

Review

Tools for genotyping human immunodeficiency virus, and implications of genetic diversity on diagnostics, treatment and prevention

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Human immunodeficiency virus (HIV) presents an unprecedented genetic variability within infected individuals and across geographical locales. Sub-Saharan Africa accounts for almost 70% of all HIV infections worldwide and represents the region with the highest diversity of genetic variants of the virus. This review looks at the current approaches and techniques for the determination of HIV genetic diversity. In addition, the rationale for a continuous periodic monitoring of the genetic landscape due to the implications of shifting genetics on the efficacy of diagnostics, treatment and prevention strategies is highlighted.

Key words: HIV, genetic diversity, genotyping, diagnostics, prevention, treatment.

INTRODUCTION

Infection with human immunodeficiency virus (HIV) is a world wide public health concern. The global spread of HIV has been rapid and several countries are dealing with huge health burdens due to HIV. The United Nations Joint Programme on AIDS (UNAIDS) estimated that 33.2 million people were living with HIV, with 2.5 million newly infected, whereas 1.3 million people died from AIDS in 2007 (UNAIDS, 2008; <http://www.unaids.org/en/KnowledgeCentre/HIVData/GlobalReport/2008/>).

Sub-Saharan Africa remains by far the worst-affected region, with an estimated 22.5 million people infected with HIV at the end of 2007, and prevalence vary between regions. Southern Africa is the hardest hit region, with adult prevalence rates exceeding 15% in most countries, reaching 30% in Swaziland and Botswana. Eastern Africa also experiences relatively high levels of prevalence with estimates above 10% in some countries, although there are signs that the pandemic is declining in this region, notably in Uganda. Elsewhere, signs of decline have been noted in Zimbabwe and Botswana.

West Africa on the other hand has been much less affected by the pandemic. Several countries in West Africa reportedly have prevalence rates around 2-3% and no country has yet reached rates above 10%. However, in two of the regions most populous countries, Nigeria and Côte d'Ivoire, between 5 and 7% of their populations respectively are reported to be infected by the virus (UNAIDS, 2008).

HIV TYPES, GROUPS, SUBTYPES AND RECOMBINANTS

Phylogenetic and evolutionary studies have shown that HIV, the causative agent of acquired immune deficiency syndrome (AIDS), (Barr-Sinousi et al., 1983; Clavel et al., 1986), is a zoonotic infection. HIV type 1 (HIV-1) is a cross species variant of simian immunodeficiency virus (SIVcpz) from chimpanzees (*Pan troglodytes troglodytes*) of West and Central Africa; and HIV-2 is a variant of SIV from sooty mangabeys monkeys (SIVsm) (*Cercocebus atys*) common in West Africa (Hirsh et al., 1989; Gao et al., 1992; 1999).

Three groups of HIV-1 namely major group M consisting of 9 distinct subtypes (A-D, F-H, J and K), group O and group N viruses have been identified in humans, on the basis of differences in the envelop (*env*),

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group associate antigens (*gag*), and the polymerase (*pol*) genes. Detailed analysis reveals subtypes A and F as comprising sub-subtypes A1, A2, F1 and F2. In addition, the epidemic in certain geographic regions is mostly driven by circulating recombinant forms (CRF), which are intersubtype recombinants and variants with mosaic genomes. To date 43 CRF have been described (www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). Recombinant viruses that are not epidemiologically linked, and do not constitute a significant amount of infections in a particular geographic locale are not considered CRF. Several cases of known circulating recombinant forms that recombine with known subtypes or other CRF have been identified and are referred to as unique recombinant forms (Delgado et al., 2008; Wang et al., 2008).

HIV-1 group M viruses are responsible for the worldwide epidemic, and over 50% of global infections are attributed to HIV-1 subtype C (Esparza and Bhamarapravati, 2000; Kantor and Katzenstein, 2004). Group O and N viruses with very low prevalence (Vergne et al., 2003; Roques et al., 2004) are mainly restricted to Cameroon, although group O viruses have been identified in several European countries and North America mainly from African immigrants (Sullivan et al., 2000; Barin et al., 2007). Apart from inter-subtype genetic differences, which could attain for example 35% in the envelope protein, strains of the same subtype can differ by as much as 20%.

Various HIV-1 subtypes have been isolated in all indigenous populations of the world with varying degrees of endemicity. For example, HIV-1 subtype B is mostly found in the Americas, Japan, Australia, the Caribbean and Europe; subtype A and D predominate in West and Central Africa, East Africa and subtype C in Southern Africa, India and China. CRF01_AE (a CRF) is predominant in the Central African Republic, Thailand and other countries of Southeast Asia. Subtype F is common in Brazil and Romania, while G and H are predominant in Russia and Central Africa; and CRF04_cpx (a CRF) is the main variant in Cyprus.

HIV-2 has a 50% homology with HIV-1 in the envelope gene sequence. In HIV-2 the accessory gene *vpx* replaces *vpu* present in HIV-1. Phylogenetic analysis delineates 7 groups of viruses (A-F) within HIV-2. It is estimated that each group arose as a result of an independent and separate sooty mangabey to human transmission (Santiago et al., 2005). Circulation of HIV-2 is restricted to West Africa, although cases have been reported in Europe (Barin et al., 2007; Costarelli et al., 2008). Although HIV-1 is responsible for the global epidemic (Gao et al., 1994; Hu et al., 1996; Hahn et al., 2000), sub-Saharan Africa represents one of the regions with the highest number of circulating HIV-1 subtypes, and intersubtype recombinants (Janssens et al., 1994; Nkengasong et al., 1994; Haesevelde et al., 1994; Simon et al., 1998; Corbet et al., 2000; Baldrich-Rubio et al., 2001), with subtypes C and A predominating (Nkenga-

song et al. 1994; Janssens et al., 1994; Kostrikis et al., 1995; Carr et al., 1996; Simon et al., 1998; Roques et al., 1999; Morris et al., 2000; Ayoub et al., 2001).

GENERATION OF HIV GENETIC VARIANTS

Genetic diversity is the hallmark of HIV biology. Several factors are known to contribute to the generation of new variants and influence the speed with which these variants evolve. Of note is the error prone nature of the viral reverse transcriptase (RT), which lacks proofreading functions, 3'-5' exonuclease activity, and introduces substitutions at a rate of approximately 3×10^{-5} nucleotide changes per site per replication cycle. A second factor is the high rate of virus production of up to 10^{10} virions per day, and the large number of replication cycles of approximately 300 per year that sustains HIV-1 infection *in vivo*. A third factor is *in vivo* selection pressure, due to host immune response and treatment interventions, which is responsible for the rapid emergence of immune escape mutants and drug resistant strains. Together, these mechanisms generate viral variants at extraordinary rates and represent a major force driving HIV-1 evolution in infected populations worldwide (Gao et al., 1996; Jetzt et al., 2000). Recombination may occur *in vivo* in tissues such as lymph nodes and spleen, whose viral burden is higher than those of other tissues. The high rate of mutation and the recombination that follows may generate quasi-species, a mixture of closely related viruses, in an infected individual or defined population. In a patient infected with multiple HIV-1 strains, recombination can produce more diverse viruses than can mutation (Zhu et al., 1995; Kuwata et al., 1997) and evidence suggests that recombination among highly divergent HIV-1 strains may occur quite frequently, indicating that this process also contributes importantly to HIV-1 diversification (Sabino et al., 1994; Diaz et al., 1995; Gao et al., 1996; Burke, 1997; Salminen et al., 1997; Takehisa et al., 1999).

METHODS FOR DETERMINATION OF GENETIC VARIANTS

Common methods used for the detection of HIV genetic variants include peptide enzyme linked immunosorbent assays; heteroduplex mobility and heteroduplex tracking assays; and sequencing and phylogenetic analysis.

Peptide enzyme linked immunosorbent assays

Peptide enzyme linked immunosorbent assay (PEIA) is used as a screening tool to obtain baseline information of the existing HIV-1 subtypes. The method makes use of a standard ELISA principle in which peptides are coated onto wells of a microtitre plate followed by incubation with

serum or plasma under investigation, with subsequent conjugation and substrate reactions. In this method viral peptides corresponding to the envelope, gag or polymerase genes could be generated and employed (Nkengasong et al., 1998; Engelbretch et al., 1999; Simparak et al., 2005). PEIA has been used to monitor the changing patterns of the circulating variants in specific locations (Nyombi et al., 2008). It is a fairly high throughput method and requires basic ELISA equipment. A limitation of PEIA is cross-reactivity among closely related viral strains, which may give an overestimate of known circulating subtypes. Nevertheless, cases of cross-reactivity can be specifically followed-up with other methods to determine the genetic make-up of the isolates. Lack of correlation between V-3 loop peptide enzyme immunoassay and sequencing has been reported (Nkengasong et al., 1998).

Heteroduplex mobility and the heteroduplex tracking assays

The heteroduplex mobility assay (HMA) is a technique devised by Delwart and Coworkers (1995) to classify unknown HIV-1 Group M isolates based on the envelope gene sequence, by comparing with known reference subtypes. Subtyping by HMA is based on evaluating the mobility of heteroduplexes formed between DNA fragments from the test sample and a subtype reference. Nested reverse transcriptase PCR is used to generate the required gene fragment from the uncharacterized HIV-1 strain. PCR products are then checked for the appropriate product length and size by agarose gel electrophoresis. Duplexes are then formed when test DNA strands anneal unto themselves (homoduplexes) or with DNA strands from the reference strains (heteroduplexes) following mixing and denaturing in a buffered environment. The mobilities of the duplexes formed (homoduplexes and heteroduplexes) are analyzed by electrophoresis on a 5% polyacrylamide gel.

Heteroduplexes formed between the unknown sample and the most closely related sequences are expected to exhibit the fastest mobilities upon electrophoresis. If the closest relatives are all from a single genetic subtype, the likely subtype of that strain is thus determined. Heteroduplexes formed when two non-identical but closely related single stranded DNA fragments anneal exhibit structural distortions at mismatch base pairs and at unpaired bases, where an insertion or a deletion in the nucleotide sequence has occurred. The structural distortions are the causes of the slower migration of the heteroduplexes compared to the homoduplexes during polyacrylamide gel electrophoresis. The extent of this retardation has been shown to be proportional to the degree of divergence between the two sequences. The presence of an unpaired base is known to influence the mobility of a heteroduplex more than a mismatched nucleotide. In order to properly assign a subtype to an

unknown isolate, it is advisable to include several references of the known subtypes circulating in the geographic region of the unknown isolate. For example, if subtype B and C are found in a particular region, to avoid unambiguous results more than one reference of subtype B and C should be included among the reference strains (Upchurch et al., 2000).

Some isolate may be difficult to subtype with HMA. This difficulty may be due to large deletions or insertions such as may occur in the V1-V2 or V4-V5 regions, which are prone to largely significant length variations (Delwart et al., 1995). A non-subtypable isolate may also indicate a genetic outlier within a known subtype, a new subtype or a recombinant virus derived from parental viruses of different subtypes (Delwart et al., 1993; Bobkov et al., 1994). The degree of variation required for good discrimination of heteroduplexes in a non-denaturing polyacrylamide gel is within a wide range of 3-20% based on mismatches. The degree of mismatches expected to be encountered should also guide the choice of fragment to use for HMA. This technique has been adapted to determine subtypes based on the *gag* and *gp41* gene sequences (Heyndrickx et al., 2000; Agwale et al., 2001).

HMA is not a definitive method in assigning subtypes when compared to sequencing and phylogenetic analysis. However, it is a very important alternative epidemiological tool for determining and monitoring the rapidly evolving HIV-1 in the course of its epidemic and infection in individuals, since it is rapid, sensitive, inexpensive and applicable in a relatively large scale (Loussert-Ajaka et al., 1998; Heyndrickx et al., 2000; Tatt et al., 2000; Upchurch et al., 2000; Sahni et al., 2008). A combination of *gag* and *env* HMA provides a good estimate of the prevalence of various genetic subtypes, as well as recombinants of HIV-1 on the *gag* and *env* regions (Bredell et al., 2000; Bessong et al., 2005). HMA reagents are freely available through the National Institutes of Health, AIDS Research and Reference Reagent Programme (www.aidsreagent.org).

An adaptation of HMA called heteroduplex tracking assay (HTA) is also used to identify viral subtypes and rapidly detect recombinant genomes (Schroeder et al., 2005). Probes that target several regions of the genome are employed and in this way intersubtype recombinants on the basis of heteroduplex mobility patterns can be identified. HTA is suitable for studies of HIV-1 diversity in areas where multiple subtypes circulate and the propensity for recombination is relatively high. HTA can resolve viruses that account for as low as 1-3% of the total viral population. However, the tool is limited since it can only be used for screening purposes. A variant of HTA incorporating biotinylated probes also allows for the detection of minority viruses within a quasi-species.

A very important application of the adaptation is that it can be used to detect drug resistant mutants which would be missed in population-based (bulk) sequence analysis which has a low sensitivity in identifying minority viruses (Schnell et al., 2008). Depending on the approach, the

use of radioactive isotopes and dedicated equipment is a limitation for poorly-resourced laboratories in terms of waste disposal and procurement of supplies.

Sequencing and phylogenetic analysis

Sequencing and phylogenetic analyses are to confirm subtype assignment either for selected regions of the genome or the full-length genome. In general, population based sequencing is performed, which identifies the majority variant among the quasi-species of an infected individual, against single genome sequencing which attempts to identify even minority populations in the quasi-species (Palmer et al., 2005). Rapid subtype assignment of partial sequences or full sequences of gene regions could be done by submitting the nucleotide sequences to an interactive database with reference sequences. Some of the subtyping programmes have features that enable the detection of recombinant sequences if the query sequence is of adequate length required for the recombination analysis. Examples of rapid subtyping programmes include REGA (<http://www.bioafrica.net/virus-genotype>), and the subtyping tools available on the HIV sequence database (<http://www.hiv.lanl.gov>).

In phylogenetic analysis, the sequences are completely aligned with reference sequences of known subtypes and recombinants using an appropriate programme. The mean genetic distances between and among the sequences under investigation and reference sequences are calculated. Sequences (tests and references) with similar genetic distances are clustered by the neighbour joining technique or other techniques and an evolutionary tree is generated. Usually, the phylogenetic tree is subjected to bootstrapping of at least 500 replicates in order to judge the reliability of the evolutionary patterns of the tree. A bootstrap value of more than 70% at a node is usually considered of high reliability. The generated tree is visualized with the TreeView programme (Figure 1). Examples of programmes used to perform phylogenetic analyses include BioEdit, ClustalX and MEGA. Despite the almost certainty of subtype attribution of samples under investigation at least for the investigated gene regions, drawbacks of sequencing include cost, and expertise in sequence editing and analysis.

Other genotyping tools

Other tools in fairly common use include restriction fragment length polymorphism (RFLP) and hybridization. In RFLP, advantage is taken of unique restriction sites on gene regions to classify different genotypes. Janini and colleagues (1996, 1998) have used this tool to detect single and dual infections. Using predefined PCR generated pol and gag DNA fragments, the electropho-

retic migration pattern of endonuclease digestion products are visualized by ethidium bromide staining or by radiolabeled probes are determined on a 10% polyacrylamide gel. A single restriction pattern indicated single infection, while two or more patterns indicated more than one type of infection.

A multi-region hybridization assay that is capable of detecting co-infection and recombinants in East Africa were subtypes A, C, D and their recombinants co-circulate has been developed by Hoelscher et al. (2002). In this method viral DNA is classified through a real-time PCR approach using fluorescent subtype-specific probes. By targeting different regions of the genome of each sample recombinants are detected. Samples reacting with more than one subtype-specific probe in a given gene region may be an indication of dual infection. Subsequent cloning and sequencing are employed to determine suspected dual infections. The method has a high throughput advantage and can be adapted to suit other regions with a different mix of co-circulating viruses, and recombinant forms.

Choice of genotyping tools

The decision as to which genotyping approach to be adopted will depend on the goal of the investigation, availability of resources and the required skilled personnel. Methods such as peptide enzyme immunoassays (PEIA), and HMA are less expensive and less technically demanding. Since one or two gene regions are employed, these tools provide a broad overview of the genetic landscape, but may not be suitable if a high accuracy of the variability of the genetic subtypes circulating in a particular region is needed for vaccine development and evaluation. Sequencing and phylogenetic analyses provide more detailed information at the molecular level which are essential for the determination of virus transmission patterns, genetic evolution over time and the selection of genes for the construction of candidate vaccines, and the formulation of reagents for immunological assays (Nkengasong et al., 1998; Smith et al., 2005; Holguin et al., 2008a). An added advantage of the availability of nucleotide sequences is the possibility to determine intra-subtype genetic variability through mean genetic distance calculations.

For countries or regions with a predominant circulating variant, subtyping tools such as HMA or partial genome sequencing followed by phylogenetic analysis could be adopted for screening purposes. In areas where there is co-circulation of different subtypes, recombinant forms and unique recombinant forms the multi-region hybridization assay should be adopted for screening purposes. However, full-length genome sequencing is the gold standard to definitely describe the genetic profile of viruses and to provide necessary data for the selection of genes for vaccine development and evaluation.

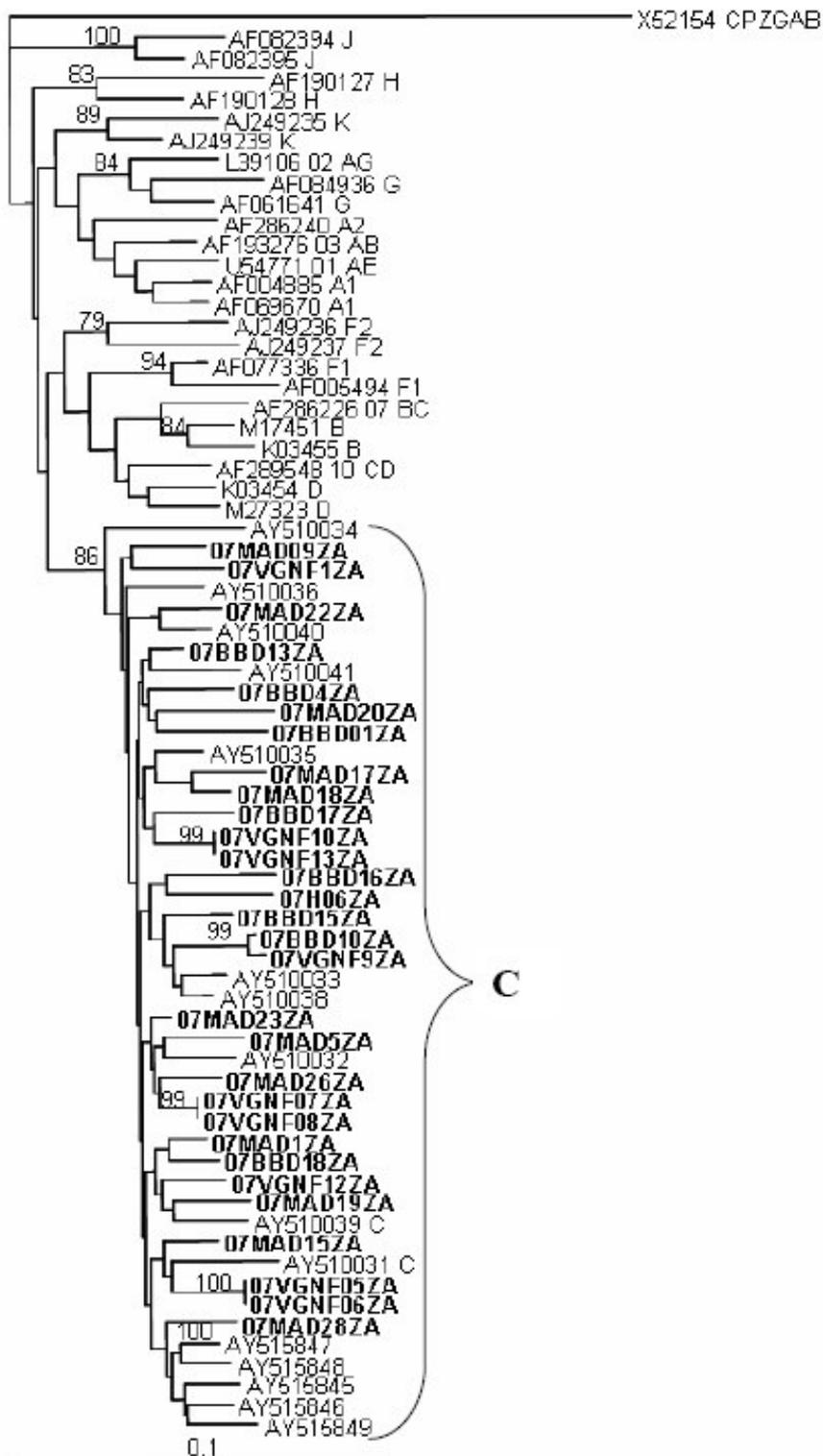


Figure 1. A representative phylogenetic analysis of HIV protease sequences to determine their subtype. In the neighbour joining tree the test sequences (shown in bold) are interspersed and clustered with reference HIV-1 subtype C protease sequences (sequences with accession numbers beginning with AY). The clustering shows that the test sequences are HIV-1 subtype C at least on the protease gene. A bootstrap value of 86% defines the reliability of the HIV-1 subtype C cluster. The tree is rooted with an outlier SIV protease sequence (X52154_CPZGAB), and the tree indicates a lineage of the test sequences to the SIV isolate.

THE NEED FOR MONITORING THE HIV GENETIC LANDSCAPE

Genetic analyses of HIV is important not only for vaccine development purposes, but also to guide treatment strategies, track the emergence of new genetic variants and to ensure that diagnostic assays are continuously able to detect circulating and emerging variants.

Implications of diversity on pathogenesis

The correlation of viral genotypes with disease progression is well known for several viral infections. This also applies to HIV. The viral set point (RNA copies/ml) for HIV-2 after seroconversion is lower than that of HIV-1 and this has been proposed as one of the reasons why individuals infected with HIV-2 progress to AIDS more slowly than individuals infected with HIV-1 (Andersson et al., 2000; MacNeil et al., 2007; Leligidowicz and Rowland-Jones, 2008). Replication is also slower for HIV-2 than HIV-1 with significantly lower viral DNA load in the former than the later (Gueudin et al., 2008). Evidence also points to the different rates of disease progression even within the HIV-1 group M variants. HIV-1 subtype A is indicatively less virulent than subtype D (Kaleebu et al., 2001). Kiwanuka et al. (2008) have also shown that generally disease progression is influenced by subtype. HIV-1 subtype C currently accounts for more than 50% of total global infections with increasing subtype C infections being reported in South America and India (Siddapa et al., 2004; Sanchez-Merino et al., 2003; Carrion et al., 2004). The reasons for the apparent high transmissibility are not understood but it is known that C viruses have genetic differences from other HIV-1 subtypes such as the possession of an extra NF κ B binding site in the long terminal repeat which may enhance gene expression. Other properties include enhanced protease stability and catalytic activity, a prematurely truncated rev protein and a relatively high diversity in the protease cleavage sites in the gag, gag-pol and nef proteins of HIV-1 subtype C viruses. This attributes impact on regulation of viral cycle and disease progression (Gordon et al., 2003). The increasing evidence that genetic variants of HIV influences the rate of disease progression may impact on the expectations by health care providers on when treatment may likely be needed taking into consideration the impact of the infecting subtype on disease progression markers such as viral load and CD4 counts or the presentation of opportunistic infections.

Implications of diversity on diagnostics

The importance and relevance of HIV genetic diversity is underscored in the initial failure to diagnose infections due to HIV-1 group O viruses in patients who showed clinical signs and symptoms of AIDS. The initial serolo-

gical tests did not contain specific antigens of HIV-1 group O and consequently antibody-based laboratory diagnosis was not possible (Reviewed in Quinones-Mateu et al., 2000). Despite progress in optimizing antibody detection assays for type O infections, difficulties in diagnosing type O viruses continue to be encountered (Zouhair et al., 2006; Henguell et al., 2008). Delays in diagnosis definitely complicate patient management. Also of note is the identification of HIV-1 subtype B variants which could not be detected by a fourth generation immunoassay (Gaudy et al., 2004). Since, the identification of infected persons is directly linked to prevention efforts through counselling in order to stem transmission, it is imperative that all infections are detected. The implication is that immunoassays need to be updated as soon as possible to capture aberrant strains causing new infections. Molecular-based laboratory diagnostic tools have also been reported to perform poorly with HIV-1 type O infection (Henguell et al., 2008). In addition, diagnostic tests for viral RNA measurements are needed for patient monitoring under treatment or to determine when to initiate treatment. There is evidence that not all HIV genetic variants, even among the major group of HIV-1, are reliably quantified by current plasma RNA determination technologies (Gueudin et al., 2007; Holguin et al., 2008b).

Implications of diversity on treatment

Anti-HIV drugs are modelled mostly against HIV-1 subtype B. Although it is expected that other HIV variants should be sensitive to these therapies, it well established that this may not always be the case as not all known HIV genetic variants have similar sensitivities to available treatment regimens. HIV-1 group O and HIV-2 viruses are naturally resistant to the non-nucleoside reverse transcriptase inhibitors with poor treatment outcomes (Drylewicz et al., 2008). In like manner, the efficacy of protease inhibitors to HIV-2 is poor (Reynolds et al., 2007; Menéndez-Arias and Tözsér, 2008). The protease gene of non HIV-1 subtype B viruses is highly variable, with indications that this may influence the efficacy of protease inhibitors. The potential impact of polymorphisms on protease inhibitors of non-B subtypes especially subtype C which is driving the epidemic in Southern Africa and responsible for more than 50% of infections worldwide, has been commented on (Bessong, 2008). In the same vein, the generation of escape mutants due to drug pressure ultimately lead to drug failure, and complicate the management of AIDS both at the individual level, and at the population level if the drug resistant variants are transmitted to drug inexperienced persons. There is evidence that HIV-1 subtype C viruses may generate resistant mutants along different genetic pathways compared to HIV-1 subtype B viruses (Grossman et al., 2001; 2004).

Implications of diversity on prevention

The golden tool to halt the spread of HIV quickly and cheaper is a vaccine to prevent new infections or to moderate disease progression for those already infected. One of the major obstacles in the development of an effective vaccine is the enormous variability among and between independent HIV isolates, and the propensity for continuous and unpredictable diversification trends. Observations of super-infection also indicate the necessity of a vaccine with broad cross neutralizing antibody or cellular immunity elicitation properties (Piantodosi et al., 2007; Bezemer et al., 2008; Holguin et al., 2008a). In as much as epitopes need to be identified for vaccine development, it is imperative that analysis of circulating variants be performed to characterize across-the-board epitopes for humoral or cell-mediated responses which could be used in vaccine development. And this is more important where the aim is geared towards a polyvalent construct. This means, a vaccine of global significance should be able to prevent new infections of all HIV types, groups, subtypes and viruses with mosaic genomes. This is a daunting task taking into consideration the need to generate sterilizing immunity for all variants. In addition, data on the viral genetic landscape are useful in the choice of populations and sites for vaccine efficacy trials.

From the epidemiological perspective, the detection of infections with new variants hitherto unknown in a particular geographical locale may indicate the need to improve or modify existing prevention strategies. As mentioned under the section on diagnostics, it is imperative that as individuals are encouraged to opt for testing which are usually performed with simple/rapid tests, the tests should be able to detect all infections no matter the infecting genetic variant.

CONCLUSION

The development of an effective vaccine against a particular virus needs to take into account the circulating genetic variants in order to ensure protection for all infected individuals with any variant of that virus. This is more pertinent in the case of HIV which has a high propensity to mutate and generate complex mosaic genomes. The evolution of new variants capable of escaping immune surveillance is an indication that diagnostic tests particularly those based on antibody detection can be compromised. The introduction and spread of variants such as HIV-1 group O and HIV-2 into regions where different HIV-1 subtypes predominate will compromise the treatment regimen if the newly introduced variant is not detected early. There is continuous diversification and redistribution of HIV variants worldwide. Therefore, it is important that each country or region devises a strategy to monitor the genetic landscape of HIV in order to detect the introduction of new variants as well as shifts in the genetic diversity of circulating viruses

with the view of optimizing diagnostic and treatment management algorithms, as well as tailors the choice of genes and reagents for vaccine development and evaluation.

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