East African Medical Journal Vol. 85 No. 11 November 2008
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LABORATORY DIAGNOSIS OF DUAL HIV-1/HIV-2 INFECTION IN GHANAIAN PATIENTS

E.Y. BONNEY, S. T. SACKEY and J. A. M. BRANDFUL

ABSTRACT

Objective: **To determine the true prevalence of HIV dual infections in a previously characterised HIV seropositive patient group due to inconsistencies between different diagnostic methods.**
Design: **A cross-sectional study of an HIV seropositive group with different diagnostic methods.**
Setting: **Three hospitals in the Northern, Ashanti and Greater Accra Regions of Ghana.**
Subjects: **One hundred and forty five HIV infected patients/individuals sampled from June to September 2002.**
Main outcome measures: **Using serological and molecular methods, the seropositive status of HIV-infected patients, previously determined by a preliminary screening process, was confirmed and discrepancies noted. The data was used to propose a more accurate laboratory diagnosis of HIV dual infections involving HIV-1 and HIV-2.**
Results: **HIV-1 infections were mostly accurately detected, but difficulties were encountered in diagnosing HIV-2 infections. To achieve a positive detection on confirmatory immunoblots, antibody concentration in some samples tested was enhanced by using larger volumes. In other cases, diagnosis of HIV infections by PCR, especially HIV-2, was possible only after increasing the DNA template or $MgCl_2$ concentrations. Such samples would otherwise have been inaccurately scored for HIV infections.**
Conclusion: **Based on the results of this study, we propose that the accurate diagnosis of HIV dual infections, especially HIV-2 component, must use an algorithm that involves PCR. Our results however underscore conclusions of a previous study that most dually seroreactive samples are predominantly HIV-1 infections with cross-reactivity to HIV-2 antigens.**

INTRODUCTION

The AIDS epidemic in Ghana was initially due to HIV Type 2 (HIV-2) (1). HIV Type 1 (HIV-1), was introduced into Ghana in the mid 90s predominates and now accounts for over 90% of all HIV / AIDS cases where HIV-2 was originally predominant (2, 3). About 2% of the infections are currently due to HIV-2 alone while 2-3% is categorised as dual infections, based on serological data.

HIV-1 and HIV-2 infections differ markedly in their prognosis and necessarily require different therapeutic approaches. For example HIV-1 subtype 0 and HIV-2 strains are naturally resistant to non-nucleoside reverse transcriptase inhibitor (NNRTI) group of antiretroviral drugs (ARVs) (4). Also, some HIV-1 group M viruses including subtype G strains and CRF02_AG recombinants are differentially susceptible to protease inhibitors (PIs) (5, 6). Estimating the true prevalence of HIV-1 and HIV-2 infections, as well as co-infections with both types is therefore necessary to successfully control the epidemic. HIV-2 is much less studied relative to HIV-1 and further work involving HIV-2 will be enhanced by the availability of molecular data on circulating HIV-2 species, as well as critical information on phenotypic properties of the virus. These will facilitate intervention efforts like development of a safe, effective and affordable vaccine to manage the pandemic. Achieving these objectives depends significantly on the accurate laboratory diagnosis of H IV-2 and, by extension, dual H IV-1/2 infections.

In Ghana, previous studies demonstrated a misdiagnosis of HIV dual infections (7, 8). This study

shows that the laboratory diagnosis of HIV-2 infection remains problematic. It is therefore necessary to optimise conditions for its detection in areas where dual infections are prevalent. We determined the proportion of dual infections among Ghanaian HIV seroreactive patients and indicated the possible sources of error that must receive attention in the accurate diagnosis of dual HIV infections.

MATERIALS AND METHODS

Patients and sample collection: Venous blood was aseptically collected from 145 HIV-infected individuals reporting to three hospitals in the Northern, Ashanti and Greater Accra Regions of Ghana from June to September 2002. These comprised asymptomatic HIV infected individuals and AIDS patients. Patient selection and sampling were done only after obtaining informed consent from the subjects. Eight mls of blood were then collected into vacutainer tubes with EDTA anti- coagulant. The samples were transported in cold boxes from the hospitals to Noguchi Memorial Institute for Medical Research (NMIMR) within 24hrs of collection. At NMIMR, the plasma was separated and stored at -20ºC. Peripheral blood mononuclear cells (PBMCs) were isolated from the rest of the blood as described (9) and stored at -70ºC until used.

Serological assays: The serological status of the patients for HIV infection was re-checked by two rapid assays, namely Determine (Dainabot Co. Ltd, Japan) and HIVSPOT (Genelabs Diagnostics, Singapore). A Serodia particle agglutination (PA) kit (Fujirebio Inc., Japan,) was used for the initial HIV typing. Dually reactive samples on PA were further analysed to confirm their dual infection status. Peptilav HIV-1/2 assay (Diagnostic Pasteur, France), and Innolia HIV-1/2 assay (Innogenetics, Belgium) were used for the diagnosis of either HIV-1 or HIV-2. Peptilav employed synthetic peptides to the mono-epitope of the transmembrane glycoproteins of HIV-1 and HIV-2 (gp 41 and gp 36 respectively) for differentiation. Dual positive samples were simultaneously reactive to both antigens (10). Innolia had several HIV antigens spanning the viral envelope and the core. The criteria for positivity by Innolia were the presence of two envelope glycoprotein bands or one envelope glycoprotein and a core protein band according to the manufacturer's directives.

Polymerase chain reaction (PCR): Genomic DNA of HIV provirus was extracted from PBMCs of PA dually reactive samples in lysis buffer composed of 10 mM Tris-HCL, pH 9.0, 50 mM KCI, 1.5 mM $MgCl_2$ and 0.5% Tween 20. Lysis was performed by incubating the specimens for three hours in a water bath at 55ºC. Iminodiacetic acid (50% Chelex-100) was added to a final concentration of 5%. The mixture was heated at 95°C for eight minutes and mixed for eight seconds. DNA associated proteins were digested with 0.6 mg/ml of proteinase-K (Sigma, USA). Samples were then centrifuged, the DNA-containing supernatant carefully taken into fresh tubes and stored at -30°C until used. Each amplification was preceded by PCR for a cellular gene, ß2- microglobulin, to assess the quality of DNA extracted.

In the quality assessment reaction, 2.5 μL of DNA template was amplified in a single round assay with 1.25 U of AmpliTaq Gold® (Roche, Molecular Systems Inc. NJ, USA) and 10x PCR buffer with 25 mM $MgCI_2$, 2 mM of each deoxy-nucleotidetriphosphates (dNTPs) and 10 μM each of forward and reverse primers in a final volume of 25 μL. Cycle conditions were 10 mins at 95ºC followed by 35 cycles of 30 secs at 94ºC, 30 secs at 45ºC, and 1 min at 72ºC in a Gene Amp PCR Systems 2400 thermal cycler (Perkin-Elmer, Norwalk, CT). A final extension step at 72ºC was performed for 7 mins. A full-length PBMC-derived clone, positive for HIV-1 or HIV-2, and a plasmid containing a known HIV unreactive sample respectively served as positive and negative controls. To guarantee primer specificity, all primer pairs (Table 1) were pre-tested on the positive HIV-1 and HIV-2 as well as negative controls in a one-round PCR assay using cycle conditions stated earlier. Adequate measures were taken to avoid cross-contamination by preparing PCR mixtures in separate dedicated safety hoods while pre- and post-PCR manipulations were performed in different rooms. Furthermore, aerosol-resistant pipette tips were used throughout the procedure.

A nested PCR was performed as previously described (11). The first round conditions were the same as for the one-round DNA quality assessment PCR referred to above. In the second round reaction, 1 μL of the first round product was amplified in a fresh 25 μL reaction mixture under the following conditions: 10 minutes at 95 °C followed by 35 cycles of 30 secs at 94ºC, 30 secs at 55ºC, and 1 min at 72°C plus an extension of 7 mins. Five μL of amplification products were eletrophoresed in 2% agarose gel (Sigma, USA) prepared in Tris-borate-EDTA (TBE) buffer alongside a molecular weight marker and viewed under UV light after ethidium bromide (10 mg/ml) staining. A positive reaction on a test sample with one or both HIV-1 (pol or env-located) primer sets was interpreted as HIV-1 PCR positive infection. Similarly a positive result with one or both HIV-2 (L TR-located) primer sets on a test sample was indicative of a PCR-reactive HIV-2 sample.

Table 1

Nucleotide sequence of PCR primers

Primer Description	Name	Nucleotide sequence (5′ - 3′)	Expected product size (bp)
ß2-	GH 20	GAAGAGCCAAGGACAGGTAC	
microglobulin	GH 21	GGAAAATAGACCAATAGGCAG	408
	HPOL 4325	CCCTACAATCCCCAAAGTCAAGG (4653-4676) *	
HIV-1**	HPOL 4538	TACTGCCCCTTCACCTTTCCA (4956-4977)	154
(Pol)	HPOL 4327	TAAGACAGCAGTACAAATGGCAG (4745-4768)	
	HPOL 4481	GCTGTCCCTGTAATAAACCC (4900-4920)	
	OA3	TGTACACATGGAATTAGGCCAGTAG (6963-6987)	
HIV	OD3	AAA TTCCCCTCCACAATTAAAACTG (7346-7371)	348
(Env)	SB	TCAACTCAACTGCTGTTAAAT (6990-7011)	
	SC	AATTTCTGGGTCCCCTCCTGAGG (7315-7338)	
HIV-2	LTRA	CTGAGACTGCAGGGACTTTCCAGAAGGG (9379-9406)	
(3′-5′ LTR)	LTRB	AAGCAAGAAGGGTCCTAACAGACCAGGGT (241-270)	
	LTRC	GGGA T ACTGCAGCAACAGCAACAGCTGTTG (7782-7811)	506
	LTRD	CCAGGCGGCGACTAGGAGAGATGGGAGCAC (184-213)	
HIV-2 **	H2L100	GCTGGCAGATTGAGCCCTG (18-35)	
(5′ LTR)	H2L 101	AAGGGTCCTAACAGACCAGGG (241-263)	
	H2L200	CAGCACTAGCAGGTAGAGCCTGGG (49-71)	150
	H2L201	GGCGGCGACTAGGAGAGATGG (191-210)	

* Figures in parenthesis indicate primer locations on HXB2 (HIV-1) or HIV-2 ROD.

**Primers were previously published in Ref 11

Statistical analysis: PCR assay was considered as the reference standard in this study. Data thereby generated were compared with the two serological confirmatory assays, Peptilav and Innolia. Sensitivity (ability to correctly diagnose true positive infections) and specificity (ability to correctly diagnose true negative infections) of the assays were analysed in order to assess the performance of the three assays on the same sample set in the accurate diagnosis of HIV infections. Sensitivity was determined according to the formula {TP/(TP+FN)} x 100 where TP was true positive and FN was false negative. Specificity was determined by {TN/(TN+FP)} x 100, where TN was true negative and FP was false positive (12).

RESULTS

Diagnosis of HIV-1 infection: One hundred and forty five HIV seropositive samples were analysed further by rapid screening with Determine and HIVSPOT and typed by Serodia PA, during which PA scored 106/145 (73%) seropositive HIV-1 infections. None was reactive for HIV-2 alone, while 39/145 (27%) were dually seropositive (Table 2). Subsequent analysis of 37 of the 39 PA dually reactive samples by Innolia, Peptilav and PCR are detailed in Table 3 and summarised in Table 4. Peptilav and Innolia respectively diagnosed 29 and 32 samples as HIV-1 only infections. The two assays identified seven and five additional HIV-1 infections respectively, which occurred simultaneously with HIV-2 as dual infections. In total, therefore, Peptilav scored 36/37 (97%) HIV-1 infections, while lnnolia diagnosed 37/37 (100%) as HIV-1 infections (Table 3). PCR, on the other hand diagnosed 26 HIV-1 only infections and 11 additional ones occurring simultaneously with HIV-2 as dual infections. Overall, PCR scored 37/37 (100%) HIV-1 infections.

Table 2
Summary of subsequent tests performed on 145 previously characterised HIV seropositive patient samples

Name of assay	Type of assay	Result of Assay: HIV Positive	HIV Negative	HIV-1	HIV-2	HIV 1/2 dual infection
HIVSPOT	HIV screening	145 (100%)	0	NA	NA	NA
DETERMINE	HIV screening	145 (100%)	0	NA	NA	NA
SERODIA PA	HIV typing	145 (100%)	0	105 (73%)	0	39 (27%)

NA = not applicable

Table 3
Diagnosis of HIV infection among 37 PA-dually reactive samples by immunoblot and PCR assays

Sample No.	Sample ID No.	Immunoblots: Peptilav	Immunoblots: Innolia	PCR
1	E2	Dual	HIV-1	HIV-1
2	E3	Dual	HIV-1	HIV-1
3	E4	Dual	HIV-1	Dual
4	E5	HIV-1	HIV-1	HIV-1
5	E6	HIV-1	HIV-1	Dual**
6	E7	HIV-1	HIV-1	HIV-1
7	E8	HIV-1	HIV-1	HIV-1
8	E9	HIV-1	HIV-1	HIV-1
9	E13	HIV-1	HIV-1	HIV-1
10	E14	HIV-1	HIV-1	Dual
11	E15	HIV-1	HIV-1	HIV-1
12	E16	HIV-1	HIV-1	Dual**
13	E17	HIV-1	Dual	HIV-1
14	E18	HIV-1	HIV-1	HIV-1
15	E19	HIV-1	HIV-1	Dual
16	E20	HIV-1	HIV-1	Dual**
17	E21	HIV-1	HIV-1	HIV-1
18	E22	HIV-1	HIV-1	HIV-1
19	E23	Dual	Dual	Dual
20	E24	Dual	HIV-1	Dual
21	E25	HIV-1	HIV-1	Dual
22	E26	HIV-1	HIV-1	HIV-1
23	E27	HIV-1	HIV-1	HIV-1
24	E28	HIV-1	HIV-1	HIV-1
25	E29	HIV-1	HIV-1	HIV-1
26	E30	HIV-1	HIV-1	HIV-1
27	E31	HIV-1	HIV-1	HIV-1

Continuation of Table 3

28	E32	HIV-1	HIV-1	HIV-1
29	E33	HIV-1	HIV-1	HIV-1
30	E34	HIV-1	HIV-1	HIV-1
31	E35	Dual	HIV-1	HIV-1
32	E36	HIV-1	HIV-1	HIV-1
33	E37	HIV-1	HIV-1	HIV-1
34	E38	HIV-1	HIV-1	HIV-1
35	E41	HIV-2	Dual	Dual**
36	E42	HIV-1	Dual	HIV-1
37	E43	Dual	Dual	Dual

* HIV-2 detected only at increased $MgCl_2$ concentration
** HIV-2 detected after increasing template concentration

Table 4

Sensitivity and specificity of PCR, Peptilav and Innolia assays in diagnosis of HIV-1 and HIV-2 infections among 37 PA dual positive samples

Assay	Infection type	Positive samples No.	(%)	TP*	TN	FP	FN	Sensitivity (%)	Specificity (%)
Peptilav	HIV-1	36	97	36	0	0	1	97	NA
	HIV-2	8	22	8	29	3	3	73	91
Innolia	HIV-1	37	100	37	0	0	0	100	NA
	HIV-2	5	14	5	32	6	6	45	84
PCR	HIV-1	37	100	37	0	0	0	100	NA*
	HIV-2	11	30	11	26	0	0	100	100

* TP = True positive, TN = True negative, FP = False positive, FN =False negative, NA=not applicable

Diagnosis of HIV-2 infection: Peptilav alone identified one case (E 41) of HIV-2 only infection. Seven additional HIV-2 infections were identified as dual infections with HIV-1. Thus Peptilav scored 8/37 HIV-2 cases. Innolia and PCR respectively scored 5/37 and 11/37 cases. In total, HIV-2 infections diagnosed were 11 (30%) by PCR, nine (22%) by Peptilav and five (14%) by Innolia.

Diagnosis of dual infection: Dual HIV-1/2 infections diagnosed by the three assays were similar to HIV-2 infections. Innolia identified 5/37 (14%) serologically dual reactive samples while Peptilav identified 8/37 (22%). PCR scored the highest number of dually reactive samples of 11/37 (30%).

Sensitivity and specificity of assays: PCR and Innolia both had 100% sensitivity in the diagnosis of HIV-1 infections, while Peptilav had 97% sensitivity. Peptilav scored a single false negative case for HIV-1 infection. Specificity could not be determined for the assays, however, because our test samples did not include HIV-1 negative cases. Sensitivity for HIV-2 diagnosis was remarkably varied among the three assays. PCR had 100%, 73% for Peptilav and 45% for Innolia. Peptilav diagnosed 3/11 false negative samples, while Innolia diagnosed 6/11 false negative HIV-2 infections. Specificity of the three assays in the diagnosis of HIV-2 infections was 100% for PCR, 91% for Peptilav and 84% for lnnolia (Table 4). Peptilav and Innolia respectively diagnosed three and six false positive cases of HIV-2 infections.

DISCUSSION

A reliable estimation of the frequency of dual infections has difficulties due to discrepancies that are peculiar to the different serological tests and genomic amplification methods employed (13). Peptilav and Innolia differed in the number of antigens used in their manufacture. Innolia possessed five HIV-1 antigens, namely the envelope transmembrane (TM) glycoprotein (gp) 41, an additional *env* antigen (gp120), as well as core antigens p31 (integrase), p17 (gag

protein) and p24 (capsid). Peptilav, on the other hand, had only one HIV-1 antigen, namely gp41. However, only 3% disparity was observed between the two assays in the diagnosis of HIV-1 infections. Innolia, not surprisingly, detected 100% HIV-1 reactive samples, while the number diagnosed by Peptilav, (97%), was not significantly different from lnnolia. There was however a discrepancy of 8% between Innolia and Peptilav in the diagnosis of HIV-2 and 28% in identification of dual HIV infections, exhibiting considerable diversity between the two assays. In both cases Peptilav correctly diagnosed more of the infections than Innolia. This observation was surprising since Innolia had extra antigens to both HIV-1 and HIV-2. For HIV-2 diagnosis, Innolia had gp 105 in addition to gp 36 and a strong reactivity to either one or both, along with a core antigen was interpreted as a positive result according to the manufacturer. Effectively, plasma HIV-2 antibodies in our samples missed out on both HIV-2 antigens in Innolia more frequently than they did on the single HIV-2 epitope in Peptilav during HIV-2 diagnosis.

The level of HIV-2 diagnosis achieved genomically on this sample set (30%) was considered to best reflect the true HIV-2 prevalence in Ghana. This is because diagnosis of HIV infections by PCR relies on the detection of proviral sequences within the host cell genome, which is a marker for viral presence. A positive PCR result thus represented a definite episode of HIV infection, hence the decision to consider this assay as the 'gold standard'. However, even by PCR, the template amount had to be doubled to improve sensitivity in, at least, three cases before achieving a positive HIV-2 diagnosis (samples E6 and E16) and a positive HIV-1 diagnosis for E41. With E20 also, HIV-2 provirus was detected only after $MgCl_2$ concentration had been doubled. These underscore the results of previous reports that it is possible to improve the detection of HIV-2 provirus in samples with a dual serological profile (14).

Discordant data between serological and genomic-based assays have been reported in other West Africa countries (14, 15). For example specimens initially diagnosed as dually seroreactive on immunoblots were subsequently shown to have only HIV-1 proviral sequences, a situation reminiscent of the cross reactivity between HIV-1 and HIV-2 antigens. Again Leonard *et al.* (16) reported the detection of HIV-2 provirus in samples confirmed as HIV-1 only on immunoblots. In our study, both HIV-1 and HIV-2 proviral sequences were detected in six samples (E6, E14, E16, E19, E20 and E24) which were earlier confirmed as HIV-1 only on immunoblots.

The disparity in the detection of H IV infections among our samples could also reflect the genetic diversity of circulating HIV molecular forms in Ghana and, possibly, the West African subregion. At the time of this study, the epidemic in Ghana was due to the co-circulation of HIV-1 subtypes A, D and G (7, 17), HIV-2 subtypes A and B (18) and possibly CRF02_AG recombinant strains, which currently predominate in Ghana (8). New recombinants such as CRF06_cpx now prevail in West Africa (19). Furthermore HIV-1 subtypes A, G, J and CRF01_AE have also variously recombined to create CRF11_cpx in Cameroon and the Central Africa Republic (20). These developments underscore the extent of genetic diversity and the potential for the emergence of new HIV variants in West and Central Africa. The interaction between these variants and the immunological characteristics of the resulting phenotypes are not altogether predictable. This study was carried out with 3rd generation kits composed of antigenic peptides designed to encompass all documented major HIV strains at that time. Indeed, all three assays were inherently capable of achieving the same diagnosis for some of our specimens. For example, apart from the several instances of a uniform diagnosis of HIV-1 infection, samples E23 and E43 were both scored as dual infections by all three assays evaluated. The inability of these assays, therefore, to correctly diagnose all the test samples at all times could be related, most probably, to unique circulating HIV recombinants. Since the assays virtually detected all HIV-1 infections, the difficulty with the accurate diagnosis of HIV dual infections was largely due to inaccuracies in the serological detection of HIV-2. This was exemplified with Peptilav, Innolia and, to an even larger extent, Serodia PA.

In conclusion, the reliable diagnosis of dual infections is characterised by some difficulties in areas where HIV-1 and HIV-2 co circulate. It appears that many assays will correctly identify HIV-1 infections. However, for the accurate serological diagnosis of HIV-2 infections, enhancement of plasma antibody levels was necessary. We also showed that a positive PCR diagnosis for HIV-2 required modification of template or $MgCI_2$ concentrations. The critical issue raised in this study is that using anyone assay on the test samples resulted in different outcomes.

Walther-Jallow *et al* (13) reported that a combination of PCR and different serological assays achieved up to 85% success in the detection of dual HIV infections. In our view, samples must be subjected to an immunoblot assay such as Peptilav, with at least two increasing amounts of the test samples. Because serology alone could overestimate the prevalence of dual infections, serologically reactive samples must be re-evaluated by PCR as prescribed earlier. The accurate diagnosis of dual infections must involve PCR. This proposed algorithm implies that accurate diagnosis of HIV-1 / 2 dual infections will necessarily require highly trained personnel and a well-resourced laboratory. This will also be expensive but unavoidable because with the increasing availability of ARVs to HIV / AIDS patients in West Africa and elsewhere, it will be critical to correctly diagnose HIV infections in these populations to guarantee optimal treatment of AIDS disease.

ACKNOWLEDGEMENTS

The specimens for this study were collected during the 3rd Infectious Diseases Project between Japan International Cooperation Agency (JICA) and Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon. The authors are extremely grateful to JICA for technical support. We also acknowledge Justice K. Kumi, N. Nii-Trebi, A. A. Hayford, S. Matrevi and W. Kumi, all of Virology Department, NMIMR and Dr. J. Ansah, Hygiene Wing of 37 Military Hospital, Accra, Ghana for their technical assistance, We are also indebted to Drs. D. Doucet, D. Candotti and Prof. J-P Allain for their review of the paper.

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