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Genetic Diversity and the Human Immunodeficiency Virus Type-1: Implications and Impact

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1. Introduction

1.1 Overview

Genetic diversity is the tendency of individual genetic characteristics in a population to vary from one another or the potential of a genotype to change or deviate when exposed to environmental or genetic factors (Dale & Park, 2010). The extent to which the trait or the genotype tends to vary describes genetic variability and at the molecular level variability is measured by determining the rate of mutation. Genetic diversity however measures the number of the actual variation of species in a population (Dale & Park, 2010).

The scope of references to the numerous microbes which demonstrate both genetic diversity and variability within their genomes are beyond the limits of this chapter. Our focus is therefore confined generally to those species which would best explain the nature, mode and factors which influence mutational changes which can be measured.

1.2 Factors influencing diversity

The genetic response of micro-organisms to host and various environmental selective pressures may vary differentially among species. The outcome characterized by the influence of these factors often result in minor or major mutational changes and host adaptability.

Organisms of one genotype such as mumps, rubella and the measles virus do not demonstrate the type variability or diversity as the influenza, human immunodeficiency virus (HIV) and hepatitis viruses. Consequently the predictability of the epidemiology of highly genetically diversified agents is often more challenging. The etiology of an influenza outbreak is invariably the result of genetic re-assortment of different influenza strains. Metzulan et al., (2004) report that genetically distinct multiple viruses can combine to cause an influenza B epidemic in a community and that the frequent re-assortment among these viruses play a role in generating the genetic diversity of influenza B viruses (Metzulan et al., (2004). There are several factors which influence diversity and are dependent on the species involved, the host with various immunological selective pressures, environmental pressures as well as treatment intervention resulting in development of resistance genes to

antibacterial, antiviral, antifungal and antiprotozoal agents. Resistance to several antimicrobials used as therapeutic agents relative to the various and specific categories of microorganisms have been well documented (Murray et al., (2007). The human immunodeficiency virus is demonstrably the most genetically diverse microorganism and is an important reference point in understanding and appreciating the impact of genetic diversity. Whatever the resulting impact of mutational changes, it is important to determine how these changes impact the definitive host, their influence on transmission of the infecting agent and whether these changes are measurable and can be used as an epidemiological tool in clinical and laboratory diagnosis.

1.3 Measuring genetic diversity

The etiological agent in an outbreak can be determined based on phenotypic characteristics which are the observable properties of the organism measured under controlled laboratory settings. Genetic characteristics are, however, more difficult to measure employing various molecular methods and highly trained individuals. The major difference between phenotypic (physiological) and genetic characteristics, is observed in altered physiological changes as opposed to properties of micro-organisms which are genetically inherited. The importance of genotyping as an epidemiological tool in determining mutant genes in drug resistance cannot be overemphasized. This involves the genome of micro-organisms and is the focal point for single or multiple mutational changes.

Behavioural changes of micro-organisms are the results of mutational changes in genes and consequently are responsible for the various features exhibited by these organisms. Included in the changing behavioral patterns of microorganisms and in particular bacteria, may range from differences in biochemical reactions, growth requirements, tolerance to various temperatures and resistance to therapeutic agents. These changes may be demonstrated under controlled laboratory conditions by assays and specific methods designed to determine phenotypic and genotypic characteristics.

2. Background of the problem

The first cases of acquired immunodeficiency syndrome (AIDS) were recognized in the USA during 1981 (Morbidity Mortality Weekly., 1981). By 1983 the human immunodeficiency virus - type 1 (HIV-1) had been identified and was later confirmed to be the aetiologic agent of AIDS (Barré-Sinoussi et al., 1983). At the end of 2000 an estimated 60 million cases of human immunodeficiency virus -type 1 (HIV-1) infection were reported globally (Barré-Sinoussi, 1983; Piot, 2001). The extensive genetic variation which characterizes HIV-1 is an important feature of the HIV/AIDS epidemic (Robertson et al., 2000). The genetic variability of HIV-1 is generated by the lack of proof reading ability of the reverse transcriptase, the rapid turn-over of HIV-1 *in vivo*, host selective immune pressures and recombination events during viral replication (Heyndrickx, 2000; Ho, 1995; Leitner, 2005; Michael, 1995; Tatt, 2001). This has several public health implications although the biological significance is not known (Loussert-Ajaka, 1998; Tatt, 2001). The HIV-1 subtypes vary in the geographical distribution of the virus (Heyndrickx, 2000; Tatt, 2001). The differences in HIV-1 subtypes and geographic diversity impact the development of efficacious vaccines, diagnostic testing and antiviral therapy (Borrow, 1997; Loussert-Ajaka, 1998; Piot, 2001). On the other hand,

the genetic heterogeneity in HIV may aid the surveillance of transmission patterns within the epidemic (Robertson et al., 2000).

The World Health Organization reports that the economies of Caribbean countries, which rely heavily on tourism components are substantially affected by the HIV epidemic (UNAIDS/ WHO, 2004). In addition, most countries in the Caribbean are limited in their capacity to control and track the evolution of the epidemic. It is therefore necessary to strategize surveillance programmes which include the determination of existing HIV-1 subtypes to understand the changing nature of the epidemic and measures to control spread of the virus in the Caribbean (d'Cruz-Grote, 1996; Delwart, 1995).

In Jamaica, the first case of AIDS was reported in 1983 and the number of reported cases increased to over 12,000 by 2007 (Ministry of Health, Jamaica, 2007). Being a tourist destination increases Jamaica's potential for introduction of different HIV-1 subtypes and circulating recombinant forms (CRFs) of the virus into the Jamaican population. The aim of this study is to determine the HIV-1 subtypes in HIV-1 infected persons and the molecular epidemiology of the virus in Jamaica. The main objectives are to:

- i. determine current subtypes and recombinant forms of HIV-1
- ii. investigate the relationships between HIV-1 subtype strains in terms of transmission and disease progression in Jamaica
- iii. evaluate the heteroduplex mobility assay (HMA) as a sub typing method in the HIV/AIDS epidemic in Jamaica

3. Literature review

3.1 Human Immunodeficiency Virus - type 1 (HIV-1)

Human immunodeficiency virus - type 1 (HIV-1), a member of the genus *Lentivirinae*, and family *Retroviridae* is closely related to its primate retrovirus HIV-2 and is the cause of acquired immunodeficiency syndrome (AIDS) (Chiu, 1985; Peeters, 2000). While HIV-1 is responsible for the vast majority of cases in the AIDS pandemic, HIV-2 is almost exclusively confined to Africa and, to a large extent, is responsible for the epidemics seen in that region (Peeters, 2000).

HIV-1 is divided into three genetic groups: group M (major or main), group O (outlier) and N (new or non-M, non-O) group M being responsible for the AIDS pandemic accounting for over 90% of HIV infections worldwide (Hemelaar et al., 2006). Nine pure subtypes of HIV-1 group M are currently known (A-D, F- H, J and K). These subtypes are further into sub-subtypes such as F (F1 and F2 and A (A1, A2 and A3). Subtypes and sub-subtypes may form additional mosaic forms when individuals are dually or multiply- infected by different strains of HIV-1 giving rise to known recombinant forms (Burke, 1997) - CRFs, - drug resistance differences). To date, over 40 CRFs are recognized (Taylor et al., 2008).

3.2 Morphology and genomic organization of human immunodeficiency virus -type 1 (HIV-1)

Structure of HIV-1 A schematic presentation of the structure of the human immunodeficiency virus - type 1 (HIV-1) as described by Hahn, (1994 - 42) is shown in

Figure 1. Like the other human retroviruses the mature virion of HIV comprises icosahedral cores containing the RNA genome, the RT enzyme and gag proteins surrounded by an envelope which is acquired as the virion buds through the host cell membrane (Folks & Khabbaz, 1998). Morphologically HIV-1 differs from HTLV-1 with respect to the nucleoid, the dense inner part of the core, and the pronounced glycoprotein spikes of the envelope. However the overall diameter of the mature particles of both viruses is 100-120 nm in diameter (Folks, 1998; Hahn, 1994). The viral core contains the viral genome, which comprise two identical copies of single stranded positive sense RNA, each of which encodes the complete structural, enzymatic and regulatory proteins (Hahn, 1994).

Fig. 1. Human immunodeficiency virus - type 1 (HIV-1) Adapted: Hahn et al (Hahn, 1994)

The structural proteins that form the nucleocapsid and matrix shell are induced by the *gag* gene. The nonstructural gene products of the HIV-1 *pol* gene region include the reverse transcriptase (RT), integrase and protease (Folks, 1994; Guatelli, 2002; Hahn, 1994). The lipid envelope encoded by the *env* gene contains structural proteins with a surface domain (SU, gp120) and transmembrane domain (TM, gp41) presented as trimeric spikes on the surface of the virion (Folks, 1994; Guatelli, 2002; Hahn, 1994; Kwong, 1998). The viral envelope contains conserved (C) and variable (V) regions, is poorly immunogenic, and contains binding sites for CD4 T cells on gp120 and chemokine co-receptors (Kwong, 1998; Wyatt, 1998a; 1998b). These, so-called, co-receptors are masked by variable loops V2 and V3 of gp120 until the CD4 molecule is engaged (Rizzuto, 1998).

3.3 Genome organization of HIV-1

The complete sequence of HIV-1 contains a 9.2 kb genome (Folks, 1994). The genomic map of the HIV-1 viral genome is well documented and is shown in Figure 2. (Hahn, 1994). In keeping with the general structure of primate lentiviruses, the HIV-1 genome comprises 9 genes, including 3 structural, *gag*, polymerase (*pol*), envelope (*env*), and 6 nonstructural, *tat*, *rev*, *vif*, *nef*, *vpu* and *vpr* genes which are flanked by long terminal repeat (LTR) sequences. The HIV-1 LTR is approximately 640 bp long and is segmented into the U3, R, and U5 regions. The genes of HIV-1, corresponding proteins and functions have been reviewed, as shown in Table 1. Guatelli et al (2002).

Fig. 2. Genome arrangement/map of the HIV-1 genome - adapted: Hahn et al (1994)

3.4 HIV-1 diversity and molecular epidemiology

3.4.1 HIV-1 diversity

The genetic diversity of HIV-1 has resulted in differences among the subtypes in the LTR sequences, transcriptional promoters, accessory and regulatory genes such as *nef*, *tat*, *rev* and *vpu* (Geretti, 2002). The basis of genetic diversity of HIV-1 subtypes is, to a large extent, the consequence of a high mismatch error rate in the reverse transcriptase (RT). In addition, there is an absence of exonuclease proof reading activity (Spira et al., 2003). Other factors which contribute to genetic diversity include the replicative rate of each viral subtype, mutational changes arising in each replicative cycle, genomic recombination and viral fitness (Robertson, 1995; Spira, 2003). Evolutionary changes in the genome may result from host, environmental and other selective pressures (Hu, 2005; Piot, 2002; Quinones-Mateu, 1999; Renjifo, 2002).

In comparison to the *env* gene the *pol* and the *gag* regions are much more intolerant to mutation primarily because they encode core protein sequences which are relatively inflexible. Genetic changes in *pol* and *gag* may give rise to drug resistance as some antiretroviral drugs are directed against the RT and protease proteins encoded by these genes (Spira et al., 2003). For example, 6.8%–10% variation has been shown in the RT of clade B isolates from Ethiopia whereas, intra-clade differences of 3.5–5% have been reported in isolates from Africa, India and South America (Loemba, 2002; Loemba, 2002a; 2002b). Differences in the degree of variation among subtypes also have been observed in LTR sequences and in the transcriptional promoters involved in HIV replication (Montano, 1997).

Other variations observed among HIV-1 subtypes include 14.4%–23.8% differences in the sequences of viral regulatory protein, *nef* (Jubier-Maurin et al., 1999). Subtype C contains a unique *rev* protein and an enlarged *vpu* product while subtype D expresses a *tat* protein with a C-terminus deletion (Gao & Robertson, 1998). Also a significantly shorter envelope V1-V2 loop sequences and fewer potential N-linked glycosylation sites have been shown in subtypes C and A compared to subtype B (Chohan et al., 2005).

Differences in co-receptor usage and syncytia inducing capacity between subtypes have also been reported (Cocchi, 1995; Deng, 1996; Fenyö, 1998; Tersmette, 1988; Worgall, 1999). For example some subtypes may exhibit enhanced susceptibility to CCR5 (R5) inhibition (Marozsan et al., 2005). Subtype B viruses for example appear as R5 viruses in early infection and emerge as X4 viruses in advanced disease while recombinant subtype BG strains appear to be X4 viruses regardless of the clinical stage of disease (Pérez-Alvarez et al., 2006). In contrast subtypes A and C appear infrequently as X4 viruses, even in advanced disease. Subtype C viruses however, are able to switch from CCR5 to CXCR4 using mechanisms similar to those used by subtype B viruses (Pollakis et al., 2004).

Variations among HIV-1 strains also have been seen in the ability to infect Langerhans cells. This tropism has been linked to vaginal/cervical transmission of the virus (Dittmar, 1997; Hu, 1999).

Subtype diversity in response to antiretroviral drugs has been reported (Loemba 2002a, 2002b; Miller, 2007; Quinones-Matue, 1998; Velazquez-Campoy, 2002). Changes in nucleotide sequences play an important role in conferring resistance to each antiretroviral drug or even entire classes of nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI) and protease inhibitors (PI) (Velazquez-Campoy, 2002; Quinones-Matue, 1998; Tantillo, 1994). While the majority of the reports on resistance to antiretroviral drugs and subtype diversity have focused on subtype B it has been noted that Y181C and Y181I mutations, respectively can render HIV-1 group O and all HIV-2 strains resistant to the entire NNRTI class of drugs (Leitner, 2005; McCutchan, 2004; Papuashvili, 2005; Tantillo, 1994).

3.4.2 Global distribution of HIV-1 subtypes, sub-subtypes, circulating recombinant forms (CRFs), and unique recombinant forms (URFs)

Majority of the subtypes, subtypes and several CRFs are found in Africa (Geretti, 2002; Leitner, 2005; McCutchan, 2004; Yang, 2005). The phylogenetic classification of HIV-1 subtypes including sub-subtypes, CRFs and URFs are well documented (Tables 1.) (Taylor, 2008). The profile of HIV-1 subtypes fluctuates in some countries. For example, in 1996 the dominant subtypes in Eastern Europe were subtypes B in homosexuals, C in heterosexuals and G in nosocomially infected patients but by 2003 subtype A1 accounted for more than 90% of infections (Papuashvili et al., 2005). In Western Europe subtype B initially dominated the epidemic but the prevalence of non-subtype B has been noted to be steadily increasing (deMendoza, 2005; Monno, 2005; Taylor, 2005). Non-B subtypes were also noted to be increasing in Canada (Akouamba et al., 2005). Emerging recombinant forms are presently seen in several regions including sub-subtypes B/CRF_O1AE, A/CRFO2_AG and A/CRFO6-cpx seen in South East Asia, Uzbekistan and Estonia, respectively (Carr, 2005; Tovanabutra, 2004; Zetterberg, 2004). A new CRF designated CRF18_cpx was recently isolated in Cuba (Thomson et al., 2005). With the exception of Cuba and Jamaica, Caribbean islands have reported subtype B as virtually the only subtype present in the other regions of

the Caribbean (Cleghorn, 2000; Gittens, 2003; Heslop, 2009; Thomson, 2005; Vaughan, 2003). Five non-B subtypes including A, C, D, E and J have been reported in Jamaica (Heslop et al., 2009). It is important to note that the predominant subtype B in the United States and other countries is associated with a homosexual epidemic, while its association with a heterosexual epidemic has been reported only in the Caribbean (Avila et al., 2002).

Classification	Definition	Examples
Subtypes or clades	Genetically related HIV-1 strains that are essentially phylogenetically equidistant, generating a starlike, rather than a treelike, phylogeny	Subtypes A, B, C, D, F, G, H, J, and K are currently known; A through D are highly prevalent, others have low prevalence and limited geographic distributions
Sub-subtypes	Distinct lineages within a subtype; genetic distance between sub-subtypes is smaller than that between subtypes	Subtypes A and F are subdivided into sub-subtypes A1 through A4 and F1 and F2, respectively; mostly these circulate in Central and West Africa
Intersubtype recombinant forms	Mosaic strains with segments from two or more subtypes alternating across the genome	Common in mixed-subtype epidemics; thought to result from infection of a person with more than one HIV-1 subtype
Circulating recombinant forms	Specific recombinant forms that are spreading in a population; new forms are defined when three people without direct epidemiologic linkage are found to be infected; the assigned name reflects sequence of discovery and subtype composition, with “cpx” indicating forms containing three or more subtypes	Currently, 43 forms are described; CRF01_AE and CRF02_AG are found principally in Southeast Asia and West Africa, respectively; others have more limited distributions
Unique recombinant forms	Intersubtype recombinant forms recovered from only a single person	Hundreds of forms have been described on the basis of partial or complete genome sequences; their potential for epidemic spread is unknown
Geographically distinct lineages	Lineages, often country-specific, that are distinguishable phylogenetically; unlike sub-subtypes, they are not phylogenetically equidistant within subtypes	Thai B, Indian C, West vs. East African D, and Former Soviet Union A (FSU-A)

Adapted from Taylor et al (2008).

Table 1. Phylogenetic Classification and global distribution of HIV-1

3.5 Molecular detection of HIV-1 infection and determination of diversification among the virus subtypes

3.5.1 Molecular detection of HIV-1 infection

The HIV-1 DNA polymerase chain reaction (PCR) assay which detects HIV proviral DNA in PBMC and HIV-1 reverse transcriptase polymerase chain reaction (RT-PCR) assay for detection of HIV-1 RNA virions in plasma are highly sensitive and independent of the host responses (Diagnostic Tests, 1998; Eisenstein, 1990). The nucleic acid amplification methods involve exponential amplification of viral nucleic acid sequences allowing detection of low numbers of HIV infected lymphocytes in patients (Schochetman, 1989).

The PCR assays enable the resolution of early HIV infection before seroconversion and have been particularly useful in the early identification of HIV infected infants born to HIV seropositive women where antibody tests may be ineffective due to passive transfer of maternal antibodies (Sheppard, 1993).

3.5.2 Human Immunodeficiency Virus -type 1 subtype determination

Several techniques for HIV-1 subtype determination have been described (Myers, 1995; Ou Cy, 1992). These comprise molecular based methods including genetic sequencing, probe hybridization assay, restriction length polymorphism (RFLP) analysis, subtype-specific PCR, combinatorial melting assay, heteroduplex mobility assay (HMA) and a phenotypic method of serotyping (Cornelissen, 2007; Delwart, 1995; Luo, 1998; Murphy, 1999; Peeters, 1998; Robbins, 1995; van Harmelen, 1999). Of these methods HMA has been shown to be reliable, rapid and inexpensive (Delwart, 1995; Loussert-Ajaka, 1998; van der Auwera, 2000). Currently the two most widely used methods for HIV-1 subtype determination are genetic sequencing and phylogenetic analysis and HMA. Molecular analysis has been used extensively in the HIV/AIDS pandemic to determine transmission patterns of HIV-1 (Leitner et al, 1999). Recently routine HIV-1 genotyping has been proposed as a tool to identify dual HIV-1 infections (Cornelissen et al, 2007).

3.5.3 DNA sequencing and phylogenetic analysis

Sequencing and phylogenetic analysis are still the most accurate approach for characterizing viral genomes in identifying new subtypes, sub-subtypes, recombinant, complex and unique forms of HIV-1 and are well documented (Leitner, 2005; Loussert-Ajaka, 1998; Tatt, 2001). These methodologies have been important tools in determining sequence relatedness, mutational changes relating to viral ancestry, the relative time of viral introduction, mutations associated with antiretroviral drug resistance, viral transmission patterns and other epidemiological information (Leitner et al., 1998). The definitive method of subtyping HIV-1 is the genetic sequencing of the envelope (*env*), group antigen (*gag*) or polymerase (*pol*). The main advantages of this method are that it allows the determination by phylogenetic analysis of the relatedness among HIV sequences, the study of the evolution of HIV quasispecies in different hosts linked to a common source of infection, and the study of virus transmission patterns among different risk groups (Diaz, 1997; Holmes, 1995; Leitner, 1999). However genetic sequencing is expensive, time consuming and requires highly qualified personnel (Hu, 2005; Leitner, 2005; Loussert-Ajaka, 1998; Tatt, 2001). In addition phylogenetic methods have been widely used to investigate alleged person to person transmission of the virus, for example, from health care professionals to their patients (Holmes, 1993; Ou, 1992). These molecular techniques have been useful in confirming transmissions in household settings, in forensic investigations including sexual offences, and cases of intentional HIV infection (Albert, 1994; APRI newsletter, 2001; Morb Mortal Wkly Report, 1994).

3.5.4 Heteroduplex mobility assay (HMA)

The heteroduplex mobility assay (HMA) has been shown to be a reliable and standardizable method of HIV-1 subtyping (Holmes, 1995; Ou, 1992). Its usefulness as a tool to study HIV diversity in different populations has been demonstrated (APRI Newsletter, 2001; Holmes, 1993; Hu, 2005). The HMA may be used to distinguish strains of HIV-1 and to trace viral quasispecies within individuals and within populations (Albert, 1994; Hu, 2005). It is cost effective and correlates well with genetic sequencing and characterization by phylogenetic analysis (Delwart, 1993; Heyndrickx, 1998). The HMA has been introduced by UNAIDS in several developing countries as a tool for monitoring subtype distribution. The combination

of results from HMA *gag* and *env* genes allows the detection of intersubtype recombinant strains. This is not possible when only one genomic region is typed (Heyndrickx, 2000; Loussert-Ajaka, 1998; Tatt, 2001).

The hypervariable V3 region of *env* is most frequently used for genetic subtyping by the HMA. This is done particularly when there is a need to investigate epidemiologically linked infections (Mulder-Kampinga, 1993; Scarlatti, 1993; Weiser, 1993). Other hypervariable regions of *env* such as V1/V2 and V4/V5 are used less in the HMA to trace epidemiological linkage (Lamers, 1993; Simmonds, 1990). As opposed to the variable region of V3, subtyping of the more conserved *tat* gene region by HMA was particularly successful in identifying epidemiological relationships in cases of person to person transmission of HIV-1 (Simmonds et al., 1990).

4. Molecular characterization and epidemiology of Human Immunodeficiency Deficiency Virus - type 1 (HIV-1) in Jamaica

4.1 Introduction

This chapter describes the study which was undertaken to determine the HIV-1 subtypes in Jamaica and assess the heteroduplex mobility assay (HMA) as a method for tracking the HIV-1 epidemic. The HMA was introduced by the Joint United Nations Programme on HIV/AIDS (UNAIDS) in several developing countries as a tool for monitoring HIV-1 subtype distribution (Heyndrickx, 2000; Loussert-Ajaka, 1998). An abbreviated account of the clinical, immunological and laboratory characteristics of a representative sample of the cohort of HIV-1 infected patients which was studied is also presented. In this chapter the terms HIV-1 isolates and HIV-1 proviral DNA are used interchangeably.

4.2 Materials and methods

4.2.1 Study population

The study population included EDTA blood samples collected from 1341 consecutive HIV-1 infected patients presenting at designated health care centres situated in several parishes throughout Jamaica were received by the Microbiology Department, University Hospital of the West Indies (UHWI), a tertiary care centre, for leucocyte immunophenotyping. Jamaica has 14 parishes from which samples were obtained from 12 parishes. Hanover and Trelawny were the only parishes that were not included. A data abstraction form was used to collect socio-demographic and clinical information from the hospital records of 94 patients who were seen at UHWI. The medical records of the patients seen at other healthcare facilities were not available for the study.

The peripheral blood mononuclear cells (PBMC) were separated from remnant EDTA blood samples of each patient using density gradient centrifugation with Histopaque® 1077 (Sigma-Aldrich Inc., St. Louis, Mo, USA) and the cells were kept frozen at - 20°C until required.

4.2.2 Immunophenotyping and T lymphocyte enumeration

Absolute CD3, CD4 and CD8 T lymphocyte counts were determined on EDTA blood samples by flowcytometry using the FACSCOUNT system and reagents (Biosciences

Immunocytometry Systems, San Jose, CA). The manufacturers' instructions were followed. The established reference ranges for T lymphocyte subsets in the Jamaican adult and paediatric populations (unpublished data) were used to evaluate the immune status of the patients. At least 1 EDTA blood sample from a healthy blood donor was included, as a control, with each test run.

4.2.3 Polymerase Chain Reaction (PCR) amplification of HIV-1 and gag genes

Polymerase chain reaction (PCR) was performed on PBMC from 318 patients. Two microlitre aliquots of PBMC was used as the DNA template for two-step nested polymerase chain reactions (PCR) carried out in a Perkin Elmer 9600 Thermal Cycler (Perkin-Elmer Corp. Norwalk, Conn.) using the PCR mixtures and amplification programs described in the HIV-1 *env/gag* HMA subtyping kit manuals, with modifications (Delwart version 5; Heslop, 2005; van der Auwera, 2000). The HIV-1 HMA subtyping kits which include plasmid clones of the complete genome of HIV-1 subtypes A-J of the major M group of HIV-1 sequences and primer pairs for PCR amplification of the HIV-1 *env* and *gag* genes were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Program (Heslop, 2005; van der Auwera, 2000). The PCR core reagents used were commercially prepared (Invitrogen, Life Technologies, Grand Island, NY).

Due to the high rate of failed amplifications with primers supplied in the HMA kit, the *gag* gene primer pairs were replaced with DT1/DT7 and DT3/DT6 as first and second round primers (Delwart, 1995; Tatt, 2000; van der Auwera, 2000). The plasmids containing cloned *env* and *gag* genes, respectively, were amplified using second round *env* and *gag* primers. A negative control in which the DNA template was replaced by reagent grade water was included with each PCR assay.

The second round PCR amplification of the *env* gene using the ED5/ ED12 primers yielded a 1.2 Kb fragment spanning the V1-V5 coding region of HIV-1 gp120, while the ES7/ES8 primer pair yielded a 666 bp fragment spanning the V3-V5 coding region of gp120. Second round amplification of the *gag* gene resulted in a 748 bp fragment of the HIV-1 p17/ p 24 gene.

The PCR products were resolved by agarose gel electrophoresis carried out in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) at 80 volts for 45 minutes and visualized by ultraviolet (UV) illumination. The appropriate kilobase markers and controls were included in each run.

4.2.4 Genotyping of HIV-1 *env* and *gag* genes by Heteroduplex Mobility Assay (HMA)

The heteroduplex mobility assays (HMA) were performed on HIV-1 isolates from 180 patients. The HMA was performed as previously described with modifications (Loussert-Ajaka, 1998).

Both the *gag*-HMA and *env*-HMA were performed on each HIV-1 isolate. The subtype reference strains were selected following a pilot run of the HMA on 28 samples. Eleven *env* and 12 *gag* reference subtypes were selected from a panel of reference subtypes to include subtypes A, B, C, D, E, F, G, H and J in the HMA procedure. All subtype B reference strains (4 from the *gag* and 3 from the *env*) included in the kit were used in the HMA due to the

dominance of this clade in the Caribbean and North America. In this method equal volumes of the patient HIV strain DNA and reference strain DNA are mixed under certain conditions to form heteroduplexes. This is followed by polyacrylamide gel electrophoresis. The HIV subtype is identified by the heteroduplex with the highest electrophoretic mobility which is the most closely matched heteroduplex formed from the most closely related strains. For the formation of heteroduplexes 5µl aliquots of a mixture containing equal volumes (5µl each and 1µl annealing fluid) of the second round PCR products of the HIV-1 isolates and the reference subtype strains were separated by polyacrylamide gel electrophoresis using Criterion™ precast gels which contained 5% polyacrylamide and 6M urea (Bio-Rad Laboratories, Hercules, CA). The heteroduplexes were visualized under UV light after ethidium bromide (0.5µg/ml) staining. The HIV-1 subtype assigned was that of the heteroduplex with the highest electrophoretic mobility.

4.2.5 Genotyping of HIV-1 *env* and *gag* genes by DNA sequencing and phylogenetic analysis

A total 63 HIV-1 isolates including 5 which were untypable by HMA were subjected to DNA sequencing and phylogenetic analysis of the purified PCR product or cloned PCR product. The sequencing primers for direct DNA sequencing were the second round PCR primers of the *gag* and *env* gene regions, respectively as described above.

4.2.6 Purification and cloning of amplicons of HIV-1 *env* and *gag* genes

The PCR products of the *gag* and *env* genes were purified using a commercially prepared kit (QIAGEN Inc. Valencia, CA) and cloned using the pGEM-T Easy Vector System Kit (Promega Corporation, Madison, WI). The manufacturers' instructions were followed.

4.2.7 Confirmation of *env* and *gag* clones by Polymerase Chain Reaction (PCR)

The recombinant clones were subjected to *env*-DNA PCR and *gag*-DNA PCR, respectively followed by agar gel electrophoresis to confirm the presence of the cloned DNA inserts, as described above.

4.2.8 Purification of HIV-1 *env* and *gag* gene clones

The plasmid vectors carrying the HIV-1 *env* and *gag* gene clones were purified using the PlasmidPURE™ DNA Miniprep kit (Sigma, St Louis, MO). The procedure followed was in accordance with the manufacturer's instructions. The final product of this procedure was highly purified plasmid DNA. The yield of cloned DNA was estimated by agar gel electrophoresis of 1-5 µl of the DNA using 1.5% agarose gel and read against an appropriate kilobase marker with ethidium bromide staining as described above. The *env* and *gag* gene clones were stored at -20°C until required.

4.2.9 Cycle sequencing of HIV-1 *env*, *gag* amplicons and clones

Commercially prepared reagents (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) were used for sequencing following the manufacturer's instructions. Briefly, sequence reactions were performed on the different HIV-1 DNA templates, including

purified PCR products, purified cloned inserts, double stranded control DNA (pGEM® 3Zf(+), 0.2µg/µl or 200ng/µl) and negative control DNA templates supplied with the kit. Sequence reactions were carried out in a Perkins Elmer 9600 thermocycler using the following parameters:

96°C for 10 seconds	↓ 25 cycles
50°C for 5 seconds	
60°C for 4 minutes	

Following purification of the sequence reactions using the DyeX 2 (QIAGEN, Valencia, CA) DNA sequencing was performed using the ABI 3100 analyser (Applied Biosystems, Foster City, CA).

4.2.10 Sequence editing and phylogenetic analysis of HIV-1 *env* and *gag* sequences

Following the cycle sequencing procedure single strand sequences generated by forward and reverse sequencing primers were assembled into contigs (a contiguous alignment of overlapping sequences) using the Sequencher™ 4.0 software program (Gene Codes Corporation, Ann Arbor, MI). The formed contigs were edited for ambiguous base pairs introduced during sequencing. The chromatograms generated were subsequently compared for relationships between the HIV-1 isolates and GenBank reference strains. All sequences were imported in the FASTA format for alignment by the ClustalX software programme (<http://inn-prot.weizmann.ac.il/software/ClustalX.html>) (Thompson et al., 1997). Alignments were carried out firstly by pairwise matching of the sequences of interest, followed by multiple alignments with referenced HIV-1 sequences from GenBank (http://hiv-web.lanl.gov/ALIGN_CURRENT/ALIGN-INDEX.html). The multiple aligned sequences were edited prior to the construction of phylogenetic trees. The PAUP 4.0 software programme (Beta version 8 for Windows 95/98/ME/NT/2000/XP, Sinauer Associates, Inc, Sunderland, MA) was used to construct rooted and unrooted neighbour-joining phylogenetic trees using the SIV_{CPZ} sequence (simian derived virus) of CPZ.GA.CPZGAB (GenBank accession number X52154, http://hiv-web.lanl.gov/ALIGN_CURRENT/ALIGN-INDEX.html) as the out-group (Swofford, 2003). The phylogenetic trees were subsequently bootstrapped (using 1000 replications) to establish confidence and statistical reliability (Hall, 2001).

4.2.11 Statistical analyses

Univariate and multivariate analyses of the patients' sociodemographic, clinical and laboratory data were performed using the Statistical Packages for Social Sciences (SPSS) Version 8 software.

4.3 Results

4.3.1 Demographic, clinical and immunological characteristics of HIV-1 infected patients

As shown in Table 2, the study population was predominantly heterosexual (90/94, 96%) with a preponderance of females (68/94, 72%) and did not contain any injecting drug users (0/94, 0%) while the only non-injecting drug used was marijuana (1/94, 1%). Other risk factors for HIV infection included inconsistent condom use (21/94, 20%), history of

ulcerative (12/94, 13%) and non-ulcerative (15/94, 16%) STI. The percentages of antiretroviral drug therapy naïve and antiretroviral drug treated patients were 56% (53/94) and 44% (41/94), respectively. The clinical manifestations in the HIV-infected persons are summarized in Table 3 and a variety was seen. Constitutional symptoms including weight loss occurred in more than three quarters of the patient population and skin rashes in almost a half while opportunistic and respiratory infections were present in over one third. Other STI were common, over 20% and weight loss was found in just under 20%. There was 1 case of Kaposi’s sarcoma while 13% of the population were asymptomatic.

The CD4 T cell counts were available for 73 patients. No trend was noted in the CD4 T cell counts by age and duration of diagnosis of HIV-1 infection for the cohort. However, when the patient population was separated into antiretroviral therapy naïve and treated categories, CD4 counts were statistically significantly higher in untreated patients ($p<0.005$, Table 4). A significant decreasing trend in CD4 counts with age in untreated patients ($p<0.05$) was observed (Table 5). In contrast no significant correlation was observed with age and CD4 counts in antiretroviral drug treated patients (Table 14). In multivariate analyses both increasing age in antiretroviral therapy naïve patients and absence of antiretroviral drug therapy were identified as independent risk factors for low CD4 counts ($p= 0. 022$; 95% confidence interval (CI), 1.014, 1.197 and $p=0.003$; 95% CI, 2.096, 35.982, respectively).

Characteristics	94
Mean age (years) ± SD	37 ±10
Gender	
Male	26(28)
Female	68(72)
Asymptomatic/Pre-AIDS	3(14)
Symptomatic/ AIDS	81(86)
Risk Factors	
Sexual practices	
Heterosexual	90(96)
MSM	4(15)
Inconsistent condom use	21(20)
History of STI	
Ulcerative	12(13)
Non-ulcerative	15(16)
Drug use	
Marijuana	1(1)
Cocaine	0(0)
IV drug	0(0)
Antiretroviral drug therapy	
Yes	41(44)
No	53(56)

*MSM = Men who have sex with men. The cohort of HIV-1 infected patients was predominantly heterosexual.

Table 2. Characteristics of 94 HIV-1 infected patients presenting at the University Hospital of the West Indies (UHWI)*

Manifestation	Frequency (%)
Constitutional symptoms	73(77)
Skin rashes	46(49)
Opportunistic infection	35(37)
Respiratory infection	34(36)
Other STI	21(22)
Vomiting/ diarrhoea	13(14)
Lymphadenopathy	11(13)
Neurological	6(6)
Kaposi sarcoma	1(1)
Asymptomatic	13(14)

* Respiratory infections include cough, dyspnea, bacterial pnepneumonia, pneumocystis carinii pneumonia, otitis media. Opportunistic infections include candidiasis, toxoplasmosis, cytomegalo virus infection, scabies, molluscum contagiosum, ulcers, hairy leukoplakia of tongue. Neurological manifestations include meningitis and neuralgia.

Table 3. Clinical manifestations in 94 HIV- infected patients*

CD4 cells/μl		
Category (N)	Mean (± SD)	Range
Untreated (32)	378 (341)	5- 1305
Treated(41)	192 (163)	1- 691
Total (73)	291(140)	1- 1305

* N = Number of patients in each category. CD4 T Lymphocyte counts were available for 73/94 patients. The CD4 cells counts were statistically significantly higher in the untreated patients (p< 0.005)

Table 4. CD4+ T Lymphocyte counts in antiretroviral drug treated and untreated HIV- infected patients*

CD4+ cells/μl		
Age group/ years(N)	Mean (± SD)	Range
≤ 20 (2)	879 (315)	656 - 1102
21 - 30 (13)	486 (345)	19 - 1305
31 - 40 (12)	284 (270)	7 - 684
41 - 50 (2)	294 (392)	16 - 571
51 - 60 (3)	8 (2)	5 - 9

* N = Number of patients in each category. A decreasing trend in CD4 counts with age in untreated patients (p<0.05) was observed.

Table 5. CD4+ T Lymphocyte counts in 32 antiretroviral drug therapy naive patients by age*

4.3.2 HIV-1 DNA-PCR assay

The PCR amplicons obtained included *env* gene, 141/318 (44.3%), *gag* gene, 170/318 (53.5%). Of the total 318 samples tested, positive results in one or both genes were obtained in 251 (79%), 113 (113/318, 35.5%) were amplified in both genes while neither the *env* or *gag* gene was amplified in 67 (21.0%) samples. The results of the HIV-1 *env* and *gag* DNA – PCR assays are summarized in Table 6. As shown in Figure 3, all *gag* (35/35,100%) and *env* (22/22, 100%) reference subtype plasmids were amplified successfully by all second round primers.

Category	Positive(%)	Negative(%)
Env-PCR	141(44.3)	177(55.7)
Gag-PCR	170(53.5)	148(46.5)
Env + gag-PCR	113(35.5)	205(64.5)

Table 6. HIV-1 DNA – PCR assays in 318 HIV- infected patients

Fig. 3. DNA polymerase chain reaction (PCR) assay: Photographs (A-D) of agarose gel electrophoresis of PCR products. Lane 1 in each gel contains the base pair (bp) marker. Gels A and B show the 748 bp PCR product of the *gag* gene region of HIV-1 isolates and reference strains. Gels C and D show the 1254 bp PCR product of the *env* gene region of HIV-1 isolates and reference strains.

4.3.3 Heteroduplex mobility assay (HMA) analysis of HIV-1 proviral DNA/isolates

The *env* and *gag* HMA were performed on the PCR amplicons of 180 samples. Of these 174 (96.7%) were unambiguously genotyped while 6 samples (3.3%) were indeterminate. The majority of HIV-1 isolates were subtype B (170/180, 94.4%) while 1 (0.6%) isolate each of subtypes A, C, D and E was found. The representative HMA polyacrylamide gel photographs in Figures. 4-5 illustrate the endpoint of the HMA procedure and comparative electrophoretic mobilities of different HIV subtypes which were found.

Fig. 4. Heteroduplex mobility assay (HMA) of HIV-1 strains. Photographs (1-III) of polyacrylamide gels showing subtype B strains which formed heteroduplexes with 3 subtype B reference strains B1 (I), B2 (II) and B3 (III). Heteroduplexes of 11 subtype reference strains including A-J strains were tested. The subtypes assigned are those of the heteroduplexes with the highest mobility formed between amplicons of the patient's isolate and subtype reference strains. The first lane (m) of each gel contains the Kb marker and the last lane (BLK) the negative control.

Fig. 5. Heteroduplex mobility assay (HMA) of HIV-1 non-B subtype strains. Photographs (IV-VII) of polyacrylamide gels showing subtypes A (IV), C (V), D (VI), E (VII). Heteroduplexes of 11 subtype reference strains A-J were tested. The subtypes assigned are those of the heroduplexes with the highest mobility formed between amplicons of the patient's isolate and reference subtype strains. The first lane (m) of each gel contains the Kb marker and the last lane (Blk) the negative control.

4.3.4 DNA sequencing and phylogenetic analysis of HIV-1 proviral DNA/isolates

A total 63 HIV-1 isolates were subjected to automated DNA sequencing which yielded 54 DNA sequences (54/63, 86%) including 26 *gag* and 28 *env* gene region sequences. Representative neighbor-joining phylogenetic trees constructed from the *gag* and *env* gene sequences, respectively are shown in Figures 6-7. Of the 54 sequences 50 (50/54, 93%) were assigned subtype B, 2 (2/54, 4%) subtype D and 1 each (1/54, 2%) assigned subtypes A and J, respectively. In the *gag* gene 87% (22/26) clustered with HIV-1 B subtype reference strains (BUS98, BFR, BTH90), 2 (8%) with D subtype reference strain (DUG94), 1 (4%) each with A subtype and CRF reference strains (A1 UG92, 18CPXCM) and J subtype reference strain (JSE93). The subtype assignments of the *gag* gene sequences were supported by bootstrap values $\geq 70\%$ in 96% (25/26) of isolates. On the other hand all (28/28, 100%) *env* gene sequences clustered with B subtype reference strains (BUS98, BFR, BTH90). The subtype assignments of the majority of *env* gene sequences (25/28) were supported by bootstrap values $> 90\%$. The relationships between the Jamaican strains were assessed by the unrooted phylogenetic trees constructed from the HIV-1 *gag* and *env* sequences.

Fig. 6. Phylogenetic analysis of HIV-1 *gag* gene sequences of Jamaican isolates: Neighbour-joining phylogenetic tree constructed from 26 HIV-1 *gag* sequences, reference subtype strains (B US98, B FR, B TH90, D UG94, J SE93, A1 UG92) and circulating recombinant forms (18CPXCM). Numbers at the nodes of the tree indicate bootstrap values expressed as the percentage of 1000 replicates supporting each subtype in the tree.

Fig. 7. Phylogenetic analysis of HIV-1 *env* gene sequences of Jamaican isolates: Neighbour-joining phylogenetic tree constructed from 27 Jamaican HIV-1 *env* sequences and subtype reference strains. The Jamaican isolates clustered with subtype B reference strains (BUS98, BTH90, BFR83). Supporting bootstrap values, indicated by numbers at internal nodes, are expressed as the percentage of 1000 replicates supporting each subtype in the tree.

4.3.5 Comparison of HIV-1 subtype assignment by HMA and phylogenetic analysis

For the subtype B viruses which were characterized by both methods there was an overall 86% (38/44) concordance between phylogenetic analysis and HMA. Of the 6 discordant subtype B isolates, 5 were indeterminate and 1 identified as subtype D in the HMA. For non-B subtypes the rate of concordance was lower (67%, 2/3) as the 2 subtype D viruses were identified as subtype E and subtype B, respectively by HMA. The HMA was concordant with phylogenetic analysis in discriminating the only subtype A virus in the sample.

4.3.6 Geographical distribution of HIV-1 subtypes in Jamaica

The geographical distribution of HIV-1 subtype strains among the parishes in Jamaica is shown in Figure 8. Multiple subtypes were found in 3 of the 14 parishes sampled.

Fig. 8. Map of Jamaica showing the geographical distribution of the different HIV-1 subtype strains in 14 parishes: Multiple subtypes were found in the parishes of St. Ann (population: 52,600), Portland (population: 33,000) and the capital city Kingston (population: 157,000).

4.4 Discussion

This study of the molecular epidemiology of HIV-1 is the largest and most recent of its kind from the English-speaking Caribbean (Cleghorn, 2000; Gittens, 2003; Vaughan, 2003). Another important difference between the present study and similar studies conducted in the other English-speaking Caribbean countries is the fact that the *gag* gene of the HIV-1 isolates was genotyped (Gittens, 2003; Vaughan, 2003).

The patients whose records were reviewed are representative of the cohort of adult HIV-1 infected patients from whom peripheral blood mononuclear cells (PBMC) were obtained for HIV-1 isolation and molecular typing. The exact time of contracting HIV infection could not be ascertained in these patients. However at the time of diagnosis a spectrum of AIDS related conditions which defined both primary and late stage HIV-1 infection were seen in the majority of patients whereas a few were asymptomatic (JAMA, 2006).

The explanation for the late presentation at hospital of some of the patients is beyond the scope of this study. What is notable, however, is the relatively long period of time between diagnosis and having the required CD4 cell counts done to assess the immune status and implement antiretroviral regimens in some patients according to current guidelines (JAMA, 2006; Office of AIDS Research Advisory Council, 2008). The late presentation, delay in immune monitoring and antiretroviral therapy are reflected in the proportion of patients whose CD4+ T lymphocyte counts were already below 350 and 200 cells/ μ l, respectively, at the time of testing. This may be due to the fact that CD4 cell determinations and antiretroviral drugs only became widely accessible to Jamaican patients under the Global HIV/AIDS Programme in mid- 2004.

The absence of intravenous drug users from the cohort is also worthy of note. Cleghorn et al (Cleghorn et al., 2000) also reported an absence of intravenous drug users from a cohort of HIV infected patients in Trinidad and Tobago. However other known risk factors for HIV-1 infection such as history of other STI, inconsistent condom use and multiple sexual partners were comparable to that seen in other cohorts including that reported from Trinidad and

Tobago and other Caribbean /West Indian islands (Cleghorn, 2000; Fleming, 1999; Gibney, 1999; Murphy, 1999).

In this Jamaican cohort increasing age in antiretroviral drug naïve patients and absence of antiretroviral drug therapy in all age categories were the only independent risk factors identified for decreasing CD4 T cell counts. This is in keeping with what is known of the natural progression of HIV infection (Fox et al., 2008). Although disease progression in HIV-1 infected patients has been linked with HIV-1 subtypes the impact of different subtypes was not assessed in this study as the overwhelming majority of patients were infected with subtype B (Baeten, 2007; Kaleebu, 2002; Kanki, 1999). The impact of antiretroviral treatment on disease progression was evident as the CD4 T cell counts were significantly lower in the group of antiretroviral drug treated patients and similar in the group of patients on antiretroviral therapy irrespective of age (Harari, 2004; Hogg, 1999).

A panel of HIV-1 primer sets from international sources was used in the PCR amplifications. Nonetheless failures in amplifications of either *gag* or *env* gene were observed in about half of the isolates and failures in both genes in about a fifth of isolates. The low sensitivity of the HIV PCR assays and the frequent non-concordance between the PCR results for the *gag* and *env* gene regions might be due to the marked genetic heterogeneity of the virus in both genes (Delwart, version 5; Swofford, 2003). This is supported by the fact that all reference plasmids were successfully amplified using the primer sets from the National Institutes of Health (NIH) prepared kits which failed to amplify a number of the HIV-1 isolates. Other authors have encountered the problem of unamplifiable HIV-1 strains with the *env* primers from the HMA kits due to the broad heterogeneity within the gp120 region of the *env* gene (Agwale et al., 2001). For the above mentioned reasons antiretroviral drug use in HIV-infected patients in Jamaica might not have contributed substantially to genetic variation in the *env* gene as reported by others (Agwale et al., 2001). Six HIV-1 subtypes were identified in this study including subtypes A, B, C, D, E and J. It was not entirely surprising to find that the majority of HIV-1 isolates in Jamaica were subtype B which accounted for over 90% of infections. This is in keeping with reports, from other English speaking Caribbean Islands, which did not include Jamaican samples (Gittens, 2003; Vaughan, 2003). Subtype B is also the predominant subtype in North America, Western Europe, Australia and South America (Distler, 1995; Laukkanen, 2000; Ramos, 1999).

The percentages of subtypes A, C, D, E and J found in this study were low. However the mere presence of these non-B HIV-1 subtypes in the HIV/AIDS population in Jamaica is of great importance as it signals high genetic diversity of the virus and the HIV/AIDS epidemic. It might be essential that these subtypes be considered in vaccine modeling. Outside of Central Africa a limited number of HIV-1 variants are usually circulating in each country, rarely more than 2 or 3, representing multiple introductions or in some cases locally generated CRFs (Pérez et al., 2006). Subtype A is found in East and Central Africa, Central Asia and Eastern Europe with a global prevalence of 12.3% (Hemelaar et al., 2006). Subtype C, the dominant subtype in India and China, is the most prevalent subtype worldwide accounting for approximately 50% of infections (388, 389). Subtype D is generally limited to East and Central Africa with sporadic cases observed in Southern and Western Africa (Vogt et al., 1986). Subtype E is a recombinant strain (CRF01_AE), and not a distinct subtype as initially thought, which co-circulates with subtype B within the intravenous drug user (IDU) population and fishermen in Thailand and also occurs in

Vietnam and South East Asia (Entz, 2000; Heyndrickx, 2000; Wasi, 1995). Subtype J occurs in various locations with a less than 1% prevalence (Archer, 2007; Hemelaar, 2006).

The DNA sequencing and phylogenetic analyses performed in the study did not allow identification of inter-subtype recombinants as the subtype of the HIV-1 isolates were assigned on the DNA sequence of a single gene region, either *env* or *gag* (Louwagie, 1993; Tatt, 2000). On the other hand genotyping by HMA analysis was performed on the *gag* and *env* gene sequences of each isolate. This method also failed to identify any recombinant strain in Jamaica except for the subtype E strain which was re-assigned subtype D by phylogenetic analysis (Heslop et al., 2005). The subtype A virus identified in this Jamaican sample appears to be related, in the *gag* gene, to CRF18_cpx which originated in Central Africa and, currently, is circulating in Cuba (Thomson et al., 2005). It has been reported that the genome of CRF18-cpx contains multiple segments clustering with subtypes A1, F, G, H and K as well as segments failing to cluster with any subtypes (Thomson et al., 2005). The multiple subtypes of HIV-1 in circulation in Jamaica should be monitored for the likely emergence of inter-subtype recombinant strains as the epidemic progresses. Recombinant strains have been observed mostly in areas where multiple subtypes co-circulate (Heyndrickx et al., 2000). The emergence of recombinant strains in other Caribbean and Latin American countries, including Cuba and Brazil, following sudden shifts in HIV-1 subtype distribution from a predominance of subtype B is well documented (Pérez, 2006; Santos, 2006; Tatt, 2000; Thomson, 2005).

The limited divergence of the Jamaican HIV-1 strains, from the subtype reference sequences, in the *gag* and *env* gene is another important observation. This was evident from the high bootstrap values supporting the subtype classification of this sample especially subtype B viruses. This might at least be partly explained by the fact that the cohort was largely antiretroviral therapy naïve. Secondly the Jamaican HIV-1 isolates which were not amplified by the NIH primer sets might include the more divergent strains and these were not genotyped (Vaughan et al., 2003). Gittens et al (Gittens et al., 2003) reported a broad genetic diversity of *env* gene sequences in Barbados suggesting multiple introductions of subtype B viruses to the island (10). Similarly Cleghorn et al reported a significant subcluster within the B subtype in Trinidad (Vaughan et al., 2003).

The discordance between HMA and phylogenetic analysis in the assignment of subtypes is not unique to the present study (Loussert-Ajaka, 1998; Novitski, 1996; Swofford, 2003; Thompson, 1994). The HMA has been shown to give excellent results for the detection of subtypes B and F, the prevalent subtypes in Caucasian patients originating from Western countries and Romania, respectively. Conversely extensive viral variation might create problems in countries like Africa where different HIV subtypes have been circulating longer (Loussert-Ajaka et al., 1998). This raises the question of whether further problems with the HMA might occur in Jamaica with its multiple subtypes of the virus. For example, in previous studies of international cohorts, the HIV-1 strains which were deemed untypeable/indeterminate by HMA and were subsequently assigned by DNA sequencing turned out to be highly divergent subtypes A-D or G related strains (Loussert-Ajaka, 1998; Novitski, 1996; Swofford, 2003; Thompson, 1994). In one study almost two thirds of the subtype D isolates were incorrectly genotyped by HMA (10). In the present Jamaican sample the 2 subtype D viruses were incorrectly genotyped, by HMA, as subtypes B and E, respectively (UNAIDS, 2004 - 12). The viruses belonging to subtypes B and D have been shown to be closely related with respect to *gag*, *env* and *pol* gene sequences and probably

diverged relatively recently. Therefore separation of subtype B and subtype D is not as well defined as between other subtypes (Tatt, 2000; UNAIDS/WHO, 2006). Nonetheless the HMA remains the recommended genotyping method second only to DNA sequencing. Consequently it has been recommended that the plasmid selection in the HMA kit be constantly revised to cover viral diversification (Loussert-Ajaka, 1998; Thompson, 1994).

Limitations of the study include the fact that a substantial proportion of samples were not amplifiable by the primer sets which were used. The failure to sequence both the *env* and *gag* genes of each isolate to indentify inter-subtype recombinants is another limitation. A more comprehensive study of the genetic diversity of the Jamaican HIV-1 isolates should include customized or more conservative primers, such as those recently described, and sequencing *pol* gene regions (Agwale, 2001; Gittens, 2003; Tatt, 2000). More extensive genomic sequence analyses which were not performed in this study might also reveal more genetic diversity and linkages among Jamaican isolates.

4.4.1 Conclusions and recommendations

Transmission of the multiple subtypes of HIV-1 in the Jamaican population is predominantly heterosexual and is not linked to intravenous drug use. HIV-1 subtype B is the dominant subtype contributing to the HIV/AIDS epidemic amidst high genetic regions of the virus in Jamaica. The results also emphasize the need for genotyping of multiple genetic regions, to be attempted, to ensure positive results against the background of frequent failures of the necessary PCR assays.

The HMA is well established as a genotyping method in the Jamaican setting. Our results confirm the value of this cost- effective and reliable method in tracking the HIV/AIDS epidemic. This does not abrogate the need for DNA sequencing and phylogenetic analysis which should be used as an adjunct methodology to resolve indeterminate HMA results and ensure the accurate assignment of non-B subtype strains of HIV-1.

The implications of HIV-1 genetic diversity and its impact on diagnosis, treatment, progression and prevention of disease continue to be better understood. Therefore it is important that the molecular epidemiology of HIV-1 in Jamaica continue to be monitored especially in light of pending vaccines and multiple subtypes of the virus found in Jamaica. We recommend the use of the HMA as a major method supported by DNA sequencing and phylogenetic analysis for tracking the transmission of the virus. A more comprehensive use of DNA sequencing which involves other genetic regions of the virus such as the *pol* gene and more extensive characterization of the genome are required to better elucidate the molecular epidemiology of this virus in Jamaica. Further studies also should include optimizing the HMA through the development of PCR primers and reference clones of HIV-1 which are customized for Jamaican strains of HIV-1.

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Genetic Diversity in Microorganisms presents chapters revealing the magnitude of genetic diversity of microorganisms living in different environmental conditions. The complexity and diversity of microbial populations is by far the highest among all living organisms. The diversity of microbial communities and their ecologic roles are being explored in soil, water, on plants and in animals, and in extreme environments such as the arctic deep-sea vents or high saline lakes. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in microorganisms. The purpose of the book is to provide a glimpse into the dynamic process of genetic diversity of microorganisms by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of microbial phylogeny, genetic diversity, and molecular biology.

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