High Level of HIV-2 False Positivity in KwaZulu-Natal Province: A Region of South Africa With a Very High HIV-1 Subtype C Prevalence

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Human immunodeficiency virus 2 (HIV-2) is found predominantly in West Africa. It is not unlikely, however, that HIV-2 may also be found in South Africa, due to the influx of immigrants into this country. It is important to distinguish between HIV-1 and HIV-2 since the clinical courses and treatment responses of these viruses are different. Routine serological methods for diagnosing HIV do not differentiate between HIV-1 and -2 infections, while rapid tests, viral load quantification and PCR are HIV-type—specific. The objective of this study was to describe the seroprevalence and molecular epidemiology of HIV-2 in KwaZulu-Natal, one of the regions with the highest HIV prevalence in the world and home of the two largest harbors in South Africa. HIV-1 positive samples were screened for antibodies against HIV-2, using a rapid test. The confirmation of HIV-2 positive samples was done by PCR. Of the 2,123 samples screened, 319 (15%) were identified as positive by the rapid test. None of these samples were confirmed positive by PCR. To explore this discrepancy in the results, a subset (n = 52) of the rapid HIV-2 positive samples was subjected to Western blotting. Thirty-seven (71%) of these were positive, yielding an overall HIV-2 seroprevalence of 10.6%. Three out of 28 (10.7%) Western blot positive samples were positive by a Pepti-LAV assay. This discrepancy between serological and molecular confirmation may be attributed to non-specific or cross-reacting antibodies. The use of rapid tests and Western blots for HIV-2 diagnosis in South Africa should be interpreted with caution. ***J. Med. Virol.***

KEY WORDS: HIV-2; prevalence; South Africa; diagnosis; serology

INTRODUCTION

While the human immunodeficiency virus (HIV) type 1 is predominant worldwide, HIV-2 is concentrated mainly in West Africa [Zeh et al., 2005]. The prominent cause of the global dissemination of HIV-2 is the population migration of infected individuals [Quinn, 1994]. It is quite possible that HIV-2 is present in South Africa in view of the high levels of immigration to the country [Anonymous, 2011] and the fact that approximately 3–4% of the total national population is foreign [Polzer, 2010]. Furthermore, KwaZulu-Natal hosts the two largest harbors in the country (Durban and Richards Bay), which are the point of entry for an increasing number of foreign individuals [Young, 2012].

HIV-2 cases have been reported in Europe, India, and the United States, as well as areas with historical and socio-economic ties to West Africa. For instance, in Portugal, HIV-2 is responsible for 4.5% of the AIDS cases, and in France, 1.8% of new infections are due to HIV-2 [Campbell-Yesufu and Gandhi, 2011]. However, there is no information on the prevalence of HIV-2 in South Africa which has the highest prevalence of HIV-1 in the world, with KwaZulu-Natal being the province which is affected the worst [UNAIDS, 2010]. The epidemic in South Africa is dominated by subtype C (i.e., more than

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95% of HIV-1 samples in KwaZulu-Natal are from subtype C viruses [Gordon et al., 2003]).

Understanding the epidemiology of HIV-2 is important because there are significant differences between HIV-1 and HIV-2. The differences are reflected at the genomic level [Bock and Markovitz, 2001], in the progression of the disease and in the response to treatment. HIV-2 infection can lead to immunosuppression and clinical AIDS, although disease progression is slower [Torian et al., 2010].

South Africa has the largest antiretroviral (ARV) therapy program in the world, with approximately 2 million patients on treatment, nearly a quarter of whom are in KwaZulu-Natal [WHO/UNAIDS/UNICEF, 2010]. HIV-1 and -2 require different treatment regimens; HIV-2 is naturally resistant to most of the non-nucleoside reverse transcriptase inhibitor (NNRTI) drugs that are presently available [Rodes et al., 2005]. Witvrouw et al. [1999] showed that in order to inhibit HIV-2 in vitro, the concentration of certain NNRTSs needs to be 50 times higher than the concentration required to inhibit HIV-1 [Witvrouw et al., 1999]. In addition, certain protease inhibitors (PIs) are intrinsically or partially resistant, or work less effectively against HIV-2 compared to HIV-1 [Kar and Knecht, 2012; Charpentier et al., 2013]. This is relevant since patients infected with HIV-2 are at risk of failing treatment and of the clinical disease progressing [Costarelli et al., 2008].

If HIV-2 is found to be prevalent, it is imperative that accurate and differential diagnostic algorithms for HIV-1 and -2 be established. This will have important epidemiological, diagnostic, clinical, and treatment implications. The objective of this study was to use available methods to differentiate between HIV-1 and HIV-2 infections, and to determine the prevalence of HIV-2 in KwaZulu-Natal.

METHODS

Patient Specimens

The estimate of the sample size (n = 2123, at a 95% confidence interval) was calculated using EpiInfo 2008 and the following statistics: the estimated population size of KwaZulu-Natal is approximately 9.4 million people [Wilson and Blower, 2005]; an estimated 39.1% of this population is HIV-infected [Noble, 2009]; an expected HIV-2 frequency of 0.2% [Zanchetta et al., 1990] together with a worst expected frequency of 0.01%.

Clinical specimens from the KwaZulu-Natal Comprehensive Care Management and Treatment Program are submitted routinely to the Department of Virology, Inkosi Albert Luthuli Central Hospital, Durban, for HIV serological and viral load testing. The 200 serum specimens that were HIV-1/2 antibody ELISA positive and 1923 HIV-1 viral load plasma samples were screened consecutively, based on the sample size calculation. The algorithm for testing is shown in Figure 1.

HIV-1 and -2 Screening

The samples were tested for antibodies to HIV-2 using the SD Bioline HIV1/2 3.0 rapid test (Standard Diagnostics, Kyonggi-do, South Korea), which is a rapid immunochromatographic test for the differential and qualitative detection of antibodies (IgG, IgM, IgA) specific to HIV-1 and HIV-2. This third-generation assay is based on the use of recombinant gp36 as the capture antigen for HIV-2.

HIV-2 Serology

The Western blot technique (New LAV Blot II, Bio-Rad, Marnes La Coquette, France) was used to confirm the rapid test findings. The Western blot was used on a subset (subset 1, n = 52) of HIV-2 rapid-positive samples (Fig. 1) for practical reasons. This assay was specifically designed to identify HIV-2 antibodies in serum or plasma samples by immunoblotting, in order to confirm the presence of positive anti-HIV-2 antibody responses and to determine its antigenic specificity.

The Western blot results were interpreted according to the manufacturer's criteria, which require the presence of at least one *env*, *gag*, and *pol* band for a positive result. A negative Western blot result is one without the presence of any bands. Any band or bands that do not qualify as positive, according to the manufacturer's criteria, are deemed as indeterminate.

In addition, a second random subset of samples (subset 2, n = 28) that showed HIV-2 positivity by the Western blot, was further tested using a synthetic peptide-based immunoassay (Pepti-LAV1-2; Bio-Rad, Sanofi Diagnostics Pasteur).

HIV-2 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used on all samples, which were HIV-2 antibody positive by the SD Bioline Rapid test, to confirm HIV-2 infection. The samples were extracted using the easyMAG extraction system (bioMérieux, Marcy L'Etoile, France). Real-time PCR amplification was then performed using an HIV-2 Real-Time RT-PCR kit (Shanghai ZJ Bio-tech, Shanghai, China), according to the manufacturer's instructions. The TaqMan primers and probes provided in this kit were designed to were designed to cover the genetic diversity of HIV-2 isolates (including subtypes A–G), spanning approximately 90 base pairs of the HIV-2 5′-LTR region. The following PCR parameters were followed for confirmation on the 319 HIV-2 antibody positive samples: reverse transcription at 45°C for 10 min, followed by 95°C for 15 min. The amplification cycle consisted of 40 of 95°C for 5 sec followed by 60°C for 30 sec.

Two additional in-house PCR protocols targeting different regions of the HIV-2 genome were used on the HIV-2 antibody positive samples and HIV-2 plasmid DNA positive control material from the

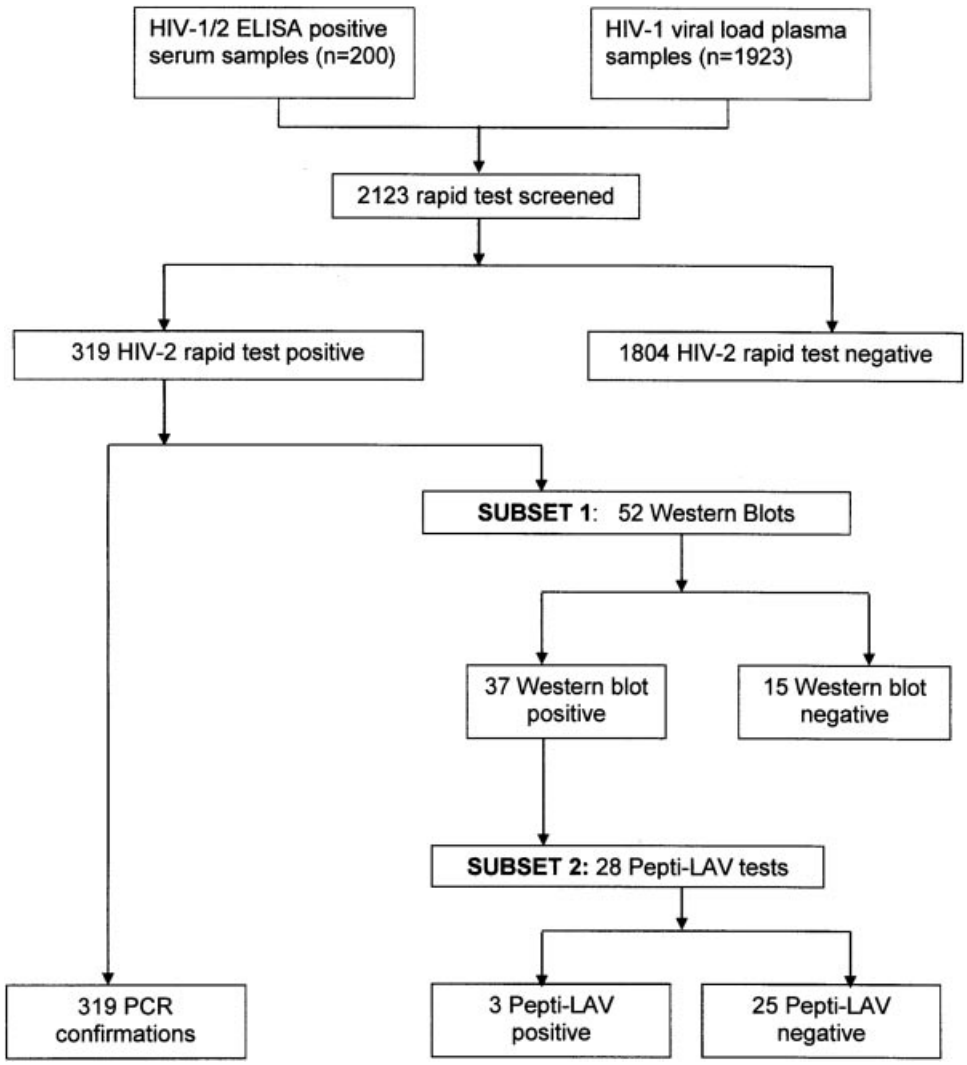

Fig. 1. Flowchart of the testing algorithm used to determine the prevalence of HIV-2 infection.

commercial kit (n = 12), to verify the sensitivity of the HIV-2 real-time PCR kit. The primer sequences of these nested PCR assays are detailed in Table I. Reverse transcription was performed using the Superscript II reverse transcriptase protocol (Invitrogen, Life Technologies, Carlsbad, CA). The first in-house protocol is from the Hospital Egas Moniz Reference Laboratory in Portugal, the European country with the largest number of HIV-2 infections [Valadas et al., 2009]. The PCR parameters consisted of denaturation at 94°C for 2 min, 30 amplification cycles of 95°C for 30 sec, 58°C for 20 sec, and 72°C for 2 min, followed by 72°C for 10 min. For the nested PCR, 2μl of first-round product was used with the same PCR parameters. The second in-house protocol was performed according to Ciccaglione et al. [2010] using the following PCR conditions: 93°C for 10 min, followed by 10 amplification cycles of denaturation at 93° for 10 sec, annealing at 55°C for 30 sec, and extension at 68°C for 4 min. The subsequent 25 cycles were performed under the same conditions, with an incremental increase in extension time (2 sec/cycle), and a last extension step of 72°C for 7 min. Five microliters of first-round PCR product was used in a nested PCR according to the following PCR profile: 95°C for 1 min, followed by 35 amplification cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec. A last extension step was performed at 72°C for 7 min. PCR products were visualized on a 1.5% agarose gel. In addition, in order to determine the suitability of serum samples for PCR, an HIV-1 PCR was performed on 20 randomly selected serum samples [Manasa et al., 2012].

Ethical Considerations

This study was conducted in accordance with the ethical principles for anonymous, unlinked surveillance published by the South African Department of

TABLE I. Alternative Nested PCR Amplification Assays Used to Confirm HIV-2 Infection in a Subset of Rapid-Positive Samples

HIV-2 Target region		Primer (Tm)	Nucleotide sequence	Reference
env, gp36	1st round	PDF1 (52°C	5′-GGTCTATTWCAGAGAAGGCAG-3′	Ciccaglione et al. [2010]
		LTR9574 (53°C)	5′-TGGTGAGAGTCTAGCAGGG-3′	
	2nd round	ENVA (68°C)	5′-GCTAGGGTTCTTGGGTTTTCTCGCGACAGCAGG-3′	
		ENVB (66°C)	5′-CAAGAGGCGTATCAGCTGGCGGATCAGGAA-3′	
pol[a]	1st round	JA218 (56°C)	5′-GAAAGAAGCCCCGCAACTTCC-3′	Brandin et al. [2003]
		JA221 (58°C)	5′-GCTCTGCTTCTGCTAATTCTGTCCA-3′	
	2nd round	JA219 (59°C)	5′-AGGGGCT(A/G)ACACCAACAGCAC-3′	
		JA220mod (53°C)	5′-GTCTTTATICCTGGGTAGAITTGTG-3′	

[a]Protocol from Hospital Egas Moniz reference laboratory in Portugal.

Health [Rees et al., 2002]. Approval was obtained from the University of KwaZulu-Natal Biomedical research ethics committee (*BREC 264/09*) and the KwaZulu-Natal Provincial Department of Health.

RESULTS

Of the 2123 HIV-1 and -2 positive samples tested, 319 (15.0%) samples were HIV-2 antibody positive by the rapid test. The first confirmatory serological assay used was the Western blot. A subset of 52 Western blots was performed on randomly selected, rapid-positive samples, 37 (71%) of which were HIV-2 antibody-positive. Selected HIV-2 Western blot results are shown in Figure 2, in which a clear reactivity to both gp36 and gp125/gp105 *env* antigens of HIV-2 was observed for sample 51. Given that 15% (319/2123) of the samples were positive by the rapid test and 71% of these were confirmed by the Western blot, the HIV-2 seropositivity as identified by rapid testing and Western Blot was 10.6%.

The second serological test, the Pepti-LAV1-2 assay, was conducted on 28 samples which were positive by the rapid test and Western blot. Three of the 28 (10.7%) were HIV-2 positive. The HIV-2 seroprevalence in this study would be 1.6%, if this result were used to calculate the overall prevalence of HIV-2 in KwaZulu-Natal. These 3 Pepti-LAV samples were negative by the commercial PCR assay, and negative or insufficient for the two nested PCR assays.

All of 319 samples tested positive by the rapid test were tested by PCR using a commercial method. None of the 319 samples were positive by PCR. The comparison of the different PCR methods using the HIV-2 antibody positive samples and the positive control material (HIV-2 plasmid DNA) all correlated with each other, resulting in no PCR positive samples. The 20 randomly selected serum samples were all amplified and detected by the HIV-1 PCR. The detailed calculations of the overall prevalence per serological assay, and the prevalence using results of the HIV-2 PCR and serological assays are outlined in Tables II and III.

DISCUSSION

The findings of this study demonstrate an unexpectedly high HIV-2 seroprevalence with the use of the rapid antibody test. PCR assays to detect the HIV-2 genome were conducted to ascertain the true infection status of the seropositive samples. None of the HIV-2 seropositive samples were amplified by PCR, even though the commercial PCR is able to detect all HIV-2 subtypes. All the internal positive controls from the commercial kit were confirmed as positive by the commercial and in-house PCR methods, which suggest that the non-amplification of the positive rapid test samples was not due to PCR factors.

The ideal sample for molecular confirmation of HIV-2 proviral DNA by PCR is a whole blood sample of peripheral blood mononuclear cells (PBMCs) to detect HIV-2 proviral DNA. Plasma and serum samples, routinely collected as part of the national HIV program in South Africa, were used in this study for RT-PCR. However, the use of serum and plasma samples has also yielded accurate results. For example, Damond et al. [2002], showed HIV-2 PCR sensitivities of 100% at viral loads of 250 copies/ml and 66% of patients with viral loads of 125 copies/ml, in plasma samples [Damond et al., 2002]. Gilleece et al. [2010] reports a median HIV-2 viral load in the plasma compartment as documented to be 3 log 10 RNA copies/ml [Gilleece et al., 2010]. In addition, the HIV-1 PCR conducted on the 20 randomly selected serum samples were all successfully amplified thereby excluding poor serum suitability for PCR. However, due to the limitations of the available sample types (serum and plasma) used in this study, the possibility of HIV-2 infection cannot be completely excluded. The HIV-2 Real-Time RT-PCR Kit (Shanghai ZJ Bio-tech Co.) used in this study can be used for quantitative and qualitative purposes. The PCR assay used for

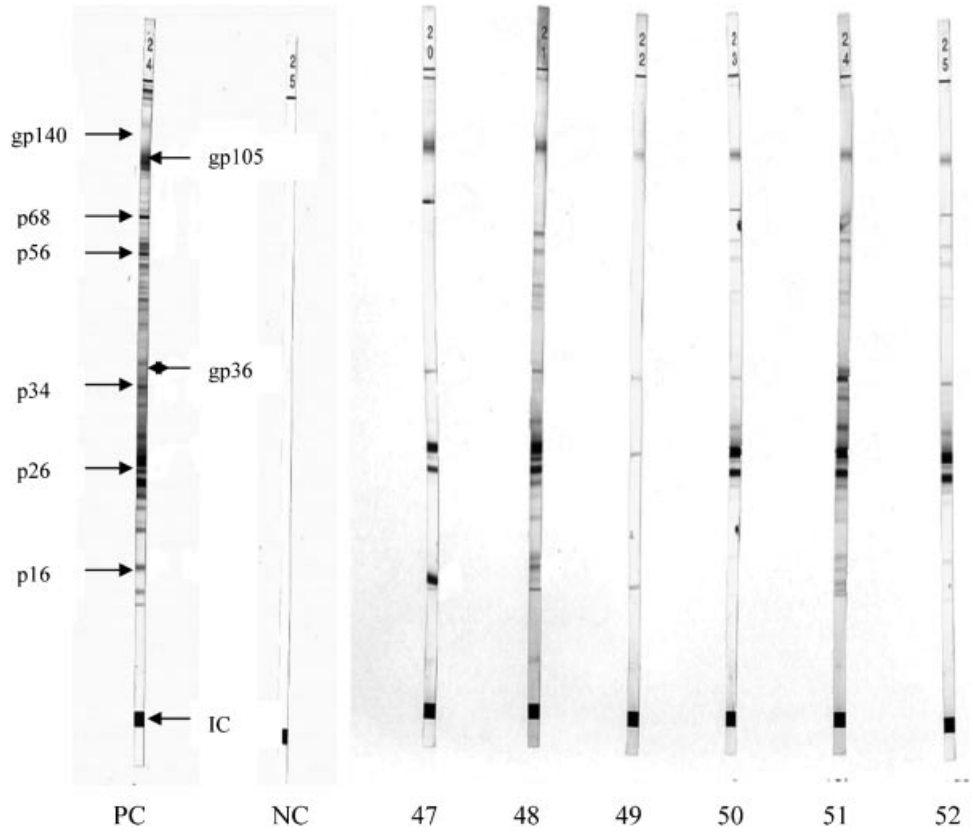

Fig. 2. Western blot profiles of a subset of rapid-positive samples showing reactivity to the major HIV-2 proteins (IC, internal control; PC, positive control; NC, negative control).

molecular confirmation was applied as a qualitative assay for the purposes of this study.

Western blots were done on selected samples to explore the discrepancy between rapid test results and PCR. It is likely that more than half of the patients who show seropositivity for HIV-2 are, in reality, infected by HIV-1 alone [Peeters et al., 1992]. The results from this study are a reflection of Qui et al. [2008], who also demonstrated the decreased specificity of Western blots [Qiu et al., 2009]. There are problems interpreting HIV-2 Western blot [WHO, 1990], even when the CDC and WHO criteria for HIV-1 are used [Mortimer, 1991; Nasrullah et al., 2011].

Dual seropositivity to HIV-1 and HIV-2 may be due to (i) infection with both virus types, (ii) dual exposure to both virus types with mono-infection, or (iii) mono-infection with cross-reacting antibodies directed at common epitopes [George et al., 1992]. Alternatively, dual seropositivity may be due to infection with a third unique virus with epitopes common to both viruses [Peeters et al., 1992]. The Pepti-LAV was used as an additional assay to further investigate the discordant serological and PCR results. This assay showed greater specificity (1.6% vs. 10.7%) when compared to the Western blot results. A more specific immunoassay will eliminate false-positive reactivity, and be able to distinguish

TABLE II. Detailed Calculations of the Overall Prevalence Per Serological Assay

	New LAV Blot II	Pepti-LAV
Total number of assay done	52 (16% of all rapid positives)	28 (8.7% of all rapid positives)
Percentage of rapids confirmed to be HIV-2 positive	37/52 (71%)	3/28 (10.7%)
HIV-2 seroprevalence	71% of 319 = 226 226/2,123 = 10.6%	10.7% of 319 = 34 34/2,123 = 1.6%

TABLE III. Results of the Various Serological and Genomic HIV-2 Assays Used to Establish the Prevalence of HIV-2

	n	HIV-1/2 dual positive (prevalence % by assay)
HIV-1/2 positive samples screened	2,123	319 (15.0%)
HIV-2 real-time PCR	319	Nil (0)
NewLAV Western blots	52	37 (71.2%)
Pepti-LAV1-2[a]	28	3 (10.7%)

[a]Additional immunoassay used to confirm HIV-2 seropositivity, not used in the seroprevalence calculation.

between HIV-1 and -2 infections with more certainty. Maximal sensitivity and specificity in enzyme immunoassays can be achieved by using short synthetic peptides [Alcaro et al., 2003], as evidenced by the results of the Pepti-LAV assays.

HIV-2 is associated with a lower plasma viral load compared to HIV-1 despite similar proviral DNA viral loads in both infections [Reeves and Doms, 2002] suggesting that HIV-2 replication is restricted in vivo [MacNeil et al., 2007]. This may account for the discordance between serological and PCR results.

The results of this study demonstrate that HIV-2 is most probably not prevalent in KwaZulu-Natal. The clinical observation of discordant CD-4 cell counts and HIV-1 viral load results of patients on treatment are, therefore, most likely not a result of infection with HIV-2. Furthermore, these results show that rapid tests and serology alone may grossly overestimate the true prevalence of HIV-2. Consequently, it is important to employ more specific, yet sensitive methods, like PCR for example, for genetic definition of the infecting HIV type. In addition, PBMCs and whole blood should be collected from patients that test positive for HIV-2 using rapid tests. Alternatively, synthetic peptide-based enzyme immunoassays may provide a more accurate and differential diagnosis. HIV-2 Western blots should be interpreted with caution, and the diagnostic utility of this assay is an avenue for further research. Future surveillance is needed to detect whether HIV-2 is present or not in this region.

POSTSCRIPT

The choice of rapid test was SD Bioline HIV-1/2 3.0; used routinely for screening at most HIV clinics in South Africa. These tests have recently been recalled by the World Health Organization (WHO), due to the large number of invalid results obtained by certain batches of these tests [WHO, 2012]. The rapid tests used for this study, however, were not part of the problematic test lots. No similar problems relating to the quality of these tests were experienced during the course of this study.

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