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Human immunodeficiency virus type 1 (HIV-1) subtype diversity in Busia, Western Kenya

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HIV infection is currently the single biggest epidemic globally. HIV the etiologic agent for AIDS is divided into two types: HIV-1 and HIV-2. HIV-2 is rare and is mainly found in some parts of West Africa. HIV-1 accounts for most cases of AIDS reported globally. HIV-1 strains can be classified into four groups: The "major" group M, group O, group N and the most recent group P. All of which may represent separate introductions of SIVs into humans. This cross sectional study determined the HIV-1 subtype diversity in Busia, Western Kenya. Briefly, participants were consented into the study based on pre-determined inclusion criteria. Viral RNA quantification was performed to select participants with virologic failure for drug resistance testing. HIV drug resistance testing (DRT) was performed and sequences obtained were used to determine circulating HIV-1 subtypes using the REGA HIV-1 Subtyping Tool Version 3.0. Phylogenetic analysis was performed using MEGA software V7.0 to confirm the circulating HIV subtypes. Out of 915 participants screened, 146 participants had virologic failure although 140 were successfully sequenced. Subtype A1 was the most prevalent subtype present in 52.9% of the participants followed by subtype D (20.7%), CRF A1_D (7.1%) subtype C and subtype B (4.3%) and subtype A2 (3.6%). Sequences within the same subtype and CRF clustered close together on the phylogenetic tree. An increase in CRFs in the population compared to previous studies. Circulating HIV subtypes should be continually monitored in Busia to determine trends in transmission and map the circulating recombinant forms for epidemiological purposes.

Key words: HIV-1, Busia county, subtype diversity, reverse transcriptase.

INTRODUCTION

The human immunodeficiency virus (HIV), can be divided into two types: HIV type 1 (HIV-1) and HIV type 2 [HIV-2] (Butler et al., 2006; Faria et al., 2014; Seitz, 2016; Sharp and Hahn, 2011; Weidle et al., 2000). The origin of HIV-1 among non-human primates has been traced to the simian immunodeficiency viruses (SIVs), which infected

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several geographically isolated chimpanzee communities in southern Cameroon and it has been postulated that both HIV types are as a result of multiple cross-species transmissions with SIVs (Faria et al., 2014; Sharp and Hahn, 2011; Taylor et al., 2008). HIV-1 accounts for most cases of HIV infections globally (Faria et al., 2014; Taylor et al., 2008). The strains of HIV-1 can be classified into four groups based on the genotypic variation in the *env* region: the "major" group M, the "outlier" group O, and the "new" group N and the most recent group P (Abongwa et al., 2019; Buonaguro et al., 2007; Faria et al., 2014; Ragupathy et al., 2011; Santoro and Perno, 2013; Tebit et al., 2016).

These four groups may represent the four separate introductions of SIVs into humans (Faria et al., 2019; Taylor et al., 2008). Within group M, there are known to be at least nine genetically distinct subtypes (or clades) of HIV-1 namely subtypes A, B, C, D, F-H, J, K (D'Arc et al., 2015; Faria et al., 2014; Taylor et al., 2008). The global HIV pandemic has been dominated by group M viruses since its discovery with the other groups N, O and P being poorly disseminated (Bbosa et al., 2019). Occasionally, two or more viruses of different subtypes can meet in the cell of an infected person and combine their genetic material to create a new hybrid virus (a process similar to sexual reproduction, and sometimes called "viral sex"), resulting in inter-subtype recombinants (Recordon-Pinson et al., 2018; Reis et al., 2019; Tongo et al., 2016). When the recombinants are transmitted and spread within a population, they are recognized as circulating strains in the HIV epidemic and are classified as circulating recombinant forms (CRFs) while unique recombinant forms (URFs), on the other hand, have been sampled only once from a single multiply-infected individual (Reis et al., 2019; Tongo et al., 2016). It is worth noting that in East Africa, where HIV-1 subtypes A, D and C are predominant, several AD and AC recombinants have been described (Giovanetti et al., 2020).

The HIV-1 subtype present in an individual has been shown to play a major role in the development of resistance towards antiretroviral therapy [ART] (Conroy et al., 2010; Kiwanuka et al., 2009; Santoro and Perno, 2013), viral transmission rates (Kiwanuka et al., 2009; Santoro and Perno, 2013) as well as progression to disease with subtype D showing faster progression to disease and higher mortality rates than subtype A1 (Baeten et al., 2007; Conroy et al., 2010; Santoro and Perno, 2013; Ssemwanga et al., 2013). The fast-increasing emergence of HIV recombinant forms has been shown to greatly impact accurate diagnosis, phylogenetic reconstruction, antiretroviral treatment and vaccine development. Several studies have demonstrated significant differences in the acquisition of HIV drug resistance, the majority noting that subtype D and C are more vulnerable to the development of drug resistance than subtype A (Santoro and Perno, 2013; Wallis et al., 2017). This study sought to determine the circulating HIV subtypes in Busia County, Kenya.

METHODOLOGY

Study site

The study was conducted at the Busia County Referral Hospital, Busia County, Kenya. All participants were on first-generation nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) anti-HIV regimens at the hospital's HIV comprehensive care center (CCC) which provides services to over 20,000 HIV infected patients. All the patients were on ART under the Kenyan guidelines for 12 months or more at the time of the study.

Study participants

The study participants were HIV-1 positive adult patients (aged between 21 and 67 years). All participants were receiving a standard triple first-line ART regimen from Busia County Referral Hospital's CCC for 12 months or more. Participants who had attended the clinic at least once within the previous 6 months were included in this cross-sectional single visit study. All participants gave voluntary informed consent to participate in the study. Study participants who demonstrated virologic failure as per the Kenyan guidelines with viral loads of >1,000 copies/mL had drug resistance testing performed.

Ethical considerations

This study was approved by the Kenya Medical Research Institute/National Scientific and Ethical Review Committees. Written informed consent was obtained from each participant before conducting any study procedure. The study was conducted according to good clinical laboratory practices.

Laboratory testing

HIV-1 RNA viral load testing

Plasma samples for HIV-1 viral RNA and drug resistance testing stored at -70°C were retrieved and thawed at room temperature. A total of 925 patients qualified for viral load testing based on the above inclusion criteria. Viral load testing was performed using Abbott M2000SP/RT (Abbott Molecular, Inc., Des Plaines, IL, USA) viral load testing assay, whose lower detection limit was 40 copies/mL.

HIV-1 drug resistance testing

Reverse transcription (RT) polymerase chain reaction (PCR), nested PCR and genotyping was performed for all participants having a viral load of >1,000 copies/mL (n=146). Briefly, blood was collected from these participants, and viral ribonucleic acid (RNA) extracted from blood plasma using Qiagen RNA MiniAmp kit (Qiagen, Valencia, CA, USA). Reverse transcription of the extracted RNA was performed to obtain complimentary deoxyribonucleic acid (cDNA) followed by nested polymerase chain reactions to produce millions of copies of the cDNA for sequencing. Successful amplification was confirmed by gel electrophoresis.

HIV-1 genotyping assay, which sequences the HIV-1 pol gene(base pairs covering PR region: codons 4 – 99 and RT region: codons 38 – 247), was performed on the amplicons using an

Table 1. Participant demographic characteristics.

REGA subtype	Mean viral load (copies/ml)	Mean age (Years)	Mean CD4	Total number of participants	Number with DRAMs
A1	97,285	38.7	159	74	43
D	97,625	39	161	29	22
CRF A1_D	93,356	38.5	164	10	4
C	11,959	40.3	342	6	0
B	184,544	38	125	6	6
A2	30,057	33.25	182	4	2
CRF A1_C	27,549	38.5	171	4	4
G	324,401	43.5	119	2	2
CRF A1_J	65,636	29	198	2	2
CRF B_C	54,456	43	282	1	1
CRF A1_F1	67,186	29	245	1	1
CRF A2_H	186,098	32	118	1	1

automated ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence quality control was performed using the Los Alamos HIV-1 sequence quality assurance tool on https://www.hiv.lanl.gov/content/sequence/QC/index.html?sample_input=1. HIV-1 subtyping was performed using the automated REGA HIV-1 Subtyping Tool Version 3.0 available on http://dbpartners.stanford.edu:8080/RegaSubtyping/stanford-hiv/typingtool/. The FASTA formatted sequences were copied and pasted into the REGA HIV-1 Subtyping Tool Version 3.0 and submitted for subtype analysis. The subtypes obtained and their proportions were downloaded in excel format. Similarly, the sequences were pasted onto the Stanford University HIVdb program version 8.8 on https://hivdb.stanford.edu/hivdb/by-sequences/ to obtain subtypes for comparative study. The HIV-1 subtypes obtained from the Stanford University HIVdb were transcribed onto the excel spreadsheet pending analysis. HIV-1 resistance-associated mutations and phenotypic drug resistance profiles were obtained from the Stanford University HIV database available on https://hivdb.stanford.edu/hivdb/by-sequences/. Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 at 1,000 bootstrap replications and the Jukes-Cantor model. Once the computation was complete, the Tree Explorer displayed two tree tabs: the original Maximum Likelihood tree and the Bootstrap consensus tree. The results were exported and saved as a .mas file for further review and analysis.

Statistical analysis

Descriptive statistics (median, interquartile range [IQRs], mean, and percentages) were used to summarize the demographic and clinical characteristics of the study participants in this study. The proportions of the different subtypes were analyzed by Pearson's Chi-squared test. Comparisons between different ages and gender were carried out using the Wilcoxon signed-rank test. Pearson's correlation coefficients were calculated. A p-value of less than 0.05 was considered statistically significant.

RESULTS

A total of 140 participants were successfully sequenced hence their subtypes determined (GenBank accession numbers MW618176-MW618315). The age range was 21 to 67 years, with a mean age of 38.96 and a median age of 38 (CI=95%, IQR=14). Seventy-eight (55.7%) of the participants were females with 44.3% being males. The participants were either on EFV or NVP based regimens with 112 (80%) and 28 (20%) being on EFV and NVP based regimens, respectively. The majority of the participants (72%) were on the TDF+3TC+EFV combination regimen.

A look at the circulating HIV-1 subtypes from the REGA HIV subtyping tool revealed that subtypes A1, A2, B, C, D, G and circulating recombinant forms (CRFs) A1_D, A1_C, C_B, A2_H, and A1_F1 were present in the study population. Subtype A1 was the most prevalent subtype in the population, present in 74 (52.9%) of the participants. Subtype D was present in 29 (20.7%), CRF A1_D was present in 10 (7.1%) of the population, subtype C and subtype B were both present in 6 (4.3%), of the population while subtype A2 was present in 5 (3.6%) of the population. CRF A1_C was present in 4 (2.9%) of the study population. Subtype G was present in 2 (1.4%), whereas CRF B_C, CRF A1_F1, and CRF A1_J were all present in 1 (0.7%) of the population (Table 1). CRFs accounted for 13.6% of the circulating HIV-1 subtypes with a majority of these CRFs (~79%) containing subtype A1. CRF A1_D was the most prevalent CRF within the study population. There were significant differences in the availability of the different subtypes and CRFs within the study population ($p=<0.0001$).

Although discordance was reported between subtypes obtained from COMET, REGA, Los Alamos National Library (lanl), and the Stanford University HIVdb subtyping software, the differences were not statistically significant ($p=0.9241$). There were subtype discordances between the REGA subtyping tool, Los Alamos National Library (lanl) and the Stanford University HIVdb subtyping software in reporting all other subtypes except subtypes G, C and D.

Table 2. Distribution of the HIV-1 subtypes circulating in the population generated from REGA, Los Alamos National Library (lanl) and the Stanford University HIVdb subtyping software.

Subtype assignment	REGA numbers (%)	LANL numbers (%)	Stanford numbers (%)
HIV-1 Subtype A1	74 (52.9)	86 (61.4)	86 (61.4)
HIV-1 Subtype D	29 (20.7)	29 (20.7)	25 (17.9)
CRF A1_D	10 (7.1)	0/1/00	0
HIV-1 Subtype C	6 (4.3)	7 (5)	7 (5)
HIV-1 Subtype B	6 (4.3)	12 (8.6)	14 (10)
HIV-1 Subtype A2	4 (2.9)	0/1/00	4 (2.9)
CRF C_A1	4 (2.9)	0/1/00	0
HIV-1 Subtype G	2 (1.4)	2 (1.4)	2 (1.4)
CRF A1_J	2 (1.4)	0/1/00	0
CRF C_B	1 (0.7)	0/1/00	0
CRF A1_F1	1 (0.7)	0/1/00	0
CRF A2_H	1 (0.7)	0/1/00	0
HIV-1 Subtype A6	0	1 (0.7)	0
CRF A1BC	0	1 (0.7)	0
CRF A1_B	0	1 (0.7)	0
CRF 01_AE	0	1 (0.7)	0
CRF10_CD	0	0/1/00	2 (1.4)
Total	140 (100)	140 (100)	140 (100)

REGA subtyping tool reported the highest number of CRFs (25), COMET reported 19 CRFs while lanl and Stanford University HIVdb reported 3 and 2 CRFs, respectively. There were however no significant differences in the number of CRFs generated by the different subtyping tools (p=0.3143). Interestingly, both lanl and the Stanford University HIVdb subtyping software did not report any CRF A1_D, the most prevalent CRF in the study population (Table 2). Only the lanl subtyping reported CRF A1_B_C, CRF A1_B, and CRF 01_AE.

It was further noted that of the 53 patients who did not possess any DRAMs hence no resistance to any of the available medications, 32 (60.4%) were subtype A1, 7 (13.2%) were subtype D, 6 were CRF A1_D, 6 (11.3%) were subtype C, while 3 (5.7%) were subtype A2. Of the 87 participants with at least one DRAM, 42 (48.3%) were subtype A1, 21 (39.6%) were subtype D, 20 (23%) were CRFs, 5 (5.7%) were subtype A2, 5 (5.7%) were subtype C while 4 (4.6%) were subtype A2. It is important to note that whereas none of the subtype C sequences had any DRAMs, all subtype B sequences had DRAMs conferring resistance (Figure 1). Interestingly, the 6 participants with E138 series mutations (mutations previously associated with ETR and RPV resistance only in subtype B) were all subtype A1. There were 13 sequences with K65R mutations (mutations selected by TDF, ABC, d4T, ddI, and rarely 3TC), of which 10 were subtype A1 while 3 were subtype D. All the 37 sequences had at least one thymidine analog mutations (TAM), 23 (62.2%) were subtype A1 or subtype A1 containing CRFs, 9 (24.3%) were subtype D or subtype `D containing CRFs while 4 (10.8%) were subtype B. For this study, there was no significant difference between the subtypes in relation to the acquisition of DRAMs (p = 0.2766). Seven participants had PI DRAMs, 5 of which were subtype A1 while the other 2 were subtype B. Of the 82 participants with NRTI DRAMs, 53 (64.6%) were subtype A1 or A1 containing CRFs, 23 were subtype D or D containing subtypes, 6 were subtype B while 2 were A2 and G, respectively. NNRTI DRAMs, present in 82 participants, 53 (64.6%) were subtype A1 or A1 containing CRFs, 25 (30.4%) were subtype D, 4 were subtype B, 2 were subtype A2 while 2 were subtype G.

While comparing the prevalence of different subtypes, female participants had significantly higher probabilities of having subtypes A1, D and A1_D, G and A2 (p=0.0211) while male participants had a higher prevalence of subtypes B and C (Table 3). Interestingly, 18 (94.7%) of the 19 participants with CRFs were females; hence, only 1 (5.3%) had CRFs.

DISCUSSION

From this study, we confirmed the complexity of the HIV-1 subtypes circulating in the population with subtype A1 being the most predominant (52.9%). Many other studies have also supported the predominance of HIV subtype A1 in most of the Kenyan HIV-positive populations. Gounder et al. (2017), in a study evaluating the complex subtype diversity of HIV-1 among drug users in major Kenyan cities also reported subtype A1 to be the predominant subtype (44.4%); a slightly lower prevalence

Table 3. Distribution of subtypes among male and female participants within the study population.

REGA subtype	Female	Male	Grand Total
A1	51	23	74
D	17	12	29
A1_D	9	1	10
B	4	2	6
C	2	4	6
A1_C	4	0	4
A2	3	1	4
A1_J	2	0	2
G	2	0	2
A1_F1	1	0	1
A2_H	1	0	1
B_C	1	0	1
Grand Total	97	43	140

There were statistically significant differences in the distribution of different subtypes between males and females (p=0.0012) with females having a high prevalence of each of the subtypes except B and C.

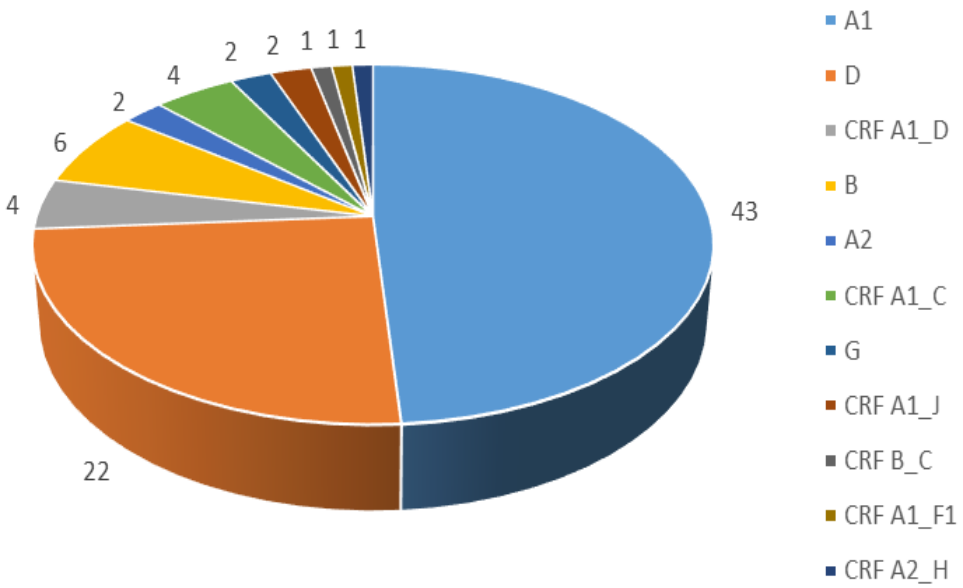

Figure 1. Distribution of different DRAMs among subtypes within the study population.

than that obtained from our current study (52.9%). According to the results of this study, subtype D was the second most predominant subtype in the population (20.7%), thereby differing with the findings by Gounder et al. (2017), who reported subtype C as the second most predominant subtype among drug users in Kenya, but agreeing with results from studies by Khoja et al. (2008), Lihana et al. (2009) and Kantor et al. (2014).

A study by Oyaro et al. (2018), on adults on ART in Western Kenya reported comparable HIV-1 subtype A1 prevalence to this study (51.4% vs 52.8% for our study) but reported lower subtype D prevalence (9.4% vs 20.7% for this study). On the other hand, Adungo et al. (2014), reported an HIV-1 subtype A1 prevalence similar to our reported figures (53.3% vs 52.9% for our study) but reported a slightly higher prevalence of subtype D (28% vs 22.7% for this study) and a comparatively lower prevalence of HIV-1 subtype A1_D (1.3% vs 7.1% for our study). This study further confirms the predominance of HIV-1 subtype A1 in Western Kenya, with an increasing

HIV-1 subtype D and CRF A1_D within the population. It was postulated that the lower subtype D proportions in our study were compensated by comparatively higher A1_D CRFs compared to the study by Adungo et al. (2014). Secondly, our higher sample size (140 compared to 75 in Adungo's study), could have been more representative of the population.

Lel et al. (2014), while looking at transmitted HIV-1 subtypes in children from Busia, reported a higher prevalence of HV-1 subtype A1 (69.8%) compared to 52.9% prevalence in this study. They also reported a higher prevalence of HIV-1 subtype D (22.6%) compared to the 20.7% prevalence reported in this study. They did not report any CRF A1_D. Important to note is the fact that the study looked at transmitted HIV-1 subtypes in infants whereas the current study looked at HIV-1 subtypes in adults hence the glaring differences in the circulating subtypes. The differences in subtype prevalence between the two studies could have resulted from participant age differences.

The HIV-1 subtype present in an individual has been shown to play a major role in the development of resistance towards ART (Chaplin et al., 2011; Santoro and Perno, 2013; Wainberg et al., 2011), viral transmission rates (Conroy et al., 2010; Kiwanuka et al., 2009; Santoro and Perno, 2013; Shaw and Hunter, 2012) as well as progression to disease with subtype D showing faster progression to disease and higher mortality rates than subtype A1 (Baeten et al., 2007; Conroy et al., 2010; Santoro and Perno, 2013; Ssemwanga et al., 2013). Several studies have demonstrated that subtypes D and C are more vulnerable to the development of drug resistance than subtype A (Clutter et al., 2016; Lessells et al., 2012; Santoro and Perno, 2013). These findings were partly supported by the current study where 75.9% of subtype D sequences had DRAMs compared to 53.4% subtype A1 sequences. None of the subtype C sequences in this study possessed any DRAMs, contradicting findings by Santoro and Perno (2013) who recorded higher resistance in subtype C than any other subtype.

Studies highlighting the differences in HIV-1 subtype diversity between males and females are scanty. One study contacted in Kenya showed marked differences in the diversity of transmitted virus between male and female participants where women from Kenya were found to be infected with multiple HIV-1 variants whereas their male counterparts were not (Long et al., 2000). The differences in the diversity of the transmitted virus could explain the emerging differences in subtype diversity between male and female participants in this study.

World Health Organization (2012), reported higher rates of mother-to-child transmission of HIV-1 in mothers having subtypes C and D than in those with subtype A and CRFs. HIV-1 subtype D has also been reported to progress faster to disease and have lower transmissibility than subtype A (Conroy et al., 2010; Ssemwanga et al., 2013). Subtype A has been shown to have higher viral transmission rates than subtype D (Kiwanuka et al., 2009). These findings imply that the high prevalence of subtype A1, D, and CRF A1_D, as confirmed in this current study, may pose a challenge to HIV-1 treatment and prevention strategies including PMTCT.

The present study demonstrated a higher prevalence of subtype D (20.7%) and CRF A1_D (7.1%) compared to most other studies in Kenya (Gounder et al., 2017; Kantor et al., 2014; Khoja et al., 2008; Koigi et al., 2014). HIV-1 subtype D has been reported to be more prevalent in Uganda (Collinson-Streng et al., 2009; Conroy et al., 2010; Kapaata et al., 2013). A higher prevalence of subtype D and its associated CRFs in the border town of Busia could therefore be indicative of cross-border infections from Uganda.

Circulating HIV subtypes in Busia county are highly diverse with reported increased subtype D and its recombinants. There is also an increased prevalence of HIV-1 CRFs within the population, the majority of which are found in females which could be indicative of higher rates of multiple infections in women than men within the study population. Continuous monitoring of the circulating HIV-1 subtypes and CRFs, therefore, is key to formulating strategies aimed at reducing the prevalence of HIV-1 within the population, reducing transmission rates, monitoring and controlling HIV-1 drug resistance testing as well as taming morbidity and mortality associated with HIV infection for the realization of WHO's 90:90:90 HIV treatment targets by the year 2030 and beyond.

Limitations of the study

The small sample size and the cross-sectional nature of the study to track virologic failure in the treated HIV patients and inclusion of infected individuals at different times and stages of HIV infection may not be representative of the epidemic drive in the region. As such the data should therefore be interpreted with caution. Additionally, recombination patterns in a heterogeneous epidemic are complex and will require another generation of genome data level to fully understand the changing dynamics of viral genotypes in this region of Kenya.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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