DEVELOPING AND APPLYING SEROLOGICAL AND MOLECULAR SKILLS TO THE VIROLOGICAL ANALYSIS OF HIV-INFECTED PATIENTS FROM KUMASI, GHANA

By

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ABSTRACT

Background : Virological monitoring is critical in the management of HIV-infected patients, providing a standard in the assessment of disease prognosis and progression, guiding the initiation of ART and treatment selection, monitoring therapeutic success and establishing treatment failure and drug resistance. The absence of viral load monitoring can impact upon individual and public health through failure to maintain viral suppression, and increased risk of drug resistance. HIV management at the KATH HIV clinic, Kumasi, Ghana, does not include virological monitoring due to the lack of laboratory infrastructure and technical skills, thus the virological response to ART among treated patients at the centre is not fully understood. Moreover, data on the prevalence of HCV infection in both the general population and HIV-positive patients in Ghana are limited, with seroprevalence estimates ranging from 0.5% to 18.7% documented among different Ghanaian populations, possibly due to differences in study populations and the serological assays employed. Furthermore, these previous studies did not attempt confirmation of HCV status by PCR or RIBA. The aim of this study is to determine the HIV virological response in a HIV/HBV co-infected cohort from KATH, ascertain the specificity and sensitivity of commercially available HCV serological assays, and develop an assay that could be used as an alternative for HCV RNA testing in Kumasi.

Methods: 247 HIV/HBV co-infected patients attending the KATH HIV clinic were recruited into a prospective HIV and viral hepatitis study, of which HIV-1 viral load was determined for 183 ART-experienced patients at study entry using the Abbott Real Time HIV-1 assay. The HIV-1 viral load detection among patients who had been on ART for at least 24 weeks was assessed. HIV positive samples from KATH with known HCV-RNA status were tested with two automated anti-HCV antibody assays, the Abbott Architect anti-HCV, Vitros Anti-HCV, and two manual EIAs, Monolisa HCV Ag-Ab ULTRA, and the ORTHO HCV 3.0 ELISA System with Enhanced SAVe. Of the last three assays the performance and the respective assay cut-offs likely to be indicative of RNA positivity were evaluated using their PCR and Architect results as reference. The development of an in-house indirect sandwich HCV core antigen EIA which could be used as an alternative for HCV-RNA testing was attempted.

Results: Overall, 58/183 (37.4%) patients who received treatment for at least 24 weeks showed a viral load >40 copies/mL with a median level of 826 copies/mL (IQR: 65 - 26752). Their CD4 T-cell counts were lower compared to patients with undetectable viral load (P= 0.002, Mann Whitney U test). Among the four HCV antibody assays the Ortho was found to be the most specific assay that could be employed in a limited resource setting such as Kumasi, and an S/CO ratio of 3.65 was found to be most likely to be indicative of HCV RNA positivity. The HCV core EIA development was not completed in time due to poor activity of commercially available agents.

Conclusion: Through this study, skills in HIV viral load and EIA development have been acquired that could be applied to improve virological monitoring at KATH with the necessary infrastructure in place. Further studies are required to identify factors that are associated with poor viral response in this cohort. The ORTHO HCV 3.0 ELISA System with Enhanced SAVe can be regarded as a suitable diagnostic tool for HCV infection in Kumasi, but further studies are required to establish the S/CO ratio most likely to be indicative of HCV RNA positivity.

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DEDICATION

I dedicate this work to Mispah, Lois, Vithel and Jude.

ABBREVIATIONS

µL	Microlitre
aa	Amino acid
Ab	Antibody
Ag	Antigen
AFB	Acid fast bacilli
ALT	Alanine transaminase
AP-1	Activator protein 1
ART	Antiretroviral therapy
AST	Aspartate transaminases
AVM-RT	Myeloblastosis virus reverse transcriptase
bDNA	Branched deoxyribonucleotide
BUE	Blood urea nitrogen and electrolytes
C/EBP	Cytidine-cytidine-adenosine-adenosine-thymidine/ enhancer binding protein
CA	Capsid
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation
CDC	Centre for disease control
cDNA	Complementary deoxyribonucleic acid
CXCR4	C-X-C chemokine receptor type 4
ddPCR	droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
DPS	Dry blood spot
EDTA	Ethylene diaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FDA	US food and Drug Administration
FRET	fluorescence resonance energy transfer
Gag	Gag-gene
GITC	Guanidine isothiocynate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCVcAg	Hepatitis C core antigen
HEPIK	HIV and viral hepatitis studies in Kumasi
HIV	Human immune deficiency virus
HRP	Horse raddish peroxidase
IDU	injection drug usage

IgG	Immunoglobulin G
IGH	Institute of infection and Global Health
IgM	Immunoglobulin M
IN	Integrase
KAA	Kwabena Adjei-Asante
KBTH	Korle-Bu teaching hospital
LTR	Long terminal repeat
MA	Matrix
MHC	Major histocompatibilty complex
mL	Millilitre
MSM	Men who have sex with men
NASBA	*Nucleic acid sequence based amplification*
NAT	Nucleic acid testing
NC	Negative control
Nef	Negative effector
Nef	Negative regulatory factor
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa beta
NHS	National Health Service
NNRTI	Non-nucleoside reverse transcriptase
NPV	Negative predictive value
NRTI	Nucleoside reverse transcriptase
NtRTI	Nucleotide reverse transcriptase
PC	Positive control
PCR	Polymerase chain reaction
PI	Protease inhibitor
pol	Pol-gene
PPV	Positive Predictive value
PR	viral protease
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RIBA	Recombinant immunoblot assay
RAM	Resistance associated mutations
RLU	Relative light units
RNA	Ribonucleic acid
RT	Reverse transcritase
S/CO	Signal : cut-off ratio
SOP	Standard operating procedure
SSA	Sub-Saharan Africa
STI	Sexually transmitted infection

TAR	Trans-activating response
Tat	Trans-Activator of Transcription
TB	Tuberculosis
TMB	Tetramethylbenzidine
tRNA	Transfer RNA
UK	United Kingdom
UNAIDS	United Nations Programme on HIV/AIDS
UNICEF	The United Nations Children's Fund
UoL	University of Liverpool
UR	Untranslated region
USA	United States of America
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VQA	Virology Quality Assurance
WHO	World Health Organisation

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CHAPTER ONE - INTRODUCTION

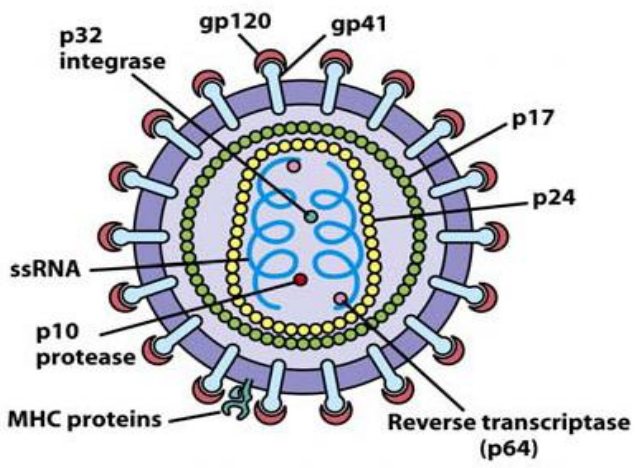
1.1 THE GLOBAL HIV EPIDEMIC

HIV infection continues to be one of the most severe global health challenges. Currently, 34 million (between 31 and 36 million) people are estimated to be living with the virus worldwide and a prevalence of about 0.8% occurs among the most productive age group, 15-49 years. The epidemic however demonstrates significant geographic variation. Sub-Saharan Africa (SSA) remains the most seriously affected region with an adult prevalence of 4.9%, accounting for 69% of the total number of people living with HIV worldwide. This reflects limited access to prevention, care and treatment in this region (WHO/UNAIDS/UNICEF, 2011). Adult prevalence rates are about 0.1% in East Asia, 0.2% in Western/Central Europe and 0.3% in South-East Asia and Oceania. Latin America, North America, Eastern Europe and the Caribbean also have $\leq$ 1% prevalence. Women represent about 50% of worldwide population living with HIV and close to 60% in SSA (UNAIDS, 2012).

Although there has been a steady rise in the global prevalence rate from 1999 to 2011 due to continuing new infections, increased life expectancy of infected people, there has also been an overall decline by more than 20% in the number of new infections since 2001. The sharpest declines have occurred in the Caribbean (42%), and SSA (25%). Moreover the rate of AIDS-related death has reduced by 24% since 2005 (UNAIDS, 2012), reflecting anti-retroviral scale up in many parts of the world. Globally, most new infections are as a result of heterosexual transmission, and this especially the case in SSA. In other regions, homosexual intercourse, intravenous drug use, and commercial sex work remain significant risk factors for infection.

1.2 THE HIV LIFE CYCLE

Figure 1.1 The HIV particle



HIV has an icosahedral structure with diameter ranging between 110-143nm (Zhu *et al*, 2003) (Fig 1.1). It is surrounded by a lipoprotein-rich bilayer which contains 72 external spikes formed by trimers of the external surface glycoprotein gp120 and the transmembrane glycoprotein gp41 non-covalently bound together (Fanales-Belasio *et al*, 2010)**.** The lipid bilayer is also studded with various host proteins including major histocompatibility complex (MHC) class I and II antigens and actins acquired during virion budding (Green *et al*, 1991).

The HIV genome is approximately 9.8kb and is composed of two molecules of single-stranded RNA enclosed by a cone-shaped capsid mainly containing p24 viral protein. External to the capsid the p17 protein forms the matrix associated with the inner suface of the lipid bilayer. The capsid also contains the viral protease (PR), reverse trascriptase (RT), integrase (IN), viral protein U (Vpu), viral infectivity factor (Vif), negative effector (Nef) and some cellular factors (Sierra *et al*, 2005). CD4+ T-lymphocytes, monocytes and macrophages are the major cellular targets for HIV infection **(**Letvin, 1990**)**. HIV enters these cells by binding to the CD4 receptor on the cell membrane (Ratner, 1993; Klatzman *et al*, 1984). Entry also requires binding to one of several co-receptors; the chemokine receptors CCR5 and CXCR4 are the main co-receptors for HIV-1 *in vivo* (Clapham, 2002). Preferential use of these two chemokine receptors identifies HIV tropism; strains that preferentially use CXCR4 are designated X4; strains that use CCR5 are called R5; and strains that use both co-receptors are designated as dually tropic (Freed, 2001).

Infection of target cells begins with virus entry, which requires the fusion of the viral envelope with the cellular membrane (Fig. 1.2). Initially, gp120 binds to the CD4 receptor (Nielsen *et al*, 2005), which causes a conformational change exposing a specific domain in the V3 loop that binds to the chemokine co-receptor.

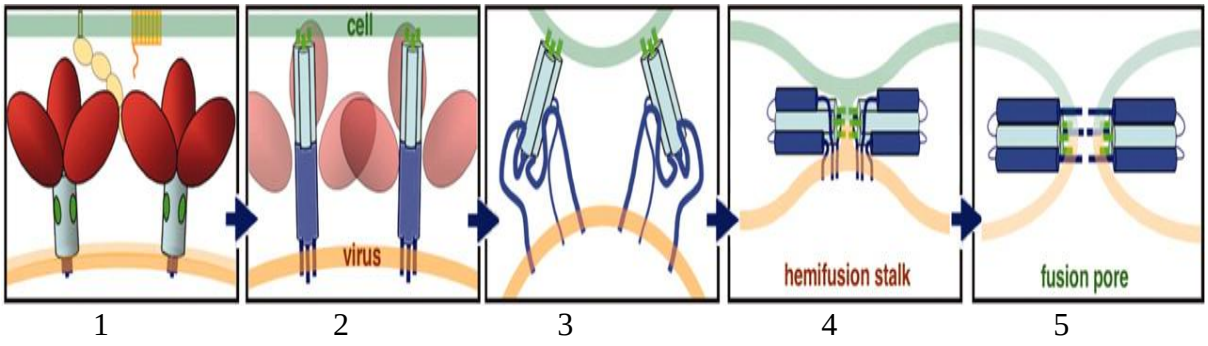


Figure 1.2 HIV entry into the host cell. 1) gp120 binds with CD4 and the chemokine co-receptor, 2) the gp41 fusion peptide springs out towards the host cell membrane, bridging the divide between the viron and the host cell membrane, 3) gp41 folds bringing cell and viral membrane close to each other, 4) gp41 forms a fusion stalk, 5) the energy derived from process 4 drives the hair-pin structure to form a fusion pore (Frey *et al*, 2006).

The interaction induces a conformational change in the transmembrane protein gp41, which exposes the ectodomain region containing a highly hydrophobic N-terminus and two heptad repeat motifs (N- and C- helices). The N-terminus inserts directly into the target cell lipid bilayer whilst the N- and C- helices fold into a stable six-helix bundle forcing the viral and cell membranes into close proximity allowing membrane fusion to occur (Freed *et al*, 2001), thereby allowing the release of the viral capsid into the cell (Kilby *et al*, 2003; Harrison *et al*, 2005).

After internalization, there is a rapid uncoating of the capsid, by a mechanism that is less well understood (Ratner, 1993; Freed, 2001; Doms and Moore, 2000) but may involve both cellular factors and viral proteins (Sierra *et al*, 2005). Uncoating is followed by reverse transcription of the viral RNA into double-stranded complementary DNA (cDNA), a process mediated by RT. The enzyme catalyses DNA synthesis from 3′ to 5′ end of the RNA, initially producing a hybrid between the genomic RNA and the complementary DNA. The genomic RNA is degraded by the RNase H activity of RT generating a minus strand DNA strand (Freed, 2001; Coffin, 1996; Joshi, 2000). A complementary plus-strand DNA is synthesised by the polymerase activity of RT, resulting in the formation of a double helix DNA molecule. The linear double-helix DNA, together with viral proteins and enzymes and cellular proteins form the pre-integration complex (PIC) (Sheehy *et al*, 2002; Miller *et al*, 1997; Jayappa *et al*, 2012), which moves actively across the host nuclear membrane into the nucleus (Freed, 2001; Jayappa *et al*, 2012).

After nuclear import, IN catalyses the insertion of the viral DNA into the host DNA. Host cellular enzymes complete integration by joining the 3′ and 5′ ends of the host and viral DNA respectively (Freed, 2001; Engelman *et al*, 1991; Hindmarsh and Leis, 1999). Once integrated, the viral DNA remains part of nuclear DNA as provirus.

Upon cell activation, transcription of the provirus into mRNA occurs. Transcription is promoted by the LTR located at the 5′ end of proviral DNA which has binding sites for cellular transcription factors including Sp1, NFκB, AP-1, C/EBP, and NFAT (Kilareski, 2009; Wu and Marsh, 2003). The initial transcription process leads to the synthesis of Tat and Rev. Tat in complex with TAR enhances the synthesis of RNA by *pol* II (Freed, 2001; Laspia *et al*, 1989) leading to the generation of three major classes of RNAs which are transported into the cytoplasm: unspliced RNAs which function as mRNAs for the synthesis of Gag and Gag-Pol polyprotein precursors (p55 and p180 respectively) and form a dimer of genomic RNA that are packaged into progeny virions; partially spliced mRNAs which encode for Env (gp160), *Vif*, *Vpr* and *Vpu* proteins; and multiply-spliced mRNAs which encode for *Tat*, *Rev*, *Vpr* and *Nef* proteins (Ratner, 1993; Freed, 2001). The splicing of the RNA is regulated by Rev and RRE proteins (Pollard and Malim, 1998).

The env glycoprotein, gp160, is synthesised and inserted in the rough ER and transported to the cell surface membrane through the Golgi. During its transportation, gp160 is cleaved by cellular protease to generate mature gp120 and gp41. Gp41 anchors the env complex in the cell membrane and binds non-covalently with gp120 (Facke *et al*, 1993). The Gag polyprotein is a 53kDa precursor which has p24, p17, p9, p7, p6, SP1and SP2 domains. Pol-gene precursor is translated from the same transcript as Gag precursor by a ribosomal frame shifting mechanism (Jacks *et al*, 1987). This precursor is cleaved into viral enzymes including RT, IN, and PR.

Gag and Gag-Pol polyproteins migrate to the plasma membrane and assemble to form the immature core of the virus. The viral enzymes, the genomic RNA dimer and the cellular $tRNA^{lys3}$ then associate with the immature core (Greene, 2002; Freed *et al*, 1994; Bryant *et al*, 1990). During the viral assembly MA forms a matrix under the viral envelope and CA proteins condenses to form a conical core surrounding the nucleocapsid coated genomic RNA. Following the assembly of viral particles specific sequences in Gag p6 promote viral release from the cellular surface (Gottlinger *et al*, 1991). During or shortly after viral release, PR cleaves the Gag and GagPol polyproteins precursors to generate mature Gag and Pol

proteins. This process in turn stimulates a series of structural rearrangement that lead to viral maturation (Wlodawer and Erickson, 1993).

1.3 ANTIRETROVIRAL DRUGS AND THEIR MECHANISM OF ACTION.

Antiretroviral drugs are classified into six major classes namely, nucleoside (or nucleotide) RT inhibitors (NRTIs and NtRTIs), non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, CCR5 antagonists, and integrase inhibitors (Table 1.1).

1.3.1 Nucleoside/Nucleotide Reverse Transcriptase Inhibitors

These are nucleoside analogues that undergo intracellular phosphorylation catalysed by cellular kinases to generate 5' triphosphate active forms (Gao *et al*, 1994); NtRTIs are already monophosphorylated and require therefore only two further phosphorylation steps to become active intracellularly. The phosphorylated inihibitors are then incorporated in the nascent viral DNA by RT, which prematurely terminates DNA chain extension (Furman *et al*, 1986) due to lack of an hydroxyl group at the 3' position which prevents the 5' to 3' phosphodiester bond necessary for DNA elongation (Mitsuya, 1986; Abu-ata *et al*, 2000).

Drugs in this class are further categorised into thymidine and non-thymidine analogues which preferentially exert their antiviral activity in activated and non-activated CD4+T-lymphocytes respectively (Miller, 2002). Currently there are 7 N(t)RTIs approved for treatment namely, Zidovudine (ZDV), Didanosine (ddI), Stavudine (d4T), Lamivudine (3TC), Tenofovir disoproxil fumarate (TDF), Abacavir sulphate (ABC), and Emtricitabine (FTC). TDF is currently the only NtRTI available.

Table 1.1 Approved antiretroviral agents available in Europe and America

Class	Agent	Abbreviation
NRTI	Abacavir	ABC
	Didanosine	DDI
	Emtricitabine	FTC
	Lamivudine	3TC
	Stavudine	d4T
	Tenofovir disoproxil fumarate	TDF
	Zidovudine	ZDV
NNRTI	Efavirenz	EFV
	Etravirine	ETR
	Nevirapine	NVP
	Rilpivirine	RPV
PIs	Atazanavir	ATV
	Darunavir	DRV
	Fosamprenavir	FOS-APV
	Indinavir	IDV
	Lopinavir/ritonavir	LPV/r
	Nelfinavir	NFV
	Ritonavir	RTV
	Saquinavir mesylate	SQV
	Tipranavir	TPV
Fusion Inhibitors	Enfuvirtide	T-20
CCR5 Antagonists	Maraviroc	MVC
Integrase inhibitors	Raltegravir	RAL
	Elvitegravir	EVG

1.3.2 Non-Nucleoside Reverse Transcriptase Inhibitors

These are a group of compounds of various structures that bind to the hydrophobic pocket near the catalytic site in HIV-1 RT (Grob *et al*, 1992) causing a distortion in the catalytic site that prevents RT activity due to the restriction of a flexible loop between amino acid positions 183 and 186 in the catalytic site. These drugs are inactive against HIV-2 (Witvrouw, 2004). Presently approved NNRTIs are Nevirapine (NVP), Efavirenz (EFV), Etravirin (ETR), and Rilpivirine (RPV).

1.3.3 Protease Inhibitors

Protease-mediated cleavage of HIV-1 polyproteins after budding is necessary for the maturation of HIV particles. PIs consist of compounds that bind to the active site of HIV-1 PR thereby inhibiting maturation and resulting in the release of immature and non-infectious viral particles. Currently available PIs are Atazanavir (ATV), Darunavir (DRV), Fosamprenavir (FOS-APV), Indinavir (IDV), coformulated Lopinavir/ritonavir (LPV/r), Nelfinavir (NFV), Saquinavir (SQV), Ritonavir (RTV), and Tipranavir (TPV). Protease inhibitors are metabolised by cytochrome P-450 CYP3A4 enzyme. Ritonavir is a potent cytochrome P-450 CYP3A4 inhibitor (Warnke *et al*, 2007). Administration of PIs accompanied by a small dose of ritonavir increases bioavailability of other PIs (Rathbun *et al*, 2002).

1.3.4 Fusion Inhibitors

The currently approved fusion inhibitor in Europe and America is Enfuvirtide (T20). T20 is a 36-amino acid peptide homologous to a segment of gp41; it binds to the heptad repeat (HR1) domain of gp41 and blocks the formation of the 6-helix bundle hairpin structure necessary to pull viral envelope and host cell membrane together for fusion, thus blocking viral entry (Clavel and Hance, 2004).

1.3.5 CCR5 Antagonists

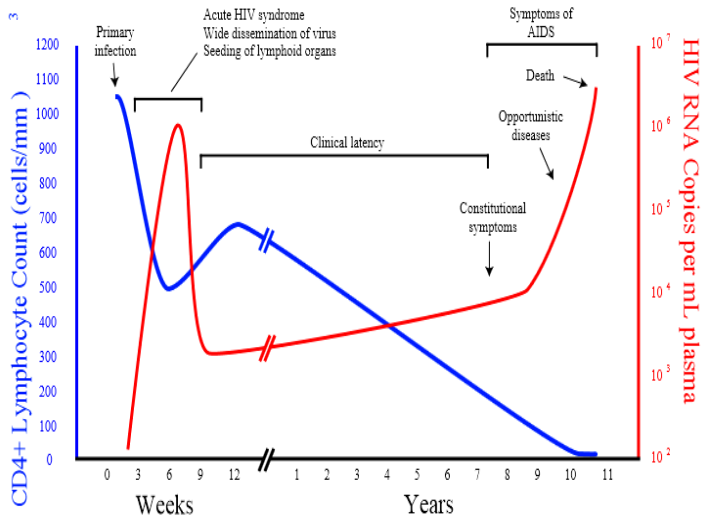
CCR5 antagonists are divided into large (e.g. PRO-140), medium-size (e.g. Met-RANTES) and small molecules (e.g., Maraviroc, Aplaviroc, Vicriviroc). Maraviroc is currently the only approved CCR5 antagonist. It binds to a hydrophobic pocket within the transmembrane region of CCR5 on the host cell membrane, causing a conformational change that prevents binding of the V_3 stem loop of gp120 (Dragic *et al*, 2000).

1.3.6 Integrase Inhibitors

Integrase inhibitors that are currently approved are Raltegravir and Elvitegravir. These drugs inhibit the DNA strand transfer step of the HIV life cycle in two ways: (i) have high affinity for the acceptor DNA site of the integrase enzyme in the pre-integration complex, competing with viral DNA for the binding to integrase thereby inhibiting the insertion of viral DNA into host cell DNA (Espeseth *et al*, 2000); and (ii) chelate the Mg^{2+} ion in the active site necessary for the activity of the integrase enzyme thereby preventing 3' processing and strand transfer (Grobler *et al*, 2002). Elvitegravir must be boosted, typically with cobicistat, which has a mechanism of action similar to that of ritonavir.

1.4 HIV VIRAL LOAD TESTING

Figure 1.3 Kinetics of viral load and CD4 T-cell counts over the course of HIV infection



The HIV viral load is a measure of the number of copies of viral RNA per millilitre of plasma. It provides an estimate of the degree of viral replication *in vivo*. There exists variation in the plasma viral load at the different clinical stages of HIV infection. Viral load levels are highest during acute infection and in late stage disease (Coombs *et al*, 1989; Ho *et al*, 1989) and are the single strongest predictor of HIV disease progression and death (Mellors *et al*, 1996; Mellors *et al*, 1997) (Fig. 1.3). The acute phase of HIV infection is characterised by a massive uncontrolled HIV replication, which results in a high viral load and rapid deletion of CD4+ T-lymphocytes (Cooper *et al*, 1985). The viral load peaks at about a week after primary infection (Lindback *et al*, 2000). During seroconversion and with the emergence of T-cell immune responses against HIV antigens the viral load declines gradually until a set-point is subsequently reached. This represents a balance between viral replication and immune-mediated virus clearance and remains relatively stable for months to years. The viral load set-point is predictive of the rate of CD4 T-cell loss, disease progression and mortality (Mellors *et al*, 1996; Touloumi *et al*, 1998, Lyles *et al*, 2000, Langford *et al*, 2007).

Baseline and follow-up measurement of viral load is important to predict the rate of decline in immunological strength and risk of disease progression. In addition, pre-treatment viral load testing is also important to decide upon initiation of antiretroviral therapy (ART) and to guide treatment selection. Patients with baseline viral load >100,000 copies/ml appear to have a greater risk of ART failure than those with lower viral load (van Leth *et al*, 2005). Certain antiretroviral agents such as abacavir and rilpivirine are especially vulnerable to loss of activity in patients with high viral load. Furthermore, the viral load retains its prognostic value during therapy, as the risk of AIDS or death in treated patients is greater in patients viral load >400 to 500 copies/mL while on ART (Olsen *et al*, 2005, Lohse *et al*, 2006).

During treatment, viral load determination is critical in the assessment of the efficacy of therapy. The goal of therapy is stated as achieving an undetectable viral load (usually <50 copies/mL) within 24 weeks of treatment. Failure to reach this target after 24 weeks of therapy or subsequent rebound, constitute treatment failure (Williams *et al*, 2012; WHO, 2006). Regular viral load testing is therefore necessary to detect and manage early therapeutic failures and prevent the emergence of drug-resistant strains.

Viral load also provides a measure of infectivity and therefore the risk of transmission including vertical HIV transmission (Kuhn, 1997; Sperling *et al*, 1996; Pitt *et al*, 1998). In a prospective study of an untreated cohort, high maternal viral load was strongly associated with both *in utero* and intrapartum vertical transmission of HIV (Mock *et al*, 1999). Knowledge about maternal viral load is therefore relevant to the early initiation of mother-to-child transmission prevention strategies. Furthermore, the viral load can be used to assess the risk of HIV transmission following an exposure, and decide upon post-exposure prophylaxis in the recipient, for example after a needle-stick injury.

1.4.1 Techniques for HIV Viral Load Testing

There are two major categories of HIV viral load testing techniques namely, nucleic acid testing (NAT) and non-nucleic acid testing (non-NAT). NAT detects and quantify viral RNA whereas non-NAT uses HIV viral enzymes and proteins as a correlate measure of viral RNA. NAT involves four major methods: Quantitative reverse transcription polymerase chain reaction (qRT-PCR), nucleic acid sequence based amplification (NASBA), branched chain DNA (bDNA), and more recently droplet digital polymerase chain reaction (ddPCR). Of these, qRT-PCR is the most commonly applied methodology

Real-time quantitative reverse transcription PCR

The technique involves first the reverse transcription of HIV-RNA into cDNA. The cDNA then undergoes PCR-amplification, the product of which is detected and measured during each PCR cycle by the use of a dual-labelled oligonucleotide fluorescent probe. The probe has a reporter fluorescent dye at the 5' end and a quencher at the 3' end. The probe anneals to the target DNA and is cleaved by the 5' nuclease activity of the polymerase enzyme during the PCR extension phase. Whilst the probe is annealed to the target DNA, fluorescence from the reporter dye is absorbed by the quencher via fluorescence resonance energy transfer (FRET) principle. Cleavage by the polymerase enzyme separates the reporter from the quencher increasing the fluorescence of the reporter.

For over a decade the second-generation Roche AMPLICOR Version 1.5 conventional PCR Assay ("AMPLICOR") was the most widely used viral load test in both clinical trials and routine practice. Clinical trials used an "undetectable" viral load by AMPLICOR (below 50 HIV-1 RNA copies/mL) as the end-point for measuring virological efficacy, and guidelines have adopted the same threshold as the goal of therapy. The Roche TaqMan Real-Time Quantitative Human Immunodeficiency Virus Type 1 RNA PCR Assay ("TaqMan") has now replaced AMPLICOR in routine diagnostic settings. There are two versions of TaqMan; version 2 has largely replaced version 1 across Europe and has recently received FDA approval in the US. Other assays based on real-time PCR technology have also been introduced in clinical settings, including the Abbott M2000SP/M2000rt RealTime HIV-1 Assay ("RealTime") and the Artus HIV Virus-1 QS-RGQ Assay. The TaqMan and RealTime assays can be regarded as third-generation assays, characterised by high sensitivity and a wide-range of quantification. The assays are highly automated. They target the LTR and Gag region and the integrase region respectively, and have a lower limit of quantification of 40 copies/ml (TaqMan version 1 and RealTime) or 20 copies/ml (TaqMan version 2).

RealTime HIV-1

The assay targets a conserved region in the integrase gene of HIV-1 group M and group O viruses (Garcia-Diaz *et al*, 2013). An internal control (IC) is introduced into each specimen during sample preparation. Purified RNA is obtained from 0.6ml of plasma using the Abbott m2000sp automated extractor, which employs magnetic particle technology, followed by amplification and detection on the Abbott m2000rt real-time PCR instrument. The duration of the assay is 5-6 hours, of which approximately 5 hours is hand-off during the automated phases of the assay. The assay lower limit of quantification is 40 copies/ml, with a dynamic range of 40 to 10,000,000 copies/ml (0.6 ml input). The assay was calibrated against a viral standard from the Virology Quality Assurance (VQA) laboratory of the AIDS Clinical Trial Group and against the WHO first International Standard for HIV-1 RNA (97/656).

1.5 HEPATITIS C CO-INFECTION IN HIV POSITIVE PATIENTS

The most recent WHO estimate indicates that about 150 million people are chronically infected with hepatitis C and at least 350 000 people die from the liver disease associated with HCV infection each year. The hepatitis C virus (HCV) has been detected in 27% and 25% of worldwide population with hepatocellular carcinoma and cirrhosis respectively. Moreover 3-4 million people are infected with the virus annually (WHO, 2012). HCV demonstrates a high level of geographic variability in its distribution worldwide. The highest prevalence rate has been reported in North Africa, particularly Egypt which has a prevalence rate of 15%-20 % (Alter, 2007). Moderately high prevalence between 2% and 2.9% occurs in Eastern Europe and Asia; low prevalence (1.0-1.9%) occurs in North America, Australia, Western Europe and Japan (Sheppard *et al*, 2005). The lowest prevalence (about 0.01-0.1%) has been reported from the UK and Scandinavian (Alter, 2006).

Establishing HCV prevalence in SSA is important because the region has been historically estimated to have a high prevalence rate of around 5.3% (Global surveillance and control of hepatitis C, 1999). A review by Madhava *et al*, 2002, estimated an overall prevalence of 3.0%, with highest prevalence (about 6%) in Central Africa; followed by West Africa (about 2.4%) and eastern and southern Africa (about 1.6%).

Age-related prevalence differs for various geographical locations and generally reflects temporal transmission patterns. For instance in Egypt, prevalence rate increases steadily with age with all age groups affected. This pattern shows a higher risk of infection in the distant past and an ongoing transmission from older to younger age groups. The existing high prevalence of HCV in Egypt is associated with the mass control campaign against the schistosomiasis, which took place between 1960 and 1980, using intravenous tartar emetic treatment (Strickland, 2006). The parenteral anti-schistosomal therapy (PAT) involved the use of reusable syringes sterilised between multiple uses which resulted in mass exposure to HCV infection (Frank *et al*, 2000). The PAT was gradually replaced with orally administered praziquantel in the mid 1980s. Hence, HCV prevalence is lower in children and young adults compared to 30 to 65 year group.

Conversely, Australia and United States have highest infection rate among 30-49 year group and sharply decrease above 50 years. This pattern indicates that transmission occurred in the previous 20 to 40 years. A different pattern has been observed in Japan, Italy, Turkey and China, where infection seems to be associated with over 50 years of age indicating that risk of acquiring infection was highest in the past 30 to 50 years (Yen *et al*, 2003; Alter, 2007).

Infection with HCV has been associated primarily with injection drug usage (IDU), and in the past transfusion of infected blood and blood products. Other routes of transmission include organ transplantation from infectious donors; medical, surgical or other interventions with contaminated needles and equipment; haemodialysis; occupational exposures to contaminated needles and equipments; and uncommonly mother-to-child transmission and sex with an infected partner. Among these factors, IDU, transfusion, and usage of contaminated needles or equipment are culpable for most infections worldwide. Appreciably high seroprevalence rates have been reported to occur in injecting drug users in USA (30%), Europe (44-80%) and Asia (36-75%) (Sy and Jamal, 2006; Alter, 2007). Screening of blood and blood products has helped to virtually eliminate transmission by this route in resource-rich countries and worldwide (Busch *et al*, 2005).

It is estimated that 4-5 million out of the 40 million persons infected with HIV are chronically infected with HCV (Alter *et al*, 2006). The number of persons with HIV/HCV co-infection differs for various geographical areas but it is generally influenced by the predominant effective route of HIV transmission in a subpopulation. HIV/HCV co-infection prevalence is high among populations where IDU is the predominant mode of HIV transmission. On the other hand, HCV prevalence is low among populations where sexual exposure is the major mode of HIV transmission. For example it is estimated that 72-95% of HIV positive injecting drug users are chronically infected with HCV, although HIV prevalence among this subpopulation is low. In addition, a HCV prevalence of up to 27% has been described among HIV positive MSM and heterosexuals (Sheppard *et al*, 2005; Alter, 2006). HCV prevalence among a cohort of HIV-positive haemophiliacs ranged between 92-100% (Francois, 2002; Shepard *et al*, 2005). In sub-Saharan Africa where HIV transmission is largely through sex and mother-to-child transfer, HCV occurs in about 3% of HIV infected patients (Ocama and Seremba, 2011).

Studies have shown that HIV/HCV co-infected patients have higher plasma HCV RNA levels, accelerated development of liver fibrosis, increased risk of cirrhosis development, high risk of hepatocellular carcinoma and increased mortality rate compared to HCV mono infected patients (Sherman, 2005; Operskalsky and Kovacs, 2011; Benhamou *et al*, 2001; van de Laar *et al*, 2010). Conversely, ART has been found to moderate progression of HCV-related liver disease hepatitis. HIV also appears to increase the risk of perinatal transmission of HCV (Gibb *et al*, 2000).

There are conflicting reports on the effect of HCV on the natural history of HIV. While some studies associate HCV infection with increased AIDS and mortality risk and decreased CD4 recovery on ART (d'Arminio-Monforte *et al*, 2009; Greub *et al*, 2000; Shepard *et al*, 2005), a publication from the USA found no difference in HIV disease progression and survival and immunological response to ART between HIV-infected and HIV/HCV co-infected patients (Sulkowski *et al*, 2002). The mechanism by which HCV impacts on HIV disease progression in co-infection is believed to involve increased T-cell activation and CD4 T –cell apoptosis leading to immunological dysfunction (Operskalski *et al*, 2011; Kaufmann *et al*, 2003).

1.6 METHODS OF HCV DETECTION

The laboratory methods used for the diagnosis of HCV infection can be placed into two main categories:

1. Serological assays to detect anti-HCV antibodies or HCV proteins in serum or plasma.
2. Molecular assays to detect, quantify and type HCV RNA.

1.6.1 Timeline and Significance of HCV Diagnostic Markers

After initial exposure, HCV RNA can be detected in blood within 1-3 weeks and is present at the onset of symptoms. Antibodies to HCV can be detected in only 50-70% of patients at the onset of symptoms, but in >90% after 3 months. Following acute infection, if HCV RNA is still present at 6 months after infection, the likelihood of spontaneous HCV clearance is extremely low, and the patient is diagnosed with chronic infection. In patients with chronic HCV infection, the HCV RNA load predicts responses to antiviral therapy and measures the success of treatment.

If a patient is HCV antibody positive but HCV RNA negative, the patient has cleared the HCV and does not have chronic HCV infection. In HIV-infected patients, the HCV antibody test result sometimes is falsely negative even in chronic infection; therefore, if HCV infection is suspected the HCV RNA is tested.

1.6.2 Serological Assays for Anti-HCV Antibody Detection

Enzyme Immunosorbent Assays (EIA)

Three generations of screening EIAs that detect antibodies against various HCV epitopes have been developed. First generation EIAs (EIA-1) were developed in the late 80s and targeted the c100-3 epitope of the HCV NS4 protein; the assays had limited sensitivity and specificity (Kuo *et al*, 1989, Gretch, 1997). Second-generation EIAs (EIA-2) were introduced in the early 90s; they contained multiple HCV antigens from the NS3 (c33c) and core l (c22-3) regions, resulting in improved sensitivity. This shortened the average 'window period' for seroconversion after transfusion as it detected HCV antibodies sooner than EIA-1, but specificity remained relatively low (Richter, 2002; Kolho, 1992). In the late 90s third generation EIAs (EIA-3) were introduced that contained reconfigured core, NS3 and NS4 antigens plus NS5 antigen; this further improved sensitivity of detection in early infection (Barrera, 1995; Kao *et al*, 1996). It should be noted however that there remains a window period of several weeks (around 6 weeks) between HCV infection and seroconversion, which may lead to negative antibody results with positive HCV antigen or RNA detection soon after infection (Cox *et al*, 2005; Stramer *et al*, 2004).

Supplemental tests

HCV EIA tests may represent false positives (Hoslina *et al*, 2012). A supplemental test is required to confirm seropositivity in patients who are HCV RNA negative. A second and third generation recombinant immunoblot assays (RIBA) were approved in 1993 and 1999 respectively by the FDA. The immunoblot is a strip immunoassay which includes the core antigen (c22p), NS3, NS4, without (in second generation) or with NS5 (in third generation) recombinant proteins plus human superoxide dismutase to detect nonspecific antibodies. Results could be negative, positive or indeterminate. With RIBA, about 95% EIA positive results are confirmed. However in low prevalence blood donors, confirmation is about 50% (Leon *et al*, 1998). RIBA indeterminate results can temporally occur in seroconversion stage (Ritcher, 2002).

1.6.3 Molecular Assays

Molecular assays are categorised into qualitative or quantitative assays and are used to detect or quantify the viral RNA. The most common tests use real-time reverse transcriptase PCR and several commercial and in house assays have been developed with variable lower limit of quantification. There are currently three tests commonly: real-time reverse transcription polymerase chain reaction (RT-PCR); branched-chain DNA (b-DNA); and transcription mediated amplification (TMA). bDNA test is less sensitive than RT-PCR and TMA, which can show a lower limit of quantification of 5-10 IU/mL.

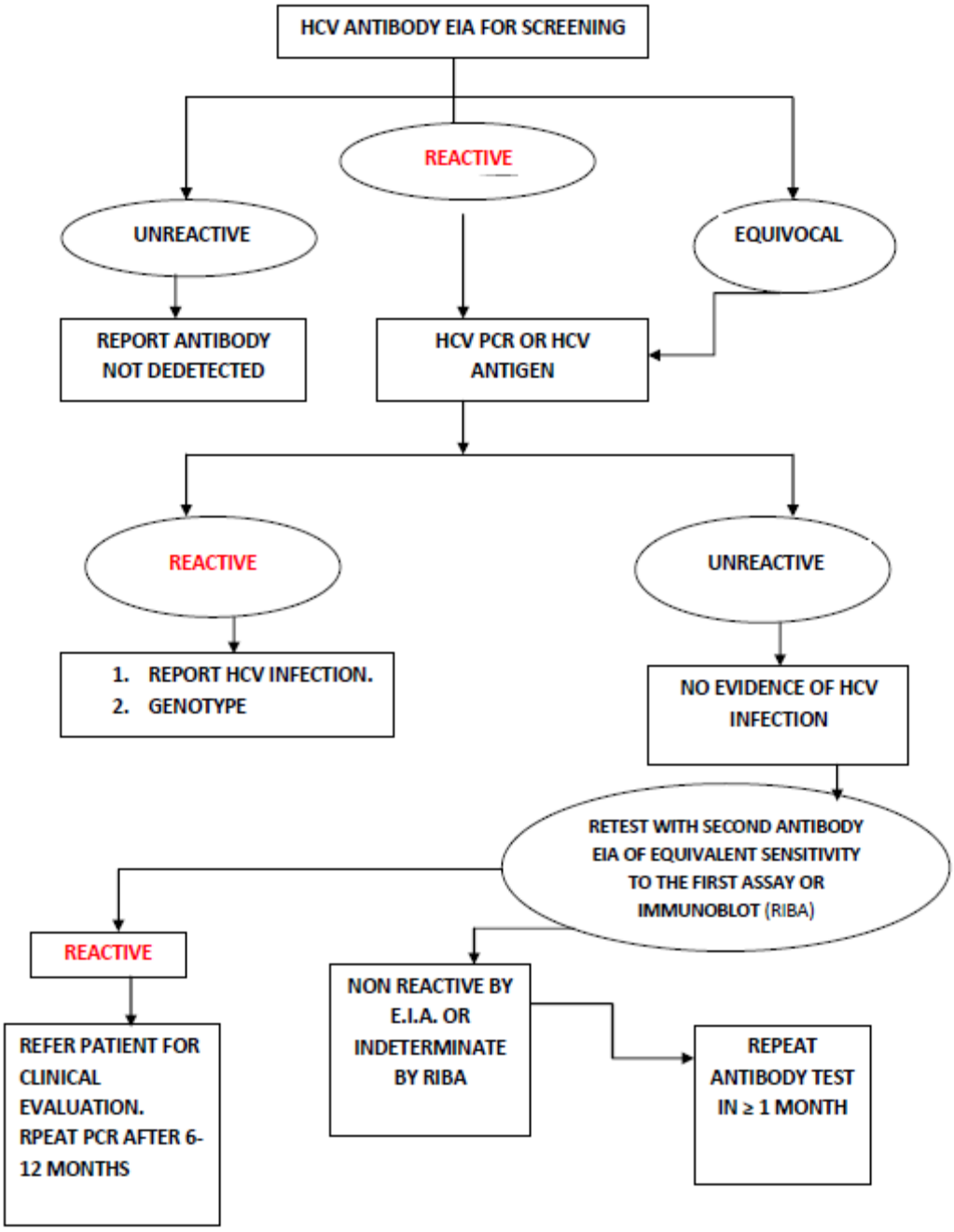
1.6.4 Genotyping

HCV is genetically heterogeneous with at least six known genotypes. Determination of genotypes is relevant in predicting the success of antiviral therapy (Schiff *et al*, 1999). Techniques for HCV genotype determination entail screening test that detects point mutations within HCV genome and confirmatory test that evaluate larger segments of HCV genes. Several HCV genotype screening assays have been described and include reverse hybridization line probe assay (LiPA, Innogenetics, Zwijnaarde, Belgium), restriction fragment length polymorphism (RFLP) of the PCR amplicons, and nested PCR with genotype-specific parameters to the core region. The optimal genotyping region is reported to be the 5' untranslated region (UR) because of high intra genotype conservation, but considerable variation between genotypes (Schiff *et al*, 1999). Gold standard confirmatory tests for HCV genotype determination include nucleotide sequencing and phylogenetic analysis of the E1 gene or NS5B gene.

1.6.5 Typical HCV Testing Algorithm

Figure1.4 shows the standard testing algorithm for HCV as recommended by the WHO, CDC and The UK Standards Unit, Microbiology Services Division.

Figure 1.4 Flow chart of HCV testing algorithm



1.7 HEPATITIS C CORE ANTIGEN AS A MARKER OF HCV REPLICATION

Though HCV antibody testing has been useful in diagnosing HCV infection and reducing viral transmission through transfusion, it is associated with lack of the ability to confirm viral infection during the pre-seroconversion window phase and in some immunocompromised patients. Molecular assays have contributed to the solution to this problem but results are obtained at a high cost and require considerable infrastructure and skill. HCV core antigen (HCVcAg) testing presents an alternative to nucleic acid testing for the purpose of early HCV detection (Zhang *et al*, 2007; Aoyagi *et al*, 1999; Icardi *et al*, 2001, Kobayashi *et al*, 1998), monitoring antiviral therapy and reoccurrence of HCV infection after liver transplant, and screening of dialysis patients, due to its strong correlation with HCV RNA levels (Tanaka *et al*, 1996; Park *et al*, 2010; Bouvier-Alias, 2002; El- Shaarawy *et al*, 2007; Kawai *et al*, 2002).

Early HCVcAg assays (Takahashi *et al*, 1992; Moriya *et al*, 1994) were found to be insufficiently sensitive. Subsequent assays used different antibodies (e.g., targeting amino acid residues 21-60), which improved sensitivity (Kashiwakuma *et al*, 1996). A first generation commercial HCVcAg assay was developed by Ortho Clinical Diagnostics, Raritan, NJ which utilized the 5F11 and 5E3 monoclonal antibodies for the capturing and detection of core antigen, respectively. The lack of its ability to detect antibody-bound antigen led to the production of a second generation assay referred to as Total HCV Core Antigen ELISA Test System or trak-C assay (Ortho Clinical Diagnostics). Improvement over the first generation was achieved by incorporating a sample pre-treatment procedure to dissociate core antigens form core antigen–anti-core antibodies complex; using two capture monoclonal antibodies (C11-3 and C11-7) and two horseradish peroxidise-conjugated monoclonal antibodies (C11-10 and C11-14) for the detection of the bound HCV core antigen.

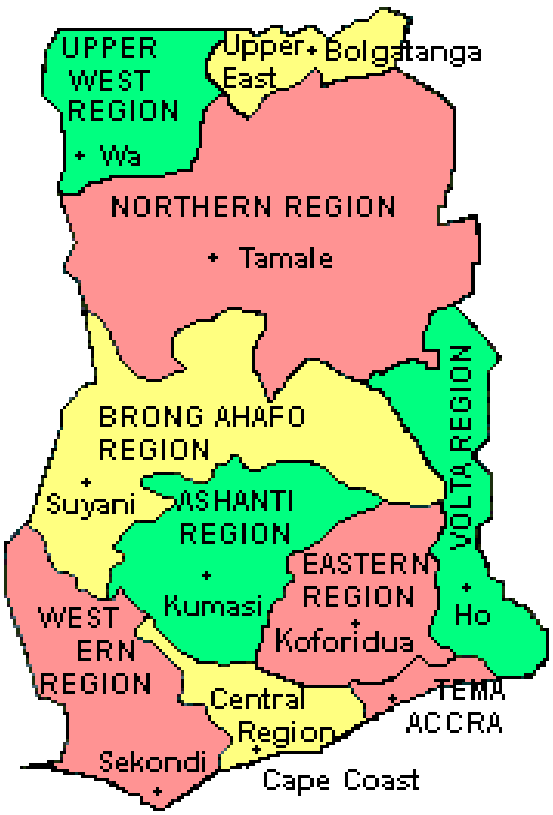
The sensitivity and specificity of various in-house and commercial HCV core antigen EIA assays have been evaluated by comparing results with NAT. Kawai *et al*, 2002 showed that the sensitivity of an in-house HCVcAg assay was lower than that of the Cobas Amplicor HCV Monitor 2.0; however, the HCVcAg levels correlated significantly with the HCV RNA levels. Other investigators (Icardi *et al*, 2001; Widell *et al*, 2002; Letowska *et al*, 2004; Gaudy *et al*, 2005; El-Shaarawy *et al*, 2007) obtained sensitivity of 95% to 99.3% and specificity of 94.2% to 99.9% with the Ortho Antibody to HCVcAg ELISA Test System. Other authors however (Reddy *et al*, 2006) described less favourable performance, with lower sensitivity and specificity (60% and 83% respectively) and a very low positive

predictive value (PPV, 14%) with the same HCV RNA and HCVcAg testing protocols. Among the factors which probably contributed to the difference in sensitivity and specificity may include difference in HCV genotypes. Assay sensitivity can be affected by sequence heterogeneity of the core protein (Saeed *et al*, 2009). Therefore the ability of the assay to detect HCV genotypes and subtypes relies on the anti-HCV core antigen capture or detection antibody used. Various studies however have reported that HCVcAg detection correlates with plasma HCV RNA levels irrespective of genotype (Kawai *et al*, 2002; Bouvier-Alias *et al*, 2002; El-Shaarawy *et al*; 2007, Park *et al*, 2010). Overall, it has been found that HCVcAg assays detects core antigen when HCV RNA levels are above about 20,000 IU/mL (Bouvier-Alias *et al*, 2002).

1.8 HIV AND HCV INFECTION IN GHANA

1.8.1 HIV Prevalence

Figure 1.5 A map showing the ten regions of Ghana.



Ghana has a generalised epidemic with a prevalence of 2.1% according to the 2011 sentinel survey report (Ghana AIDS commission, 2012**)**. It is estimated that 80% of infection in Ghana occur through sexual routes (Ghana AIDS commission, 2012).The most affected age group is 30-34 years of age with a prevalence of 2.9% in 2011. Prevalence shows geographical differences ranging from 0.3% in the Northern region to 4.7% in the Central region. The most urbanised areas – namely the Greater Accra Region and the Ashanti Region – had a prevalence of 3.2% and 3.1% respectively in 2011. This compared with prevalence below 0.4% in rural communities (Ghana AIDS commission, 2012).

1.8.2 Antiretroviral Therapy

ART was introduced in the country in 2003 and overall, a total of about 65 000 people living with HIV have received treatment since the onset of the programme and about 56 000 adults and 3000 children are still receiving therapy. As of 2011, close to 4 000 men and 10 500 women were on ART together representing about 60% of those who need ART (Ghana AIDS commission, 2012). There are currently over 150 treatment centres nationwide which comply with a national guidelines (National HIV/AIDS/ STI Control Programme - Ghana, 2010).

The current antiretroviral drugs recommended in Ghanaian national guidelines for HIV treatment are as follows:

1. NRTIs and NtRTIs: Zidovudine, Lamivudine, Abacavir, Emtricitabine
2. NNRTIs: Nevirapine, Efavirenz
3. Pis: Ritonavir boosted Lopinavir (LPV/r) ritonavir boosted Atazanavir (ATV/r).

The regimens indicated for treatment includes 2 NRTIs and 1 NNRTI; or 2 NRTIs and 1 ritonavir boosted PI. Table 1.2 shows the choice of drugs used as first and second line as recommended by the national guideline.

Table 1.2 Recommended antiviral combinations for HIV treatment in Ghana

FIRST-LINE	
First Choice	ZDV + 3TC + NVP or EFV
Second Choice	First option: TDF + (3TC or FTC) + NVP
SECOND LINE	
First Choice	TDF+ FTC or 3TC + (LPV/r or ATV/r)
Second Choice	ZDV+3TC+ (LPV/r or ATV/r)

1.8.3 Treatment Monitoring

Monitoring of ART in Ghana as indicated in the national guideline involves:

1. Clinical evaluation to be done at 2-3 months during follow-up to monitor adherence, drug toxicity, allergies, adverse effects, opportunistic infection and efficacy of therapy. Laboratory monitoring to be conducted every 6 months during follow-up to establish side effects and drug toxicity: includes haematological test (full blood count); urine routine examinations; biochemistry (fasting blood sugar, BUE/Creatinine, AST, ALT, and lipid profile); microbiology (sputum test for AFBs to rule out TB infection if symptoms of respiratory infection are observed); immunological assessment (CD4 T-cell count).

- Viral load testing is recommended before and at every six months of therapy. However, routine viral testing is not available at the majority of treatment centres due to lack of laboratory facilities and funds. Finally, tests for diagnosing viral hepatitis are only recommended for the purposes of ruling out liver damage attributable to ART.

1.8.4 HIV/HCV Co-Infection

Data on the prevalence of HCV among HIV positive patients in Ghana are limited. HCV seroprevalence of 3.6% and 17% (2/12) has been reported among ART-naive adults and women who visited the obstetrics and gynaecology unit, respectively at Korle-Bu Teaching hospital (KBTH) in Accra (Sagoe *et al*, 2012; Apea-Kubi *et al*, 2006). A definitive HIV/HCV co-infection prevalence rate in a Ghanaian cohort is yet to be published. No treatment algorithm is presently adopted for the treatment of HIV/HCV co-infection in Ghana. Therefore accessing treatment outcomes in Ghanaian HIV/HCV cohort will be necessary to provide basis for a review of treatment protocols for coinfected patients.

1.9 The Kumasi HIV Cohort

The Kumasi cohort consists of adult HIV positive patients who were attending the Komfo Anokye Teaching Hospital (KATH) HIV clinic for routine medical care. Kumasi is the capital city of the Ashanti Region and the second largest city of Ghana. Of the 4 million people in the Ashanti region (Ghana Statistical services, 2010), 3.1% are estimated to be living with HIV (Ghana AIDS commission, 2012) and the major treatment centre is KATH in Kumasi. ART was introduced at KATH in 2004 and over 7000 patients have since been enrolled of which over 3000 are currently receiving therapy. The adult clinic runs weekly seeing an average of 100 patients per day and between 700 and 1000 newly diagnosed patients annually.

Adult treatment and treatment monitoring at KATH HIV clinic are in compliance with the National HIV Treatment Guidelines (National HIV/AIDS/ STI Control Programme-Ghana, 2010). Treatment monitoring involves clinical evaluation and CD4+ cell count as a measure of treatment efficacy, and full blood count, creatinine and hepatic transaminases (AST/ALT) to detect treatment toxicity. Though recommended by the national guidelines, HIV viral load testing is not included in the routine monitoring of HIV patients in Kumasi.

Whereas HBV prevalence has been well characterised in Kumasi HIV patients (Geretti *et al*, 2010) data on the prevalence of HCV in co-infected patients is currently limited in Kumasi as in the rest of Ghana. Although diverse HCV seroprevalence rates ranging from 0.5% to 18.7% have been published among different Ghanaian populations (Sagoe *et al*, 2012, Apea-Kubi *et al*, 2006, Acquaye *et al*, 2000, Owusu-Ofori *et al*, 2005, Nkrumah *et al*, 2011), the rate of HCV co-infection among HIV positive patients in Kumasi is currently unknown. The diverse seroprevalence rates reported may be due to the various populations studied but potential differences in the specificity and sensitivity of the anti-HCV screening assays employed cannot be ruled out. Typically the available studies did not employ HCV RNA detection or RIBA for the confirmation of infection or antibody positivity.

As more patients benefit from the scale up of ART in Ghana, the implication is that life expectancy in patients with chronic viral hepatitis will be improved and the evaluation of the overall burden of co-infection becomes critical. Given the limited infrastructure, there is also a need to evaluate screening assays that can provide a specific diagnosis of HCV infection and guide appropriate treatment strategies for HIV/HCV co-infected patients in Ghana.

1.10 AIMS OF THE STUDY

The aim of this project was to develop serological and molecular skills that can be transferred to Kumasi, Ghana in order to improve the care of the local HIV-infected population. The serological and molecular skills were applied to identify HBV co-infection and determine ART outcomes in the Kumasi cohort, and to evaluate the best approach for diagnosing HCV co-infection in the same population.

1.11 OBJECTIVES

- Become proficient in HIV viral load testing.
- Become proficient in HCV serology testing.
- Describe the HIV virological response in the Kumasi cohort.
- Determine the performance of HCV antibody screening assays in relation to HCV PCR results.
- Develop an EIA to detect HCV core antigen as an alternative to HCV RNA testing in Ghana.

CHAPTER TWO – MATERIALS AND METHODS

2.1 STUDY SITE

Figure 2.1 The study area and site

Location of the study area


Komfo Anokye Teaching Hospital

The study site is the HIV clinic at Komfo Anokye Teaching Hospital (KATH) in Kumasi, the second largest city in Ghana located in the Ashanti Region, which is approximately 300 miles north of the equator. KATH is a thousand-bed teaching hospital which administratively consists of eleven directorates. The HIV clinic at KATH is under the Medicine Directorate and it is one of the major HIV treatment centres in Ghana in terms of numerical status of clients and infrastructure. After clinical evaluation and treatment monitoring at the clinic, patients are sent to the Diagnostics Directorate for laboratory services. Laboratory services available at KATH include HIV serology, CD4 cell count, biochemistry, microbiology, haematology, and radiology.

2.2 STUDY POPULATION

Consecutive adult HIV seropositive patients who attended the KATH HIV clinic for routine medical care between October 2010 and June 2012 and consented to take part in the 'HEPIK' study (HIV and viral hepatitis study in Kumasi) were prospectively recruited in this study. Demographic and clinical data were extracted from the clinical record files. Serum and plasma samples were collected and stored at -80˚C at KATH. In addition to undergoing local testing, frozen serum and plasma on dry ice and dry plasma spots (DPS) were shipped to the University of Liverpool (UoL) for additional investigations.

Ethical approval was obtained from the Committee on Human Research Publications and Ethics at the Kwame Nkrumah University of Science and Technology of Kumasi, Ghana.

2.3 SAMPLE COLLECTION AND STORAGE

2.3.1 Plasma and Serum Samples

Two trained phlebotomists at KATH took venous blood into serum and EDTA tubes labelled with study identification information and were taken to the 'HEPIK' laboratory for testing and storage. The samples were spun at 3000 rpm for 10 minutes to obtain the supernatant serum and plasma. The recovered serum and plasma were aliquoted into cryovials and stored at -80˚C. Four cryovials each were used to store the plasma and serum per patient to avoid freeze-thawing of a particular sample.

2.3.2 Dry Plasma Spots Preparation

Dry plasma spot (DPS) samples were prepared from stored plasma at KATH using Whatman 903 protein saver cards according to the following procedure:

1. Frozen plasma was thawed at room temperature for 30 minutes.
2. The working surface was decontaminated with 70% ethanol and Whatman cards were labelled with sample identification details.
3. The sample was vortexed for 6 seconds.
4. Using a micropipette with filter tips, 75µl of plasma was applied to the centre of each of 4 pre-printed circles on the card, making sure that cards from different patients do not come into contact with each other and tips were changed after producing each card. Care was also taken avoid touching the cards with pipette tips when dispensing the serum.
5. The cards were placed face up on dry, ant-free surfaces to dry at room temperature overnight.
6. The dry cards were closed such that the dry spots faced inside.
7. Each DPS was placed in a single dry plastic bag together with three desiccant sachets. The bag was squeezed to displace air and then sealed.
8. Sealed bags were stored in a dry box and transported to Liverpool within 1 week after preparation. At UoL the DPS were stored at -80˚C.

2.4 LABORATORY TESTS

The following assays were performed (location; person performing the experiments):

1. Hepatitis B surface antigen (HBsAg) testing (KATH; KAA)
2. Hepatitis C antibody testing (KATH and UoL; KAA).
3. HIV-1 RNA load testing (UoL, KAA)
4. HCV RNA testing (UoL, Simon King).

2.4.1 Hepatitis B Surface Antigen Testing

Samples were screened for HBV surface Antigen (HBsAg) using the Determine HBsAg lateral flow test (Inverness Medical, Japan) HBsAg. The Determine test was previously shown to have excellent specificity but relatively low sensitivity in the Kumasi HIV-infected population (Geretti *et al*, 2010). Therefore, samples that tested HBsAg negative by Determine were retested by the Murex HBsAg 3.0 EIA (Abbott Diagnostics Division, UK).

Determine assay

Determine HBsAg is an immunochromatographic test for qualitative detection of HBsAg. Freshly collected serum (50µl) is added to the sample pad on the test card. The sample mixes with the selenium colloid-antibody (mouse, monoclonal) conjugate as it migrates through the conjugate pad. The mixture then migrates through immobilised antibody at the patient window site. If present, HBsAg in the sample binds to the antibody-selenium colloid and to the antibody at the patient window site forming a red band at the patient window site. If absent the conjugate flows past the patient window and no red band is formed at the patient window site. A red line is formed at the control window site to validate assay. Results are read 15 minutes after serum application. Red bars appearing on both patients and control window sites were interpreted as positive whereas only one red bar at the control site was interpreted as negative and no bar at both sites was interpreted as invalid.

Murex HBsAg EIA

The sample is pre-incubated in coated microwells with a mixture of mouse monoclonal specific for different epitopes on the ‘a’ determinant of HBsAg. Affinity purified goat antibody to HBsAg conjugated to horseradish peroxidase is then added to the sample in the well. During the two incubation steps any HBsAg present in the sample is bound to the well in an antibody-antigen-antibody-enzyme complex. After washing, a solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide is added to the wells. Wells

which contain HBsAg and hence bound conjugate will develop a purple colour which is converted to orange when the enzyme reaction is terminated with sulphuric acid, and colour absorbance is read at 450nm.

The main steps in the assay were:

1. 25 µl of sample diluent added to each well and 75µl of sample were added to each well.
2. After changing the gloves, 2 negative controls (NC) and 1 positive control (PC) were added to the wells
3. The plate was incubated at 37˚C for 60 minutes
4. 50 µl of conjugate was added to each well and the plate was rocked gently for 10 seconds, followed by incubation at 37˚C for 30 minutes.
5. The plate was washed in 5 cycles to remove unbound components. 100 µl of freshly prepared substrate was added to each well, followed by incubation for 30 minutes at 37˚C.
6. The reaction was stopped with 50 µl of sulphuric acid and the absorbance of each well was read at 450nm wavelength.

Absorbance cut-off value (COV) was calculated as follows:

COV = Mean NC absorbance + 0.05

Samples with absorbance below and above 10% of the COV were recorded as non-reactive and reactive respectively, whereas absorbance within +/- 10% COV was recorded as equivocal. Equivocal samples were retested. The assay was accepted as valid if the NC absorbance was less than 0.2 and the PC absorbance was greater than 0.8.

2.4.2 Hepatitis C Antibody Testing

Serum samples were obtained from the Kumasi cohort and tested for HCV RNA by Simon King (PhD student in the group). Samples with known HCV RNA status – either positive or negative – were included in the HCV serology study. Four assays were employed which included two automated assays, the Abbott Architect anti-HCV (Abbott Diagnostics) and the Vitros Anti-HCV assay (Ortho Clinical Diagnostics), and two manual plate EIAs, the Monolisa HCV Ag-Ab ULTRA (BIO-RAD) and the ORTHO HCV 3.0 ELISA System with Enhanced SAVe (Ortho Clinical Diagnosis). The Architect and VITROS assays were performed in London by the diagnostic staff of the NHS virology laboratories of Charing Cross Hospital, and the Royal Free NHS Foundation Trust, respectively.

Architect anti-HCV assay

The assay is an automated two-step chemiluminescent microparticle immunoassay for the detection of serum or plasma anti-HCV antibodies. If antibody is present in the sample, it binds to a solid phase made of recombinant HCV antigen coated paramagnetic particles. After a wash step, a murine anti- human IgG/IgM acridinium-labelled conjugate is added. Following a further wash, hydrogen peroxide and sodium hydroxide are added. The resulting chemiluminescent reaction is measured in relative light units (RLU) which are directly proportional to the amount of anti-HCV present in the sample.

The assay cut-off is computed using the mean RLU of a triplicate of the manufacturer's calibrator which is included during the assay. The assay cut-off is calculated by adding 0.074 to the mean RLUs of the calibrator.

Results are calculated as signal/cut-off (S/C) value.

$$\text{Result (S/C)} = \frac{\text{Signal for test sample}}{\text{Cut-off value}}$$

Specimens signal to cut-off ratio (S/CO) values <1 are considered non-reactive whereas S/CO values ≥1 are considered reactive. Results of S/C ≥ 0.9 but <1.0 are classified as equivocal.

VITROS anti-HCV assay

The VITROS anti-HCV test is performed on the VITROS Eci/EciQ Immunodiagnostic System, the VITROS 3600 Immunodiagnostic System and the VITROS 5600 Integrated System using Intellicheck® Technology. The assay involves a two-step immunometric technique which involves the binding of HCV antibody to HCV recombinant antigen–coated wells at the first stage. Following a washing step which removes the unbound sample, a HRP-labelled murine monoclonal anti-human IgG conjugate binds to any human IgG bound to the wall. After removal of unbound conjugate by washing, a luminogenic substrate is added to the wells followed by the addition of an electron transfer agent. The HRP catalyses the oxidation of the luminal derivative and the intensity of the emitted light is increased and prolonged by the electron transfer agent. The light signal is read by the system which is a measure of the amount of HRP conjugate bound which in turn directly correlates with the concentration of the anti-HCV present in the sample. The instrument is calibrated prior to testing using a calibrator supplied by the manufacturer. During the calibration process, a lot-specific parameter is used to determine a valid stored cut-off value for the VITROS Immunodiagnostic System. Results are calculated as signal/cut-off (S/C) value.

$$\text{Result (S/C)} = \frac{\text{Signal for test sample}}{\text{Cut–off value}}$$

A result of <0.9 S/C is interpreted as non-reactive whereas a result of ≥ 1.00 S/C indicates reactive for anti-HCV. Results of S/C ≥ 0.9 but <1.0 are classified as equivocal.

Monolisa HCV Ag-Ab ULTRA

The assay involves initial addition of conjugate 1, which contains biotinilated monoclonal anti-HCV capsid, and samples/controls to the wells. The solid phase of is coated with monoclonal anti-capsid antibodies, 2 recombinant proteins from NS3 1 and 3a, one recombinant antigen from NS4 region and a mutated peptide from the capsid area of hepatitis C genome. If present, anti-HCV antibodies bind to antigens fixed on the solid phase and capsid antigens bind to the monoclonal antibodies coated on the microplate and the biotinilated capsid antibody. After incubation and washing peroxidase labelled antihuman IgG and Streptavidin-peroxidase conjugate are added. Streptavidin peroxidase conjugate reacts with biotinilated monoclonal antibodies against HCV capsid antigen if present. This reaction takes place at 37˚C for 30 minutes and a washing step removes all unbound

enzymatic conjugate and the antigen-antibody complex is detected by the addition of substrate (citric acid and sodium acetate solution plus TMB). The reaction is stopped with 1N sulphuric acid and absorbance read at 450nm. The assay was carried out with adherence to kit SOP as follows.

1. 100µl of conjugate 1 was added to the formatted microplate and 50 µl of samples were added to the wells starting from the sixth well and the reaction mixture was homogenised by 5 time aspiration with the micropipette.
2. With new gloves on, one negative control, 3 antibody positive and 1 antigen positive controls were sequentially added to the plate starting from the first well.
3. The microplate was covered with adhesive sealer and incubated for 90 minutes at 37˚C. The microplate was then washed in 6 cycles with working concentration of washing buffer.
4. 100 µl of conjugate 2 was dispensed into each of the wells and the plate was covered and incubated at 37˚C for 30 minutes. The plate was washed in 6 cycles with washing buffer.
5. 80 µl of freshly prepared TMB substrate was applied to each well. Then 100 µl of stop solution was added to the wells to stop the reaction.
6. The absorbance of the wells was then read at 450nm using Mutiscan Spectrum, Thermo Electron Corporation, Thermo fisher, UK (SR# 1588-522) within 30 minutes of stopping the reaction.

The optical densities of the three positive antibody controls (PabC) were used to calculate COV by the following equation.

$$\text{COV} = \left[\frac{Total\ of\ PAbC}{3}\right] \div 4$$

The following assay validation criteria were used based on the assay protocol.

1. Negative control absorbance less than 0.6 x COV
2. 0.8 <mean absorbance of PAc ≥ 2.400
3. Absorbance of antigen positive control greater than 0.5

2.4.3 Analysis

True positivity was defined as a sample reactive by the EIA and HCV-RNA positive or HCV-RNA negative but positive by Architect whereas true negative sample was defined as negative by the EIA and negative by PCR or Architect. HCV-RNA and Architect results were used as reference because HCV-RNA testing is the gold standard for HCV diagnosis and Architect has been validated to have highly sensitive and specific in the UK (HPA-MiDAS & NBS-NTMRL, 2008). Sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of each the Vitros, Monolisa and Ortho were calculated using the following formulae:

$$\text{Sensitivity} = \frac{\text{Number of True positive}}{\text{Number ofTrue positive+Number of False negative}}$$

$$\text{Specificity} = \frac{\text{Number of True Negative}}{\text{Number of True Negative+Number of False positive}}$$

$$\text{NPV} = \frac{\text{Number of true negatives}}{\text{Number of true negative+number of false negatives}}$$

$$\text{PPV} = \frac{\text{Number of true positives}}{\text{Number of true positive+number of false positives}}$$

Samples with absorbance less than 10% of COV were negative whereas samples with absorbance greater than 10% COV were considered positive. Signal to cut-off ratio (S/CO) for each sample was calculated by dividing sample absorbance by the COV.

2.4.4 Real-Time PCR for Serum/DPS HCV-RNA Determination

The process involved off-board lysis of HCV in serum and DPS and internal control (Phocine Distemper Virus, PDV), automated extraction of HCV-RNA using NucliSENS® easyMAG automated Extractor (Biomerieux SA, France) and Real-Time PCR determination of HCV-RNA load using ABI Prism 7500 Real Time PCR System.

2.4.5 Plasma HIV-1 RNA Load Testing

The process involved the following steps:

1. Automated HIV-1 RNA extraction and master mix preparation using Abbott m24sp™ System (Abbott Molecular Inc. USA) and Abbott m2000sp™ System (Abbott Molecular Inc. USA).
2. Real-Time PCR on Abbott *m*2000rt Instrument System (Abbott Molecular Inc., USA).

HIV-1 RNA extraction

System specific reagents used were:

1. Abbott *m*™ Sample preparation System, which comprises:
   a. Lysis buffer (100mM Tris solution containing Guanidine thiocyanate and 10% Tween)
   b. Wash buffer 1 (50mM Acetate solution containing Guanidine thiocyanate and detergent)
   c. Wash 2 (Nuclease free water)
   d. Elution buffer (20mM Phosphate buffer solution with preservative)
   e. Microparticles (1.5% Maghemite (g-Fe_2O_3) particle in Lysis buffer)
2. Internal control (IC), controls (negative, low positive and high positive) and calibrators

Lysis buffer containing the GITC lyses viral particles by denaturing the capsid and inactivating RNAses whereas the Tween detergent destroys the membrane proteins. The negatively charged viral RNAs are released which are captured by positively charged magnetic particles under a basic and high salt concentrated medium. The magnetic particle-RNA complex are captured in a magnetic field and are washed efficiently using wash buffer 1 and 2 to remove cell debris and proteins. The elution buffer containing negatively charge phosphate competitively removes RNA from the Fe^{2+} magnetic particles.

Sample preparation

1. Plasma sample, IC, calibrators and controls were removed from freezer and allowed to thaw to room temperature.
2. Instrument daily maintenance was performed which included nucleic acid decontamination and disinfection of instrument platforms, and extensive flush to remove trapped air bubbles in fluidic system.
3. Sample tubes were labelled with respective sample ID.
4. The samples and controls were vortexed for 6 seconds to homogenise.
5. 1 mL of the plasma was aliquoted into the sample tubes and centrifuged for 5 minutes at 3000rpm.
6. The samples and controls were appropriately arranged in sample racks according to the instrument protocol.
7. Reagents were loaded onto the machine in appropriate positions.
8. Samples and control positions were appropriately assigned and machine was started to run extraction.

Master Mix Preparation

System specific reagents used were Abbott Real-Time Amplification reagents comprised:

a. A thermostable rTth DNA polymerase enzyme (rTth polymerase)
b. Activator reagent which contains 30mM manganese chloride solution
c. Oligonucleotide reagent which contains 4 primers, 2 probes, 1 quencher oligonucleotide and 4dNTPs in a buffered solution, with a reference dye.

Master mix was manually made up for the Abbott m24sp by putting together per sample 11.29µl, 39.54 µl and 5.88 µl of activator reagent, oligonucleotide reagent and rTth polymerase respectively. Master mix preparation was an automated process on the Abbott m2000rt. At the end of the process 50µl of RNA extract was added to 50 µl master mix in a 96-well optical plate. The plate was sealed with optical plate adhesive sealers and transferred to the Abbott m2000rt instrument for viral load testing by Real-time PCR. Abbott m24sp and m2000sp instruments used the Evoware and m2000sp v5.0.186.0 software respectively to run extraction and master mix preparation. New instrument calibration was run each time there was a change in lot numbers of sample preparation and amplification reagents

2.5 HEPATITIS C CORE ANTIGEN ELISA DEVELOPMENT

2.5.1 Reagents

Reagents used for the HCV core antigen ELISA development are indicated in Table 2.1. In order to develop a HCV Ag EIA, a literature search was performed and the format as indicated by Kawai *et al* (2002) and Aoyagi *et al* (1999) were initially considered. The format was that of a direct sandwich EIA, comprising a monoclonal capture antibody bound to the plate and a labelled monoclonal secondary antibody for detection. An indirect sandwich method was explored for its effects on the sensitivity and specificity of the assay. The assay format comprised: i) two murine monoclonal antibodies targeting HCV core amino acid (aa) residues 21-40 and 70-90 were used for capturing HCV core Ag, ii) a rabbit polyclonal anti-HCV core antibody targeting aa 9-21 as primary detector antibody, and iii) anti-rabbit IgG conjugated with horseradish peroxidase (HRP) as secondary detector antibody. TMB was used as enzyme substrate.

Table 2.1 Reagents used for the HCV core antigen EIA development

Capture Antibodies	Murine monoclonal antibodies to HCV core antigen; target epitopes: aa21-40 (Thermo Scientific). This is a21kDal protein well conserved among different HCV genotypes Clone: C7-50 (Bukh *et al*, 1994).
Detector Primary Antibody	Rabbit polyclonal antibodies to HCV core antigen; target epitopes: aa 9-21 (Antibodiesonline.com).
Detection Conjugate	Stabilised Goat anti-rabbit IgG (Heavy and Light Chain) Antibody- HRP (Thermo Scientific)
Positive Control	Hepatitis C core antigen protein; recombinant fragment corresponding to amino acids 2-119 of hepatitis C.
Substrate	1 step Ultra TMB – ELISA (Thermo Scientific)
Washing Buffer	PBS /0.05% Tween 20
Stop Solution	1N H_2SO_4
Blocking Buffer	BSA/PBS
ELISA Plate	96-well flat bottom ELISA plates (Thermo Scientific)

2.5.2 Antibodies Selection

Antibodies were selected based on:

1. Antibodies used in the HCV core Ag EIA by Kawai *et al* and Aoyagi *et al*.
2. HCV core amino acid sequences well conserved among known HCV genotypes.
3. Commercial availability of antibodies.

Kawai *et al* used antibodies that recognised HCV core aa 21-40 for capture and 41-60 for detection, whereas Aoyagi *et al.* Used antibodies targeting HCV core aa 100-120 and 120-140 for capture and 21-40 and 41 to 50 for detection. Among these antibodies, the monoclonal antibody targeting aa 21-40 was found to be commercially available. Conserved HCV core aa sequences for all HCV genotypes were obtained from the Los Alamos HCV Sequence Database (Table 2.2).

Table 2.2 Conserved HCV core amino acid sequences for all HCV genotypes on Los Alamos

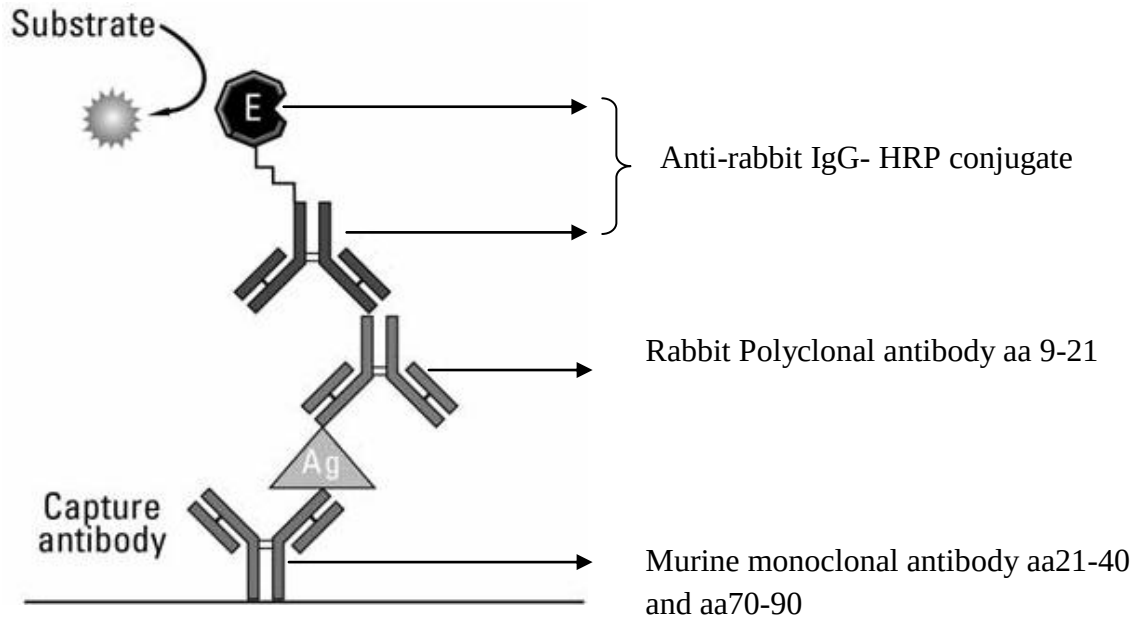
Epitope	**Sequence**
9-21	R- TKRNT-RRP DV
21-48	DVKFPGGGQIVGGVY LPRR-GPRLGVRA
50-67	RKTSERSQPRGRRQPIPK
80-90	RKTSERSQPRGRRQPIPK
92-105	GWAGWLLSPRGSRP
115-129	RSRNLGKVIDTLTCG
165-181	ATGNL-PGCSFSIFLLAL

Within the 21 to 48 conserved region, the monoclonal antibody targeting aa 21-40 was available commercially. However, an amino acid substitution was identified at position 36 in published HCV genotypes 3 sequences. The leucine to valine substitution could have potentially led to diagnostic escape for HCV genotype 3 (Saeed *et al*, 2009) if only anti-HCV core antibody aa 21-40 was used for capture. Therefore I first identified conserved HCV core aa sequences for HCV genotype 3. These were:

1. aa 77-99 (WAQPGYPWPLYGNEGCGWAGWLL);
2. aa111-140 (NDPRRRSRNLGKVIDTLTCGFADLMGYIPL).

Among these, the closest antibody found commercially was a monoclonal antibody targeting HCV core aa 70- 90.

Figure 2.2 Diagrammatic representation of the indirect sandwich ELISA format used for the HCV core antigen detection



2.5.3 Microplate Coating

1. Capture antibodies were removed from freezer/fridge and allowed to thaw to room temperature. Equimolar concentration of the two capture antibodies (monoclonal HCV core antibodies aa21-40 and aa70-90) was prepared by diluting the same volume of each capture antibody in PBS (pH=7.4) to the desired concentration (refer to table 2.7).
2. 100 µl of the mixture was pipette into the microplate wells and covered with adhesive plate sealer and the plate was incubated overnight (18 hours) at 4˚C
3. The plate was then washed with PBS/0.05% Tween in 6 cycles.
4. The wells were blocked for 2hrs at room temperature with 250 µl of 1% BSA/PBS.
5. The blocking buffer was then discarded and the plate was blotted on tissue to remove excess blocking buffer.

2.5.4 ELISA Optimisation

Table 2.3 shows concentrations of the various capture and detection antibodies that were used during the assay optimisation. A checkerboard system was used to as a guide for optimisation of reagents used (Table 2.4).

Table 2.3 Reagent concentrations used for the HCV optimisation

Reagent	**Concentration**
Both capture antibodies	1 µg/ml, 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml,10µg/ml Prepared from 1mg/ml stock
Second primary antibody	1:250, 1:500, 1:1000, 1:1500 Prepared from 1mg/ml of stock
Anti-rabbit IgG-HRP conjugate	1:60, 1:120, 1:250, 1: 500, 1:1000, 1:2000, 1:5000, 1:10000 Prepared from 1mg/ml stock
Positive control	0.2µg/ml Prepared from 1mg/ml stock

Table 2.4 Checker board used for HCV core antigen EIA optimisation. The yellow and blue shaded areas indicated two different concentrations of anti-rabbit HRP conjugate.

	1	2	3	4	5	6	
	Various Capture Antibodies concentrations pipetted into each column						
	← Equal concentrations of second primary antibody (aa9-21) into all wells →						
A	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	
B	Negative Control	Negative Control	Negative Control	Negative Control	Negative Control	Negative Control	
C	Positive Control	Positive Control	Positive Control	Positive Control	Positive Control	Positive Control	
D	Serum Sample	Serum Sample	Serum Sample	Serum Sample	Serum Sample	Serum Sample	Various concentrations of Anti-rabbit-HRP conjugate pipette into each row
E	Serum Sample	Serum Sample	Serum Sample	Serum Sample	Serum Sample	Serum Sample	
F	Serum Sample	Serum Sample	Serum Sample	Serum Sample	Serum Sample	Serum Sample	

Verification of Antigen-Antibody interaction

An experiment was performed to verify whether various antigens are recognising their targets. This involved:

1. Antigen-capture antibody interaction: Microplate was coated with HCV core protein (positive control) and incubated with each of the capture antibodies and then both. The complex was then detected with anti-mouse IgG-HRP conjugate.
2. Antigen-second primary antibody interaction: Microplate was coated with HCV core protein (positive control) and incubated with the second antibody (aa 9-21) and the complex was detected with anti-rabbit HRP.

First and second primary antibody interaction: Microplate was coated with second primary antibody (rabbit polyclonal antibody aa 9-21) and then incubated with first primary antibodies. Detection was performed with anti-mouse IgG-HRP

Sample Preparation – Antigen Retrieval (Kawai et al, 2002)

Reagents used:

a. 24% polyethylene glycol 4000
b. 20mM sodium citrate in 5% NaCl.
c. 10M Urea in 0.5M NaOH
d. 0.5M NaH_2PO_4 in 5% Triton-X 100

Procedure:

1. To 200 µl of serum 40µl of 24% polyethylene glycol 4000 in was added in a 1.5mL Eppendorf tube.
2. The mixture was then incubated for 1 hour at 4^oC.
3. Centrifugation at 4000g for 15 minutes.
4. The supernatant was decanted and the precipitate formed at the bottom of the tube was dissolved with 25 µl of 20mM sodium citrate in 0.5% NaCl.
5. 50 µl of 10 M urea in 0.5 M NaOH was added to the solution and incubated at 37 ^oC for 30 min.
6. 50 µl of 0.5 M NaH_2PO4 in 5% Triton X-100 was then added to the sample.

CHAPTER THREE – RESULTS

3.1 ART IN THE KUMASI HIV INFECTED COHORT

Within the study period, 1780 HIV-positive patients attending KATH for routine care were screened for HBsAg by the Determine and Murex assays, of which 254 were HBV infected. Overall 247 patients representing 97.2% of identified HIV/HBV co-infected subjects were recruited in the HEPIK study. Their characteristics are summarised in (Table 3.1).

Table 3.1 Characteristics of the HIV/HBV co-infected cohort at study entry

Characteristic	Result
Total number	247
Age, median years (IQR)	40 (34-46)
Gender, female n (%)	165 (66.8)
Duration of HIV diagnosis, median years (IQR)	3 (1-5)
CD4 count, median cells/µL (IQR)	478 (298-659)
ART status, experienced n (%)	202 (81.8)
Number of drugs received, median (IQR)	4 (3-4)
Duration of ART, median years (IQR)	1 (1-3)
ART at study entry (among ART-experienced patients), n (%)	
2NRTIs + EFV	116 (57.4)
2 NRTIs + NVP	74 (36.6)
2 NRTIs + LPV/r	5 (2.5)
Other[a]	5 (2.5)
Undergoing ART interruption	2 (1.0)

[a]includes 2NRTIs+NFV and 1NRTI+NFV+LPV/r IQR = interquartile range; ART = antiretroviral therapy; NRTIs = nucleos(t)ide reverse transcriptase inhibitors; EFV = efavirenz; NVP = nevirapine; LPV/r = ritonavir-boosted lopinavir; NFV = nelfinavir.

At the time of recruitment, 202 patients (133 females and 69 males) were ART-experienced, having received a median of four (range: 3-8) antiretroviral drugs for a median of 67 weeks (IQR: 42-147 weeks). All the 202 patients were NRTI-experienced, 201 (99.5%) were

NNRTI-experienced and 10 (5%) were PI-experienced. Howeever two patients were undergoing ART interruption at study entry. The ARVs received at study entry are summarised in Table 3.2.

Table 3.2 Antiretroviral drugs received at study entry among 200 patients

Class	Drug	n	%
NRTI	3TC	194	97
	ZDV	148	74
	d4T	33	17
	TDF	12	6
NNRTI	ABC	6	3
	DDI	5	3
	NVP	74	37
PI	EFV	116	58
	NFV	5	3
	LPV/r	5	3

At study entry the majority of patients were receiving the standard first-line ART regimen in use at KATH, consisting of two NRTIs plus one NNRTI. Only a small number of patients were receiving PI-based ART. The most common ART regimen at study entry was ZDV, 3TC plus either EFV or NVP. Several patients were receiving d4T although this is no longer part of national treatment guidelines.

3.2 TREATMENT CHANGES PRIOR TO STUDY ENTRY

Prior to study entry, 102/202 (50.5%) of ART-experienced patients had undergone changes in the composition of the ART regimen at least once (range: 1-3). The most common involved NRTI to NRTI substitutions (n=93; 91.2%) including d4T to ZDV (n=73; 78.5%), ZDV to d4T (n=12; 12.9%) and ZDV to TDF (n=7, 7.5%); 16 patients underwent NNRTI to NNRTI substitutions including NVP to EFV (n=11; 64.7%) or EFV to NVP (n=7; 41.2%). The reasons for these changes were not systematically recorded, and are believed to reflect toxicity, variable drug supply and pregnancy. Only nine patients had changed from an

NNRTI- to a PI-based regimen as a result of treatment failure, defined as either a clinical event or a poor CD4 T-cell count response to ART.

3.3 VIROLOGICAL RESPONSES TO ART

In the overall 247 HIV/HBV co-infected cohort, plasma HIV-1 RNA levels were obtained at study entry in 227 (91.2%) patients, of which 183 (80.6%) were ART-experienced and 44 (19.4%) were ART naive. Among the 183 treated patients, plasma HIV-1 RNA levels were undetectable (<40 copies/mL) in 111 (60.6%) whereas 72 (39.3%) had detectable HIV-1 RNA with a median load of 1061 copies/mL (IQR: 68-21476 copies/mL).

3.3.1 Virological Responses in the Population on ART for at Least 24 Weeks

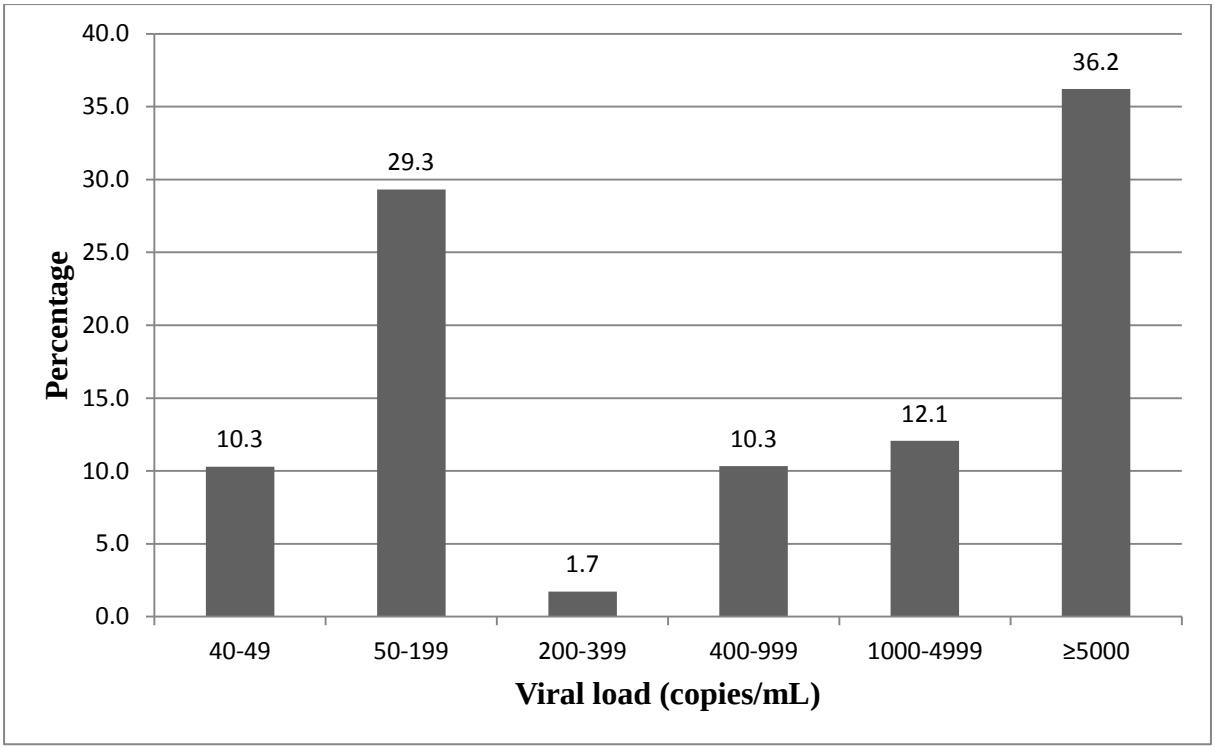
As treatment guidelines recommend that plasma viral load should be undetectable within 6 months of starting ART, virological responses were analysed among 155 patients who had been on ART for at least 24 weeks at study entry. Overall, 58 (37.4%) patients showed a viral load >40 copies/mL (Table 3.3). The median level was 826 copies/mL (IQR: 65 – 26752). CD4 T-cell counts were lower in patients with viral load >40 copies/mL compared with patients showing a viral load <40 copies/mL (P= 0.002, Mann Whitney U test). Overall the proportions with viral load <40 copies/mL were 59 (39.8%) and 38 (66.7%) among women and men respectively, and 56 (65.1%), 36 (60%) and 5 (55.6%) among patients receiving EFV, NVP and a PI respectively. The viral load levels observed in the 58 patients with >40 copies/mL are shown in Figure 3.4.

Of 189 patients on NNRTI-based regimens at study entry, 146 had received ART for at least 24 weeks prior to study entry. Plasma HIV-1 RNA levels were <40 copies/mL in 92 (63%) patients and >40 copies/mL in 54 (37%). Among the 10 patients who were on PI-based regimens, five had HIV-1 RNA >40 copies/mL whereas five had a viral load <40 copies/mL respectively. The proportions of patients who showed virological failure are as shown in figure 3.1

Table 3.3 Characteristics of patients who had received ART for at least 24 weeks with HIV-1 RNA levels above or below 40 copes/mL

	HIV-1 RNA copies/mL	
	<40	>40
Number (%)	97 (62.6)	58 (37.4)
Age, median years (IQR)	41 (36-48)	40 (37-47)
Gender, female n (%)	59 (60.8)	39 (39.8)
Duration of HIV diagnosis, median years (IQR)	4 (3-6)	3 (2-5)
CD4 count, median cells/µL (IQR)	528 (398-727)	421 (230-637)
Number of ARVs received, median (IQR)	4 (3-4)	4 (3-4)
Duration of ART, median years (IQR)	4 (3-5)	3 (2-5)
ARVs at study entry, n (%)		
EFV	56 (57.7)	30 (51.7)
NVP	36 (37.1)	24 (41.4)
PI	5 (5.2)	4 (6.9)
One ARV change prior to study entry, n (%)	33 (34.2)	28 (48.3)
More than one ARV change prior to study entry, n (%)	16 (16.5)	3 (5.2)

Figure 3.1 Percentage of patients (n=58) meeting different thresholds for defining virological failure after at least 24 weeks of ART.



3.4 Anti-HCV Serology Results

A total of 34, 24, 78 and 71 serum samples of known HCV RNA status were tested for the presence of anti-HCV antibody by two automated assays – the Architect and the Vitros, and two manual EIAs, the Ortho and Monolisa. Testing was based upon sample availability. Four HCV RNA positive samples were tested by three assays; the Vitros assay included only two of the four HCV-RNA positive samples (Table 3.7). Twenty-three samples were tested by all the four assays. The HCV-RNA positive samples were reactive by all assays. In addition, three HCV-RNA negative samples were reactive by Architect. These three samples were also reactive by the other tests, yielding a total of seven samples that were scored as "True positive".

Table 3.4 Anti- HCV detection by four serology assays

	Tested			Assay results		
	HCV RNA^+ n (%)	HCV RNA^- n (%)	Total n	Reactive n (%)	Non-reactive n (%)	Equivocal n (%)
ARCHITECT	4 (12)	30 (88)	34	7 (21)	27 (79)	0
VITROS	2 (8)	22 (92)	24	8 (33)	15 (63)	1 (4)
ORTHO	4 (5)	74 (95)	78	9 (12)	69 (89)	0
MONOLISA	4 (6)	67 (94)	71	21 (30)	47 (66.2)	3 (4)

The mean (range) signal/cut-off ratios (S/CO) obtained with reactive, non-reactive and equivocal sample by each assay are shown in table 3.5

Table 3.5 Mean signal/cut-off ratios obtained with each anti-HCV assay

	Mean S/CO (range)		
	Positive	Negative	Equivocal
Archictect	8.80 (1.01-12.75)	0.16 (0.07-0.43)	None
Vitros	12.62 (1.65- 29.60)	0.49 (0.19- 0.84)	0.91*****
Monolisa	3.77 (1.21-6.62)	0.45 (0.22- 0.84)	1.02 (0.94-1.09)
Ortho	3.97 (1.05-5.29)	0.12 (-0.01-0.68)	none

***** One sample

3.4.1 Assay Performance

The sensitivity, specificities, NPVs and PPVs were calculated using the number of true positive and negative samples as defined in chapter 2 (page 30). The performance of each assay based on the results for the HCV-RNA tested samples which were tested by the assay and Architect was analysed. A further analysis based on the 23 samples that were tested by all four assays was done. In a resource-limited setting a more specific rather than sensitive screening assays which have the potential to minimise the number of samples that require confirmatory test will be the cost-effective assay. Therefore in order to restrict the specificities of the assays the equivocal results with Vitros and Monolisa were classed with the reactive results.

Table 3.6 Assay performance with respective number of tested samples

Assay (n)*	True +ve	False +ve	True -ve	False -ve	Sensitivity (95% CI)	Specificity (95% CI)	NPV (95%CI)	PPV (95% CI)
Monolisa (31)	7	16	8	0	100 (59.0-100)	33.3 (15.6-55.3)	100 (62.8-100)	30.4 % (13.2 -52.9)
Ortho (31)	6	1	23	1	85.7 (42.1-99.6)	95.8 (78.9-99.9)	95.8 (78.9-99.9)	85.7 (42.1-99.6)
Vitros (23)	5	3	16	0	100 (47.8-100)	84.2 (60.4-96.6)	100 (79.4-100)	62.5 (24.5-91.5)

*Indicates the number tested by the EIA and Architect plus HCV PCR.

The Vitros assay showed a sensitivity of 100% whereas the sensitivity for the manual assays Monolisa and Ortho were 100% and 85.7% respectively. This reflected the fact that Ortho showed no reactivity with a HCV- RNA sample that gave reactivity by the Architect and two other assays. Specificity was highest with Ortho (95.8%), followed by Vitros (84.2%), and was lowest by Monolisa (33.3%). An analysis of the assay performance based on 23 samples tested by all four assays showed a slight reduction in sensitivity and PPV with Ortho whereas sensitivity with Monolisa and Vitros remained at 100%. Specificities and NPVs of Monolisa, Ortho and Vitros were marginally reduced as indicated in table 3.7.

Table 3.7 Assay performance with same samples tested by all four assays.

Assay (n)	True +ve	False +ve	True -ve	False -ve	Sensitivity (95% CI)	Specificity (95% CI)	NPV (95%CI)	PPV (95% CI)
Monolisa	5	14	4	0	100 (48.7-100)	22.2 (6.4-47.6)	100 (39.8-100)	26.3 (9.2-51.2)
Ortho	4	1	17	1	80.0 (28.4-99.5)	94.4 (72.7-99.9)	94.4 (72.7-99.9)	80.0 (36.0-98.0)
Vitros	5	3	15	0	100 (47.8-100)	83.3 (58.8-96.4)	100 (78.2-100)	62.5 (24.5-91.5)

The lowest (S/CO) that corresponded to a true positive result was 1.65 with Vitros, 1.52 with Monolisa and 1.15 with Ortho. The S/CO ratio obtained with true positive samples by Monolisa was significantly higher than that obtained with false positive results. The mean S/CO obtained with true positives, false positives, and true negatives are as shown in table 3.8

Table 3.8: Mean (range) signal/cut-off ratios according to the definition of true and false positive and negative.

	Mean S/CO (range)			
	True positives	False positive	True negatives	False negative
Vitros	15.2 (1.65-29.60)	4.38 (0.91-10.43)	0.49 (0.19-0.84)	—
Monolisa	5.06 (1.52-6.45)	2.2 (0.94-5.99)	4.35 (1.15-5.29)	0.29*****
Ortho	4.35 (1.15-5.29)	1.49*****	0.17 (-01-0.7)	—

***** One sample

The mean S/CO ratios obtained with true positive results was higher than that obtained with false positive results by Monolisa (P=0.0006, t test). However, 10/16 (65%) of false positive samples showed an S/CO ratio within the range obtained with true positive S/CO ratios. Similar observations were made with Ortho, as the S/CO of the only false positive sample was within the range of values obtained with true positive samples. Also, there was no significant difference between the mean S/CO ratios obtained with true positive and false positive results by Vitros.

3.4.2 Comparison between Monolisa and Ortho

Given the lack of automated platforms in Kumasi, we had interest in evaluating manual EIAs for their performance. Therefore the performance of Monolisa and Ortho was assessed. Of the 71 and 78 samples tested by Monolisa and Ortho respectively, the rate of HCV seropositivity was 29.6% (24/71) by Monolisa and 11.5% (9/78) by Ortho. The median S/CO ratio for seropositive samples was 3.05 (range, 1.21-6.62) for Monolisa and 4.89 (range, 1.05-5.29). The median S/CO ratio with antibody negative results were 0.38 (range, 0.22-0.84) for Monolisa and 0.05 (range, -0.01-0.68). Three samples were identified by Monolisa as equivocal with average S/CO of 1.02 whereas there was no equivocal result by Ortho.

Monolisa was found to be more sensitive for HCV antibody detection in the Kumasi HIV cohort compared to Ortho (100% vs. 85.7%) whereas specificity of Ortho was higher than Monolisa (95.8% vs. 33.3%). There was a higher rate of false positivity by Monolisa compared to Ortho (51.6% vs. 2.8%) resulting in a lower specificity by Monolisa than Ortho (33.3% vs. 95.8%). Monolisa was found to have a negative predictive value higher than Ortho (100% vs. 95.8%) but had a lower positive predictive value compared to Ortho (30.4% vs. 85.7%). The median S/CO ratio of the true HCV seropositive samples was 5.92 (range: 1.52-6.45) for Monolisa and 4.97 (range: 1.15-5.29) for Ortho. These results indicated that the Ortho assay is better suited for use in Kumasi, as it would result in fewer samples requiring unnecessary additional investigations due to false positivity.

3.4.3 Assay performance in relation to HCV RNA results

The results of the HCV molecular epidemiological studies performed by Simon King in the Kumasi cohort showed a HCV RNA prevalence of 0.64% (8/1249). Four of the positive samples were available for antibody testing by the Architect, Monolisa and Ortho and two had sufficient volume to also allow testing by Vitros.

The mean S/CO ratios with for HCV RNA detection by the four assays were as shown in table 3.9.

Table 3.9: Mean (range) signal/cut-off ratios according to HCV RNA detection

	Mean S/CO ratio (range)	
	HCV RNA Positive	HCV RNA Negatives
Architect	11.03 (7.24-12.75)	0.5 (0.07-5.15)
Vitros	26.65(23.70-29.60)	2.36 (0.19-19.3)
Monolisa	6.28 (5.92-6.45)	1.02 (0.22-5.99)
Ortho	5.10 (4.87-5.29)	0.26 (-0.01-4.53)

Possible assay cut-offs that are likely assay to be indicative of HCV RNA positivity were obtained by using the lower S/CO value obtained by each assay with HCV RNA positive samples, minus 25%. The values obtained were Architect, 5.43, Vitros, 17.77, Monolisa, 4.44, and Ortho, 3.65. The results with Vitros may have been different if all the RNA positive samples were tested.

3.5 HEPATITIS C CORE ANTIGEN ELISA DEVELOPMENT

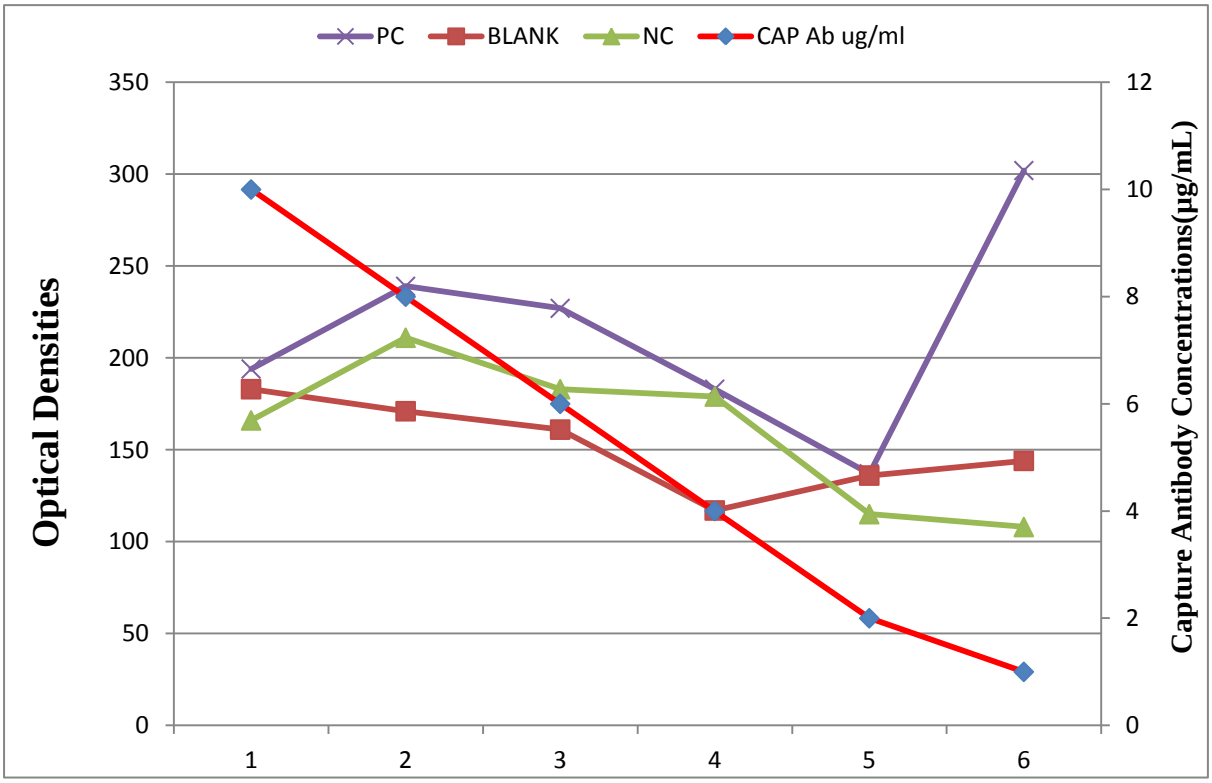
3.5.1 ELISA Optimisation

Repeated experiments using reagents at the various concentrations as indicated in Table 2.7 of methodology, blanks, control and sample wells were reactive with a low signal to noise ratio (<1) even at the lowest concentrations of all the capture and detection reagents indicated. In an attempt to minimise the background reactivity, the experiment was repeated with increased blocking of the antibody-coated wells. This was done by experimenting with

1. detection primary antibody (rabbit polyclonal aa 9-21) and the anti-rabbit IgG-HRP conjugate diluted in 0.05% Tween 20 in 1% BSA/PBS,
2. blocking coated plate overnight with 1% BSA/PBS at 4˚C
3. increasing the conjugate titrating range from 1:60 – 1: 250 to 1:1000 to 1:10 000

No signal was obtained in all of the above experiments.

Figure 3.2 Optical density comparisons for blanks, controls and samples during ELISA optimisation.



3.5.2 Verification of Antigen-Antibody Interaction

A verification of the reactivity of the various antigens or antibodies with their respective targets was then performed as outlined in Methods. Results are summarised in Table 3.11.

Table 4.0 Interaction of HCV core proteins with the various anti-HCV core antibodies employed in the ELISA development

Interaction	Results
HCV Core protein (0.2µg/mL) and capture antibodies 1. aa21-40 and 2. aa70-90 3. Equimolar concentration of both	All reactive with high background
HCV core protein and detection primary antibody (aa 9-21)	Non reactive
Anti- HCV core antibodies (aa21-40 and aa70-90) and detection primary antibody (aa 9-21)	Non reactive

The HCV core ELISA development was discontinued at this point and the indirect sandwich format regarded as unsuitable for further development with commercially available reagents. Further steps that could have been undertaken include.

1. Test an alternative commercial source of antibodies
2. Identification of antibodies targeting a different aa residue
3. Optimise concentrations of capture and detection antibodies to obtain the minimal concentrations at which positive and controls are appropriately identified with and the minimal background noise.

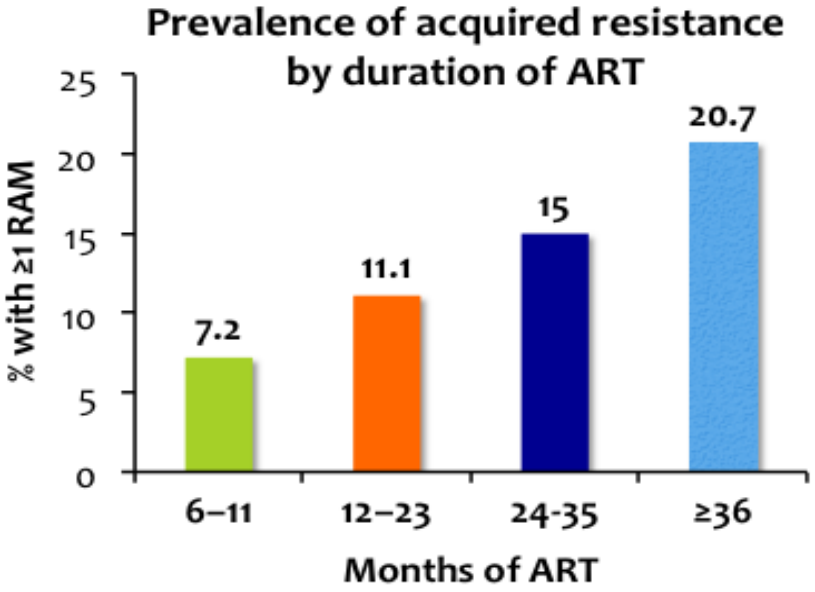
CHAPTER FOUR – DISCUSSION

Molecular testing plays a critical role in the management of HIV-infected patients, including confirming diagnosis, assessing prognosis and disease progression, guiding initiation of ART and treatment selection, monitoring therapeutic success and detection failure and drug resistance. However, access to molecular testing is limited in Kumasi and the rest of Ghana. Thus, there has been lack of routine virological monitoring in the management of HIV patients since the inception of ART at the KATH HIV clinic in Kumasi. This is mainly due to the lack of laboratory infrastructure and expertise for molecular testing. One aim of this research work was to develop proficiency in the area of HIV and HCV molecular testing. This was achieved over three months through observation and practice, followed by successful testing of a blinded proficiency panel. The techniques applied, included both manual and automated nucleic acid extraction, assay specific pre-PCR master mix preparation, quantitative real-time PCR, and results interpretation. These skills acquired will be applied to provide molecular testing to help improve the management of HIV in Kumasi once the necessary infrastructures are put in place, namely purchasing a real-time PCR machine and identifying suitable laboratory space to allow a gold-standard PCR work flow to be set in place. The knowledge acquired will also be shared with laboratory and non-laboratory colleagues at work, with the objective of building capacity for molecular testing in infectious diseases.

With the limited resources for routine viral load testing in Kumasi and other treatment centres in Ghana, clinicians depend on clinical evaluation and CD4 T-cell counts to monitor responses to ART and decide when to change to an alternative regimen. However, this approach has been proven to be less sensitive for determining treatment failure (Rawizza *et al*, 2011, Boullé *et al*, 2013). Using CD4 T-cell counts in particular may lead to treatment being inappropriately changed in some patients, whereas others may experience prolonged virological failure before treatment is changed, leading to the accumulation of drug resistance and other negative consequences of uncontrolled HIV replication. Waiting for the CD4 T-cell count to drop or for a clinical event to occur may increase the risk of mortality in those patients. Moreover, uncontrolled viral replication under drug pressure may have negative public health implications, including persistence risk of HIV transmission, including the transmission of resistant strains. The latter is likely to compromise first-line ART regimens given the absence of resistance testing prior to starting ART in Ghana.

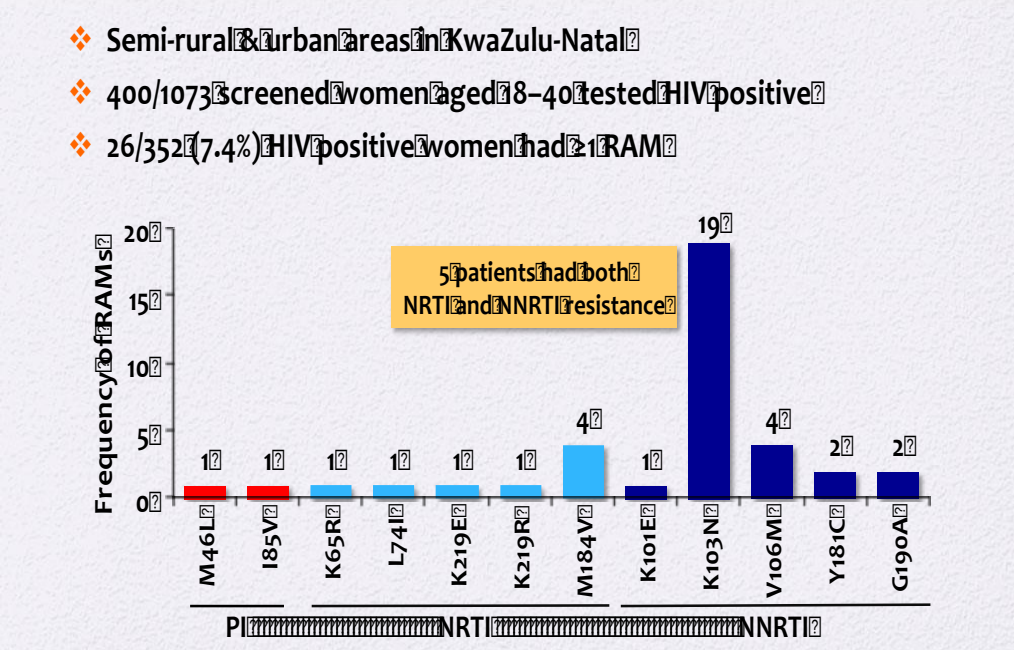
This study found that among HIV-infected patients receiving ART at KATH, whereas the majority had undetectable plasma HIV-1 RNA after at least 24 weeks of ART, 37% had a detectable viral load (< 40 copies/mL). Of these, 36.2% showed virological failure based on the WHO definition (WHO, 2010). Multiple factors may have resulted in this outcome, including suboptimal adherence, use of less favourable ART regimens, and baseline clinical, immunological and virological determinants (Kwobah *et al*, 2012; Harrigan *et al*, 2005; Datay, 2010). We also detected a high number of intra-class drug changes in this population, which resulted from problems of toxicity or drug supply, which in turn may have affected adherence. Furthermore, the impact of transmitted and acquired HIV drug resistance has not been evaluated in this population. Recent surveys have shown that in resource-poor settings ART-induced resistance is about 7.2 % 6-11 months of therapy, increasing to 21% in patents on ART for 36 months or longer.(Fig 4.1) (Stadeli *et al*, 2013). In parallel, rates of transmitted drug resistance have also increased, and are about 5.7 % in Africa (Stadeli *et al*, 2013).

Figure 4.1. Prevalence of treatment-associated HIV drug resistance in Africa, Asia, and Brazil in studies published before November 2011*(adapted from Stadeli et al, 2013)*



A cross-sectional study conducted among women participating in a HIV prevention trial in South Africa showed that 7.4% of those newly identified as HIV positive at screening already had HIV drug resistance, especially to the NNRTIs (Parikh *et al*, 2013).

Figure 4.2 MTN-009: HIV drug resistance in women screened for HIV prevention trials. The frequency of specific resistance-associated mutations (RAMs) for the three main ART classes is shown.



This observation underscores the need for access to regular viral load and resistance testing both at baseline and during ART in Kumasi. Currently CD4+ T-cell count testing is performed every 3 months during follow up at the KATH HIV clinic. If the right facilities are installed viral load testing could be done every 6-12 months to replace CD4 count for patients on ART. This will enable clinicians to detect early virological failure and initiate a timely change in regimen before extensive drug resistance accumulates, and the risk of HIV transmission and clinical and immunological deterioration increase. However to make the results more useful to patients' management, it will be necessary to have a wider range of alternative regimen at the centre for patients who fail with the initial drugs. An additional consideration will be the cost effectiveness of implementing viral load testing as an ART monitoring strategy. Although early diagnosis, CD4 count testing and clinical monitoring increase the potential life expectancy of people infected with HIV (Nagakawa *et al*, 2011; Phillips *et al*, 2008; Bendavid *et al*, 2008), modelling studies for resource-limited settings have shown relatively higher incremental life expectancy with viral load monitoring (Phillips *et al*, 2008; Bendavid *et al*, 2008). However higher lifetime cost of running viral load produces a higher incremental cost effectiveness ratio (the ratio of the change in costs to incremental benefits of an intervention or treatment) compared to CD4 count and clinical monitoring which will be economically less favourable in the dominant low-income settings in SSA including Ghana. In a South African model, an addition of viral load testing to CD4

count monitoring at every 6 months cost $5414 per life-year gained (Bendavid *et al*, 2008). Furthermore, the cost effectiveness of viral load is markedly influenced by the cost of second-line treatment (Kimmel *et al*, 2010). In the South African model CD4 count testing showed life improved life years two months lower than virological monitoring. This indicates that CD4 count monitoring still shows as a good strategy for improving life expectancy. However, the use of CD4 count and clinical monitoring without virological monitoring will eventually lead to a higher risk of HIV transmission and onward transmission of resistant strains. There is therefore the need to explore cheaper viral load monitoring techniques in Ghana that will help mitigate the impact of the lack of virological monitoring on public health. Point of care viral load testing could be the cost-effective strategy to explore in resource-limited settings (Estill *et al*, 2013). Some viral load techniques that can be explored include the *Exavir Load* assay from Cavidi (Sweden). This technique has been shown by a study in Botswana to be five times cheaper compared to the standard techniques currently available and has limit of detection of 1000 copies/mL (Cairns, 2009). In anticipation of the infrastructure for viral load testing in Ghana, it is important to keep the widening of access to ART as a priority.

The results of this work provide the basis for further characterisation of the ART response among treated patients in Kumasi. A follow-up to this work will focus on measuring virological responses longitudinally and detecting HIV drug resistance among patients with detectable HIV-1 RNA to assess the rate of emergence of drug resistance by treatment duration. We hope that some of these tests will be conducted in Kumasi where samples and clinical data are still being collected. Furthermore, we wish to perform a formal statistical analysis of factors associated with detectable viral load by logistic regression. Finally, given that our population was HBV co-infected, we wish to explore whether rates of virological failure are similar in HBV-positive and HBV-negative patients.

Documented HBV prevalence ranges from 6% to 16% in the general population in Ghana (Acquaye, 1991; Acquaye and Mingle, 1994; Martinson *et al*, 1996) whereas a prevalence of 16.8% has been found in HIV co-infected patients in Kumasi (Geretti *et al*, 2010). Despite this high rate of HBV co-infection, screening for viral hepatitis in HIV patients is performed only in selected cases to exclude liver toxicity due to ART. As more HIV patients survive with increased access to ART, chronic liver disease in co-infected patients will emerge as a threat and therefore characterisation of viral hepatitis among co-infected patients will be of grave importance. A previous research by Geretti *et al*, 2010 is performing detailed HBV

prevalence studies in the Kumasi HIV cohort and monitoring liver disease in this population using laboratory tests and fibroscanning.

Among the currently available antiretroviral drugs in Ghana, those that are known to be active against both HIV and HBV are FTC, 3TC and TDF (Dore *et al*, 2010). 40% of HBV infected patients receiving 3TC monotherapy risk HBV resistance after 2 years of therapy and the risk is higher with HIV co-infection and duration of therapy (Benhamou *et al*, 1999; Cooley *et al*, 2003). FTC also demonstrates similar efficacy and resistant pattern to 3TC. TDF is currently the preferred ARV drug having dual activity against HBV and HIV and has an additional potency against 3TC/FTC resistant HBV (WHO, 2011). An interesting result from this study however indicates that minority (6%) of the HIV/HBV cohort from Kumasi were treated with TDF whereas the majority (97%) were treated with 3TC. This indicates a high risk of drug resistance as a result of poor treatment of HBV infection in coinfected HIV patients. 3TC induced mutations in HBV polymerase, the target of therapy, could also lead to mutations in the HBsAg, the target of vaccine-induced protective antibodies and diagnostic assays , due to the overlap of the polymerase and surface open reading frames, leading to loss of recognition by either or both (Sheldon *et al*, 2008; Torresi *et al*, 2002). The screening for HBV in HIV patients prior to treatment and including TDF in the regimen for the treatment of co-infected patients in Kumasi will be important to avert HBV related diseases and the spread of HBV mutant strains which could impact negatively on public health.

On the other hand, data on the prevalence of HCV infection in both the general population and HIV-positive patients in Ghana are limited. Seroprevalence rates ranging from 0.5% to 18.7% have been documented in different Ghanaian populations (Acquaye *et al*, 2000; Owusu-Ofori *et al*, 2005; Nkrumah *et al*, 2011). Two studies showed 3.6% and 17% seroprevalence among HIV patients (Sagoe *et al*, 2012; Apea-Kubi *et al*, 2006).

A problem with the uneven results from the various HCV seroprevalence studies is the difference in the specificities and sensitivities of the assays employed and the fact that none of them attempted HCV RNA detection by PCR or confirmation of seropositivity by RIBA. In this study, samples with known HCV RNA status were screened with four commercial serological assays, namely the Architect, the Vitros, the Monolisa and the Ortho. The HCV PCR was performed using serum and DPS. The DPS provided the benefit of cheap and easy means of sample transportation, and worked well indicating that centralised testing in a laboratory with molecular testing facilities may be feasible.

The accurate detection and treatment of hepatitis C in a limited resource setting requires the use of a sensitive but more importantly, specific serological assays which have the highest probability to capture HCV RNA positive samples. This is in order to reduce the number of samples that require HCV RNA confirmation. Architect is a well-established automated assay that has been proven to have high specificity and sensitivity (99.8% and 100% respectively) in the UK; however this automated system is currently not available in Ghana. The performance of two manual EIAs that could be implemented in Kumasi, the Monolisa and the Ortho, was therefore evaluated. Ortho was found to be less sensitive but more specific than Monolisa and would be the preferred choice to be used in Kumasi. To be able to use the Ortho as a reliable assay to differentiate between HCV RNA positive and negative samples, the cut-off for positivity could be set at around 3.65, which is 25% lower than the smallest S/CO ratio associated with HCV RNA positivity in this study. However this cut-off should be interpreted with caution and further studies are required to confirm this cut-off since a limited number of HCV RNA positive samples were available. In this context it is worth noting that results from Simon King's molecular epidemiological studies indicate a low HCV prevalence of 0.64% in the Kumasi HIV cohort. This indicates that documented seroprevalence in Ghana must be interpreted with caution.

The Monolisa assay showed a lower specificity than reported in UK evaluations (HPA-MiDAS, 2006) and the manufacturer's insert. The reason for this poor specificity is unclear. Suboptimal specificity of some manual EIA assays has been previously observed in Africa, including most notably the variable performance seen with type-specific herpes simplex virus (HSV) serology studies (Biraro *et al*, 2011). In these HSV studies, increasing the assay cut-off for positivity improved specificity, although assay performance showed geographical variability (Mujugira *et al*, 2011)

HCV core antigen (Ag) has been shown to have a linear correlation with plasma HCV RNA (Ayoyagi *et al*, 1999, Kawai *et al*, 2002, Widell *et al*, 2002) and can be detected with a simple manual EIA. However there are currently no commercial assays for HCV Ag detection, although combine HCV antibody/antigen assays have been introduced. We attempted to develop a HCV Ag assay for its potential to be used as a surrogate for HCV-RNA detection where resources for molecular testing are limited, and as a one-step screening test for HCV.. An indirect sandwich method was attempted, which was contrary to the direct sandwich principle used by two previously published in- house HCV core EIAs. The reason was because the former is assumed to have a higher specificity than the latter. The assay was troubled by high background. Standard strategies were followed to resolve the non-specific

binding without success. It should be noted that among the primary and secondary antibodies used in previously published studies only one, recognising aa21-40 of the HCV core, was commercially available at the time this study was started. Surprisingly, after several failed attempts, the primary detection antibody (recognising the HCV core amino acid residue 9-21) was found not to react with the HCV core protein used as positive control under the conditions used. Further steps that could have been taken to develop the assay were to obtain a new source of antibody and revert to a direct sandwich format.

Currently treatment of hepatitis C is not widely accessible in the general population of Ghana and there is no nationally adopted treatment algorithm. This may be due to high cost of treatment but possibly due to the fact that the burden of HCV in the general population including HIV co-infected patients has not been systematically assessed. As more HIV/HCV co-infected patients survive with ART implications are that more co-infected patients will be at a higher risk of HCV associated liver fibrosis, cirrhosis, hepatocellular carcinoma and mortality. Regular screening of hepatitis C for people with HIV will be important for the evaluation of the need and cost-effectiveness of HCV treatment inclusion in the management of co-infected patients.

Conclusions

This study gave me the opportunity to develop molecular and serology skills, and to apply myself to trouble-shooting the development of an EIA assay. Research findings were that the majority of patients receiving ART at KATH were responding well to treatment. A substantial number however had plasma HIV-1 RNA detected after at least 24 weeks of therapy. There are important implications in terms of health status and risk of emerging HIV drug resistance and onward HIV transmission. Furthermore, given that the findings specifically involved patients co-infected with HBV, there are also potentially severe implications for the progression of liver disease in this subset. The issue of liver disease is important in HIV-infected patients, as improved survival through ART means that chronic hepatitis has the opportunity to progress and cause disease and mortality. Neither HBV nor HCV screening are routinely available among HIV-infected patients in Kumasi. HBV appears to be the bigger problem however as prevalence rates are significantly higher than those for HCV. There are no rapid tests for HCV available in Ghana and well validated tests are

lacking elsewhere. We found that the performance of HCV serological assays varied with the Kumasi samples, with some tests showing better specificity than others. Furthermore we suggest a cut-off that may help to identify the patients that are most likely to test HCV RNA positive, although further data are required. Although development of a HCV Ag test did not succeeded, the experimental work was highly educational because of the trouble-shooting that was undertaken at each step of the assay development.

Future Work

The planned follow up to this work will include:

1. In the treated HIV cohort in Kumasi, we will i) ask the statisticians for help in conducting a formal statistical analysis to identify factors associated with a detectable HIV-RNA on treatment; ii) obtain follow-up samples for longitudinal evaluations; iii) perform HIV drug resistance testing; and iv) compare ART response between patients with or without HBV infection.
2. Continue to collect DPS for future molecular tests including further HCV RNA testing that will increase the number of HCV RNA positive samples available, allowing further refinement of serology cut-off values that are predictive of HCV RNA positivity in this cohort.

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