env* fragment) in the general population and sex workers

	Cotonou		Yaoundé		Kisumu		Ndola	
env	*n*	%	*n*	%	*n*	%	*n*	%
General population								
A[a]	47	82	89	86	71	71	0	0
C	0	0	0	0	6	6	114	100
D	1	2	4	4	20	20	0	0
G	9	16	5	5	2	2	0	0
Other	0	0	6[b]	6	1[c]	1	0	0
U	1				3			
Total	58		104		103		114	
Sex workers								
A	47	82	51	86	39	72	1	2
C	0	0	0	0	7	13	54	96
D	0	0	2	3	7	13	0	0
G	10	18	2	3	0	0	0	0
Other	0	0	4[d]	7	1[e]	2	2[f]	2
U					2			
Total	57		59		56		57	

U, Unclassified. [a] Using *env* HMA, it is not possible to differentiate between subtype A and CRF02_AG. [b] Two CRF01_AE, two subtype F, one subtype H and one group O. [c] Dual A–D infection. [d] Two subtype F, two group O. [e] Possible triple infection A + C + D. [f] One dual A–C infection, one $C_{outlier}$.

positive samples from the general population from Cotonou and Yaoundé were used for subtyping. For Kisumu and Ndola, a random sample of sera was taken for subtyping. An *env* fragment was obtained from 87 to 95% of the samples (Table 1). The proportion of samples that could be classified by HMA was 83% for Cotonou, 85% for Yaoundé, 74% for Kisumu, and 93% for Ndola.

Table 2 and Figure 1 show the distribution of subtypes according to classification of the *env* fragment in the general population and sex workers in the four cities. In Cotonou and Yaoundé, over 80% of HIV-1 infections were of *env* subtype A. In Cotonou most non-A infections were subtype G, while in Yaoundé subtypes D, G, F and H were identified as well as CRF 01_AE and three

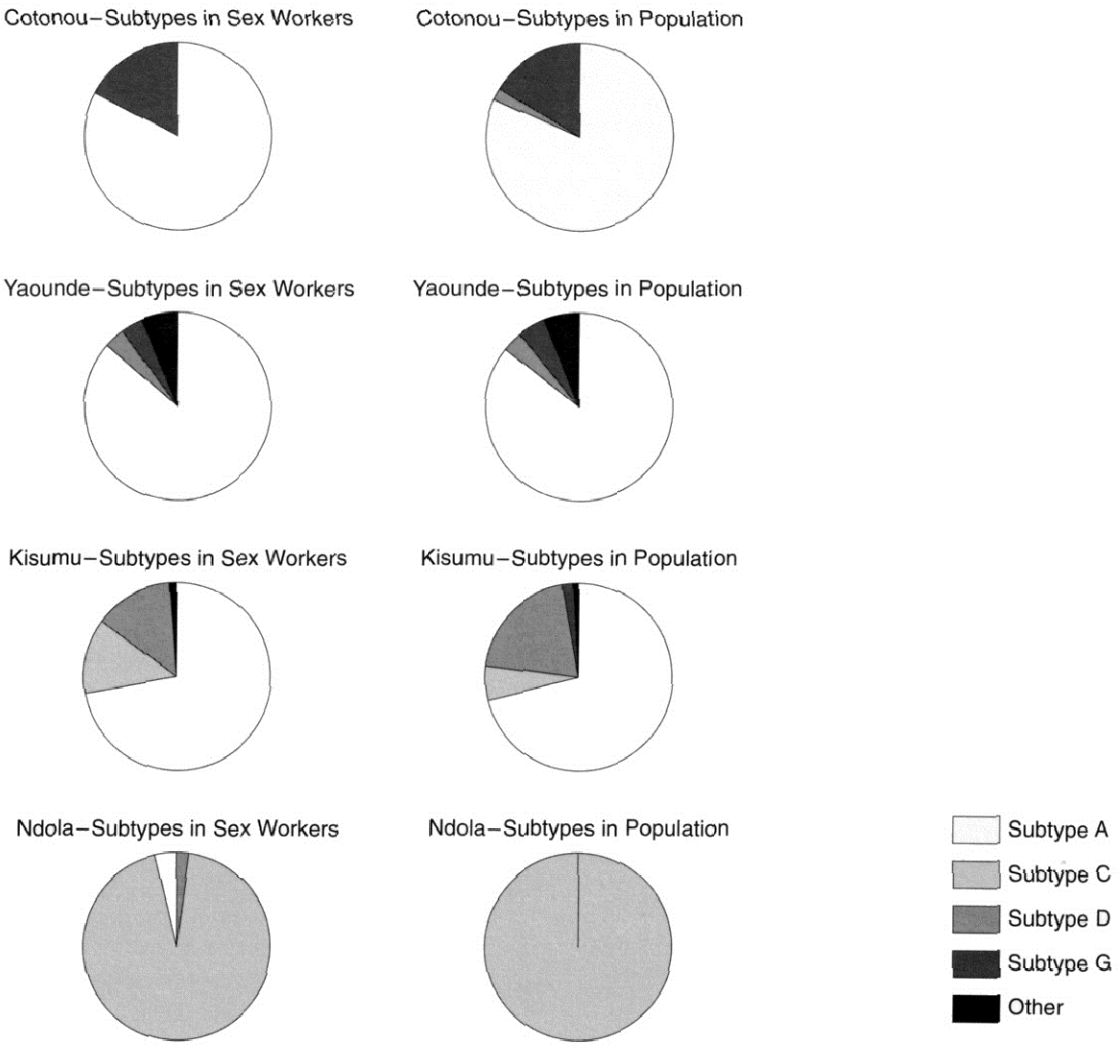

Fig. 1. Distribution of subtypes (based on *env*) in sex workers and the general population in each city.

isolates belonging to group O. In Kisumu, around 70% of HIV-1 infections were *env* subtype A. The majority of non-A infections were subtype D, although C and G were also found. In Ndola, all but three people were infected with subtype C. One individual from the general population was infected with an 'outlier C', one sex worker was infected with subtype A, and one had a dual (A + C) infection.

If sex workers and their clients formed distinct sexual networks within the populations in the four cities, then the distribution of subtypes might be expected to differ between sex workers and the general population. However, the distribution of subtypes (based on analysis of the *env* fragment) was similar in sex workers and the general population for all cities. A higher proportion of subtype C was found among sex workers than the general population in Kisumu, but this was not statistically significant (Fisher's exact test $P = 0.355$).

The distribution of subtypes (A versus non-A) in different groups within the general population was then compared for each of the cities except Ndola, where only subtype C was found. The different factors examined included: ethnicity, age, educational level, number of lifetime sexual partners, travel outside the city in the past year, residence in the city in the past 5 years and, for those who had lived in the city for less than 5 years, the previous place of residence. Within each city, the distribution of non-A subtypes was not significantly different across any of these factors (Table 3).

Using subtype information from the *gag* and *env* fragments, a minimal estimate was obtained on the proportion of recombinant strains (see Tables 4 and 5). Of those samples identified as subtype A in *env* in Cotonou and Yaoundé, roughly one-half (42% in sex workers, and 58 and 60% in the general population) were actually CRF02_AG. The prevalence of isolated recombinants

Table 3. Prevalence of non-A subtypes in different subgroups of the general population in Cotonou, Yaoundé and Kisumu

	Cotonou		Yaoundé		Kisumu	
	Non-A/total	%	Non-A/total	%	Non-A/total	%
Ethnic group						
Main ethnic group	5/22	23	9/54	17	25/82	30
Other ethnic groups	5/35	14	5/45	11	3/17	18
	($P = 0.485$)		($P = 0.565$)		($P = 0.382$)	
Age						
15–24 years	2/15	13	3/31	10	9/33	27
25–34 years	7/27	26	8/38	21	11/37	30
35–49 years	1/15	7	4/31	13	8/29	28
	($P = 0.291$)		($P = 0.464$)		($P = 1.00$)	
Education						
Less than primary	6/36	17	3/17	18	11/40	28
Primary	3/18	17	10/71	14	14/41	34
Secondary or higher	1/3	33	2/12	17	3/18	17
	($P = 0.704$)		($P = 0.744$)		($P = 0.392$)	
Residence						
Resident > 5 years	6/42	14	11/71	15	15/51	29
Resident ≤ 5 years and previously resident in:						
Other urban area	3/7	43	0/14	0	4/16	25
Other rural area	1/5	20	4/13	31	9/32	28
Out of country	0/3	0	0/1	0	–	–
	($P = 0.207$)		($P = 0.123$)		($P = 1.00$)	
Travel						
Travelled in past year	4/20	20	5/30	17	11/26	42
Did not travel in past year	5/34	15	10/60	17	12/57	21
	($P = 0.712$)		($P = 1.00$)		($P = 0.064$)	
Number of lifetime partners						
1	3/8	38	0/5	0	3/8	38
2–4	4/27	15	4/25	16	14/50	28
5+	3/22	14	10/67	15	11/41	27
	($P = 0.285$)		($P = 1.00$)		($P = 0.838$)	

P values are from Fisher's exact test.

was 0% in Ndola, although one case of dual infection was found. The highest prevalence of unique recombinants was found in Kisumu (22% in the general population and 31% in sex workers), with the next highest in Yaoundé (11% in the general population and 8% in sex workers). There was no significant difference in the prevalence of recombinant strains between the general population and sex workers in Cotonou, Yaoundé or Kisumu.

Discussion

The distribution of HIV-1 strains that we found in the general population and among sex workers in Cotonou (Benin), Yaoundé (Cameroon), Kisumu (Kenya) and Ndola (Zambia) is in line with the pattern that has been found by several other researchers [12–16]. In Uganda and Kenya, the predominant subtypes are A and D, with a smaller proportion of people in Kenya being infected with subtype C. Throughout the rest of East Africa and Southern Africa the picture is dominated by subtype C. The predominant strains circulating in most of West Africa are subtype A, CRF02_AG and subtype G. In Senegal and The Gambia, however, a broad variety of HIV-1 subtypes has been found, including subtype B in Senegal [17,18]. Subtype A and CRF02_AG are the most common strains in West Central Africa, but they circulate alongside a large variety of other strains [19]. All known HIV strains, with the exception of CRF03_AB and CRF04_cpx, have been isolated from patients in West Central Africa. There are two possible explanations for the high variety of strains in this part of Africa that are not mutually exclusive. Co-circulation of many different subtypes could be the result of multiple introductions of the virus in the population and/or a

Table 4. Distribution of subtypes and recombinants in the four cities (based on *env* and *gag*) in the general population and sex workers

	Cotonou, Benin		Yaoundé, Cameroon		Kisumu, Kenya		Ndola, Zambia	
gag and *env*	*n*	%	*n*	%	*n*	%	*n*	%
General population								
Same subtype in *gag* and *env*	11	58	21	48	40[a]	78	40[b]	100
CRF02_AG	7	37	18	41	0	0	0	0
IRF	1	5	5	11	11	22	0	0
Total	19		44		51		40	
Sex workers								
Same subtype in *gag* and *env*	12	60	12	57	22	69	22[c]	100
CRF02_AG	7	35	7	33	0	0	0	0
IRF	1	5	2	8	10[d]	31	0	0
Total	20		21		32		22	

[a] One was a dual infection: A + D in *env* and A in *gag*. [b] One sample was classified as an outlier C based on V3–V5 sequence. [c] Included dual infection: A + C in *env* and A in *gag*. [d] One was a dual or triple infection: A + D in *env* and D + C in *gag*.

Table 5. Details of isolated recombinants found in the general population and sex workers in three cities

	Cotonou, Benin	Yaoundé, Cameroon	Kisumu, Kenya
General population	G/A	A/G (× 2)	A/C (× 2)
		D/A	A/D
		F/CRF02_AG	D/A (× 4)
		H/A	D/CRF02_AG
			D/C (× 2)
			G/A
Sex workers	G/CRF02_AG	A/G	A/D (× 4)
		F/A	A/C
			C/A (× 2)
			D/A (× 2)

Data presented as *env/gag*.

long-standing HIV epidemic with differentiation of HIV strains in subtypes and CRFs. The recent discovery of zoonotic transmission of primate immunodeficiency viruses in Cameroon suggests that the HIV epidemics in this part of Africa are among the oldest, if not the oldest, on the continent [20].

In recent years, subtype C has been put forward as the driving force behind the explosive epidemics in East and Southern Africa [21–23]. In our study, we found subtype C only in Kisumu and Ndola, the two high HIV prevalence cities. In Kisumu, however, subtype C was isolated from 6% of HIV-infected individuals only. In the past, subtype C has been isolated from patients in Cameroon on at least three different occasions [11,14,24]. If subtype C were a major driving force behind the explosive epidemics in certain regions in sub-Saharan Africa, then we should also have seen a high prevalence of HIV infection in the general population of Yaoundé. So far, the HIV prevalence in Yaoundé has increased slowly. While it is possible that there are differences in transmission efficiency and pathogenicity between different subtypes, our data on the distribution of HIV strains suggest that different subtypes and CRFs do not play a major role in determining the differences in prevalence of HIV infection between different regions in sub-Saharan Africa.

We did not find any significant differences in the distribution of subtypes between the general population and sex workers. Differences in distributions have been found where there are different routes of introduction of the virus into the general population, for instance via sex workers and via intravenous drug users, as has been shown in Thailand [25] or in homosexuals and heterosexuals in South Africa [26]. In well-established epidemics, as in the four cities we studied, differences in distribution of strains might suggest a massive recent introduction of new strains (for instance, through migration) or separation between sexual networks of clients of sex workers and other men in the general population. The lack of difference in distribution of HIV-1 strains between sex workers and the general population suggests that there is no important separation between the sexual networks of clients of sex workers and other men. There were also no significant differences in the distribution of subtypes within different subgroups of the general population (Table 3). While sample sizes were small, resulting in low power for the tests, there were also no obvious consistent patterns. This suggests that, in the four cities, sexual mixing is not assortative in terms of any of the factors we examined.

We used a newly developed HMA for the *gag* fragment to obtain a minimal estimate of the prevalence of recombinants circulating in the general population and among sex workers. In South West Uganda, 19% of circulating strains were found to be A/D recombinants [27]. In Dares-Salam, Tanzania, 15% of HIV-infected infants were infected with a recombinant strain [21]. Out of 77 strains from Senegalese patients, 12% were found to be discordant in *env* and *gag*, and were IRFs [17]. In the Gambia, four out of 28 strains were found to be IRFs [18]. Intersubtype recombination can be expected to occur more frequently in those populations where there is already a high variety of HIV-1 subtypes circulating and where the probability of being infected by different strains is high. The highest prevalence of unique intersubtype recombinant forms was found in Kisumu (over 20%), with the next highest in Yaoundé (around 10%). These were also the two cities with the most variety in subtypes, supporting the proposed hypothesis. In a similar vein, it might be hypothesized that, because sex workers are likely to be exposed to a greater variety of subtypes, they would have a higher prevalence of recombinants than the general population. In our study, there was no significant difference between the two groups in Cotonou, Yaoundé or Kisumu. However, our sample sizes were too small to detect a statistically significant difference in (recently formed) recombinants between sex workers and the general population, unless it was large.

Roughly one-half of the strains from Cotonou and Yaoundé that were originally labelled as subtype A based on the *env* HMA were actually CRF02_AG. This strain was first isolated from an individual in Nigeria [28]. Since then a high prevalence of CRF02_AG has been documented in Nigeria, Senegal, Cameroon and Gabon [16–19]. There is also evidence that this CRF has been circulating in West and West Central Africa for many years already. By reanalysing *env* sequences, encoding the region C2V3 until the start of gp41 (900 base pairs) of subtype A samples from Côte d'Ivoire and Cameroon, in combination with *gag* HMA, it was found that CRF02_AG strains were already prevalent in these countries in the early 1990s [11]. There are several possible explanations for the high prevalence of CRF02_AG in West and Central Africa. A first possible explanation is that this recombinant emerged in one place and was introduced in other populations in the early stages of the HIV epidemic via infected individuals; for instance, sex workers who are known to be very mobile in these parts of Africa. A second possible explanation is that CRF02_AG has some advantage over subtypes A and G. If this is the case, then the proportion of infections with CRF02_AG is expected to increase further.

Conclusion

In conclusion, the pattern of distribution of subtypes that we found does not suggest differences in circulating subtypes play a major role in explaining the differences in prevalence of HIV infection between the two low HIV prevalence cities (Cotonou and Yaoundé) and the two high prevalence cities (Kisumu and Ndola). We found a high prevalence of recombinants in Yaoundé and Kisumu (at least 10 and 20% in the general population) that, considering the techniques used, was an underestimate of the real prevalence of recombinants. The emergence of recombinants requires close surveillance as it is not yet clear what this means in terms of testing strategies, vaccine development and possibly future spread of HIV.

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