DETECTION OF HIV-1 RNA/DNA AND CD4 MRNA IN FECES AND URINE SAMPLES OF THE MULTICENTER AIDS COHORT STUDY VOLUNTEERS

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HIV infects and depletes CD4+ T cells in Gut Associated Lymphoid Tissues (GALT) of the Gastrointestinal (GI) tract at a very early stage of infection. Furthermore, GALT are the major reservoirs of HIV-1 and may constantly shed virus and CD4+ T cells into the intestinal lumen throughout the entire course of infection. We hypothesize that the dynamic changes of HIV-1 and CD4+ T cell quantities in feces are linked to disease progression and can be used to predict disease prognosis. The aims of this study are to establish sensitive methods for detection and quantitation of HIV-1 and CD4 mRNA in feces, and to use the methods to monitor the amount of HIV-1 RNA/DNA and CD4 mRNA in feces samples of HIV infected patients and to correlate the findings with disease progression. In addition, since urine may potentially serve as a vehicle for HIV-1 transmission we have also measured HIV-1 RNA/DNA in the urine samples from the same population used for the feces study. Our results showed that using normal feces spiked with known copies of DNA and RNA, as low as 2.5 copies of HIV-1 DNA and 40 copies of HIV-1 RNA were detected per input in both nested PCR and RT-nested PCR reactions respectively. Human CD4 mRNA was also detected in feces. From HIV-1 infected volunteers of the Multicenter AIDS Cohort Study (MACS), HIV-1 DNA, RNA and human CD4 mRNA was detected in 8%, 19% and 31%, respectively, in the feces samples from patients with detectable viral load in blood. In the urine samples from the same study population, HIV-1 DNA was

detected in 26% of HIV-1 infected donors and this detection is not always correlated with the presence of detectable viral load in blood. This study has major Public health significance as it demonstrates that HIV-1 RNA/DNA could be detected in feces and urine samples, which may lead to the development of a future non-invasive approach to evaluate disease progression and prognosis. In addition, our study demonstrated, for the first time, the presence of human CD4 mRNA in fecal specimens of infected donors, which could be used as a valuable tool in the future to assess the pathogenesis of Gut Associated Lymphoid Tissue over the course of HIV-1 infection.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

1.1 HIV-1 infection:

Human immunodeficiency virus-1 (HIV-1) is a lentivirus, which can lead to Acquired Immunodeficiency Syndrome (AIDS), a condition in humans in which the immune system begins to fail, leading to life-threatening opportunistic infections.

1.1.1 Epidemiology of HIV-1 infection:

UNAIDS and WHO estimate in 2007 that AIDS has killed more than 25 million people since it was first recognized in 1981, making it one of the most destructive pandemics in recorded history. Despite recent improved access to antiretroviral treatment and care, in 2007 alone, AIDS killed an estimated 2.1 million people including 330,000 children and there were 2.5 million new infections with total 33.2 million people living with HIV[1, 2]. Sub-Saharan Africa remains by far the worst-affected region, with more than 22.5 million of total infected people. South and South East Asia are second-worst affected with 4.0 million infected people.

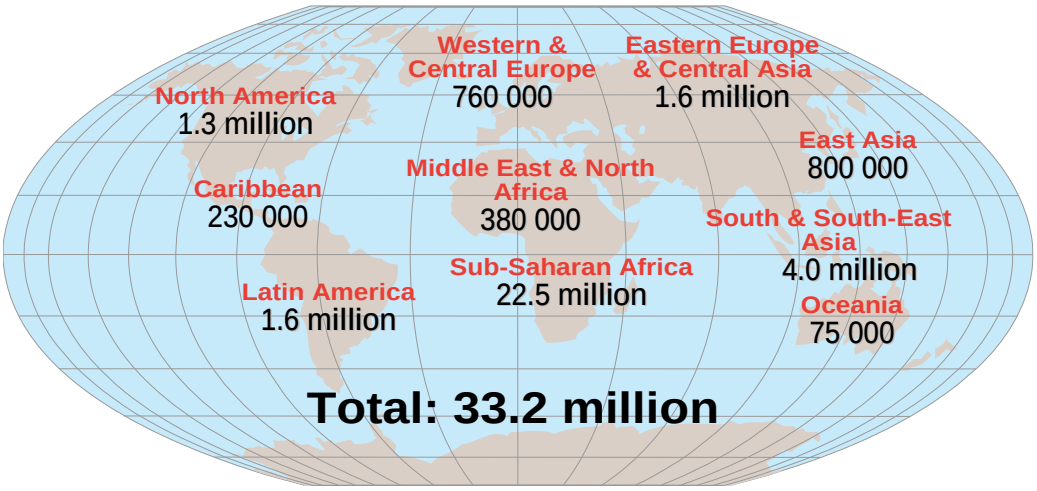


Figure 1: The Global Summary of the HIV/ AIDS Epidemics

Source: www.unaids.org

1.1.2 Transmission of HIV-1:

The majority of HIV-1 infections are acquired through unprotected sexual contact. Sexual transmission can occur through the genital, rectal or oral mucous membranes with the risk of female-to-male transmission of 0.04% per act and male-to-female transmission of 0.08% per act. For various reasons, these rates are 4 to 10 times higher in developing countries[3]. HIV-1 is also transmitted through contacting HIV-1 infected blood and/or blood products. This transmission route can account for infections in intravenous drug users, hemophiliacs, recipients of blood transfusion and health-care workers. HIV-1 could also be transmitted from infected mother to the child during pregnancy (through placenta), child birth (exposure to mother blood) and breast feeding[4]. HIV-1 has been found at low concentrations in the saliva, feces, tears and urine of infected individuals, but the potential risk of HIV-1 transmission through these vehicles is currently considered low[2, 5] .

1.1.3 HIV-1 replication cycle:

The primary target cells for HIV are human CD4 positive T cells. HIV enters CD4+ T cells or other target cells through interaction of HIV-1 envelope proteins gp120 with human CD4 (HIV receptor) and CCR5/CXCR4 (HIV-1 co- receptors) on the cell surface and release viral capsid into the cells. Once the viral capsid enters the cell, viral reverse transcriptase (RT) transcribes the single positive stranded viral RNA genome into a complementary DNA followed by formation of double stranded viral DNA intermediate which is integrated into the host genome by viral integrase. Progeny virions are produced from the infected cells through transcription of HIV-1 genes from integrated viral DNA, leading to production of HIV proteins and full-length copies of the HIV-1 RNA genome. To produce

mature viral particles, viral envelope glycoprotein gp160 is cleaved by viral protease into glycoproteins gp120 and gp41 and then transported to the plasma membrane of the host cell. The Gag-pol polyprotein gets associated at the inner surface of the plasma membrane with HIV-1 genomic RNA and undergoes a maturation process where HIV-1 protease cleaves the polyproteins into individual functional HIV proteins and enzymes. These various structural components then assemble to produce mature HIV virions by budding out of the infected cells (Figure 2) [6].

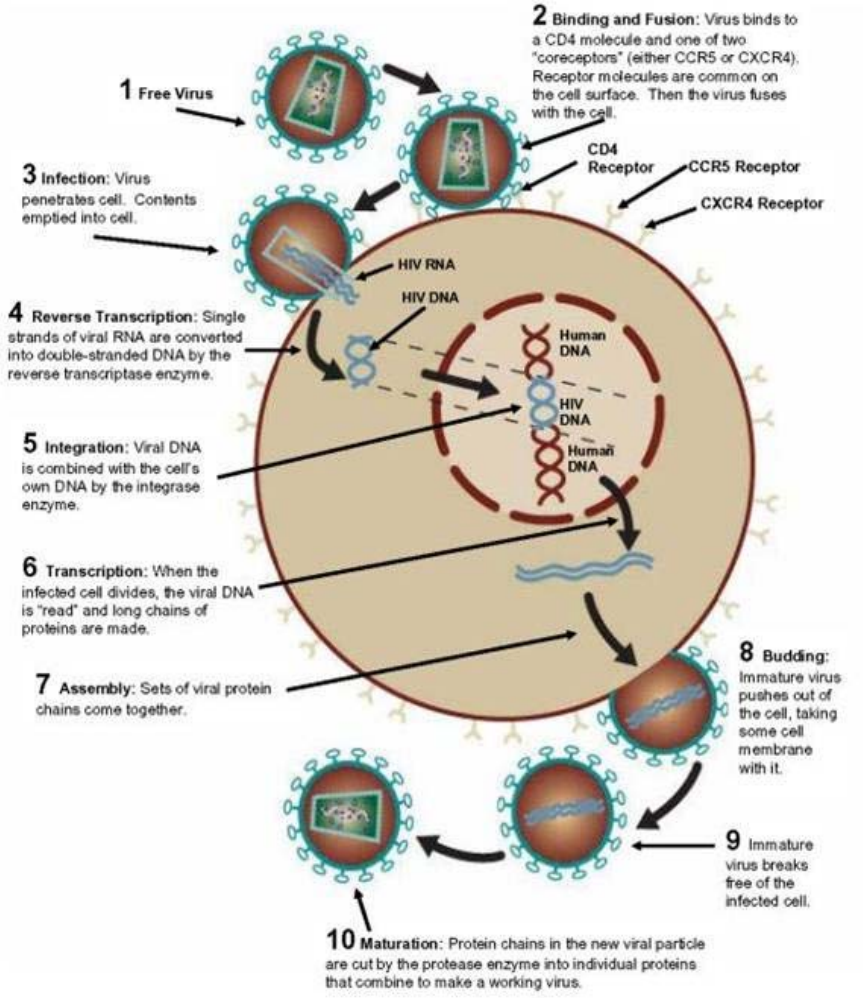


Figure 2: HIV-1 Life Cycle

Source: Coffin, J., 1995. Science 267:483-489

1.1.4 The clinical course of HIV-1 infection:

Natural infection of HIV-1 infection has four stages: incubation period, acute infection, latency stage and AIDS (figure3). The initial incubation period is asymptomatic and usually lasts between two and four weeks following HIV infection. The second stage, acute infection, lasts an average of 30 days. During this period patients generally show symptoms such as fever, swollen lymph nodes, rash, mouth and esophageal sores and malaise. In the latency stage, patients show few or no clinical symptoms and this stage can last anywhere from two weeks to twenty years and beyond. In AIDS, the fourth and final stage of HIV-1 infection, patients show symptoms of various opportunistic infections, weight loss, & diarrhea.

An acute HIV infection is associated with a burst of HIV-1 viremia and an abrupt decline of CD4+ T cells in the peripheral blood due to HIV-1 cytotoxicity and immune destruction. Within a few weeks of primary infection, HIV-1 viremia decreases and CD4+ T cell counts rebound due to anti-HIV specific immune response in the body. At this stage, patients are often unaware of the HIV-1 infection since it causes only mild flu-like symptoms in clinic. However, in the acute stage of infection, HIV disseminates extensively and establishes multiple viral reservoirs in the body, which sets up clinically latent phase of infection. During the clinical latency, the CD4+T cell counts continue to decrease. When CD4+ T cell counts fall below a critical level, the infected person becomes susceptible to opportunistic infections and AIDS develops [7].

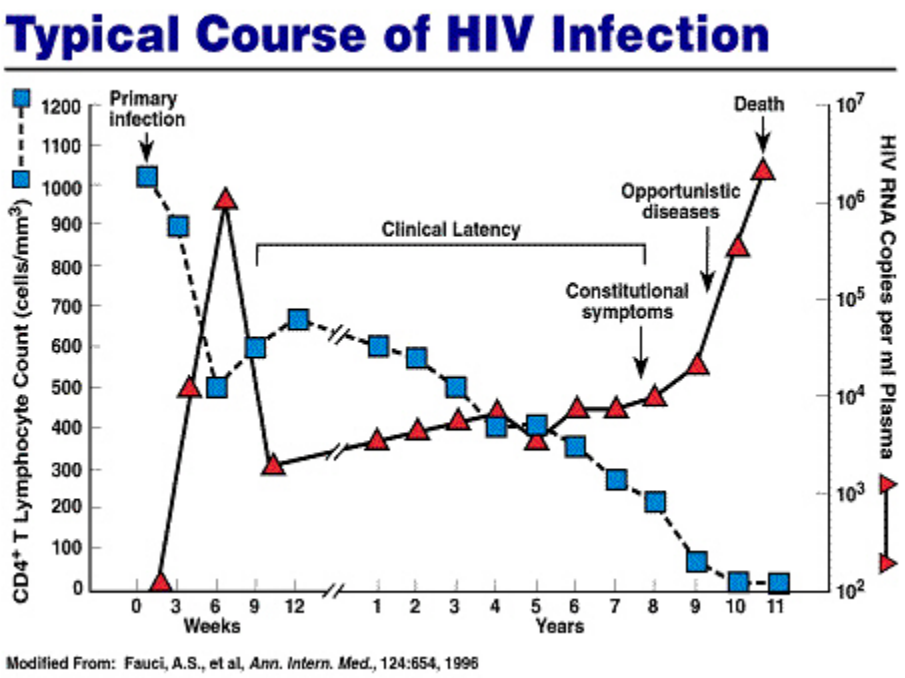


Figure 3: Clinical courses of HIV-1 infection

Source: Fauci, A. S., Ann. Intern. Med., 124:654,1996

1.1.5 Current methods for diagnosis of HIV-1 infection:

Blood is the most commonly used specimen for testing and diagnosis of HIV-1 infection. The primary tests for diagnosing HIV and AIDS include:

Serologiocal assay Test: Enzyme-linked immunosorbent assay (ELISA), is used to detect anti-HIV antibodies. If an ELISA test is positive, the Western blot test is performed to confirm the diagnosis. ELISA is quite sensitive for testing chronic HIV infection. However, because antibodies aren't produced immediately upon infection, the test may be negative during a period of a few weeks to a few months after being infected.

Viral Load Test: This test measures the amount of HIV-1 RNA genome in blood and is used to detect early HIV infection or to monitor therapeutic efficacy. The

three most commonly used technologies for measuring HIV viral load in the blood are reverse transcription polymerase chain reaction (RT-PCR), branched DNA (bDNA) and nucleic acid sequence-based amplification assay (NASBA). The basic principles of these tests are similar: HIV is detected by amplification of viral DNA or RNA.

CD4+T Cell Count: CD4+T cells are depleted at very early stage of HIV-1 infection. The CD4+T cell count in a blood sample is commonly measured by a flowcytometry based method and used to assess the disease progression. The CD4+ T cell count correlates with a person's risk of developing opportunistic infections and is a useful marker for HIV disease staging and treatment planning. The Center for Disease Control and Prevention (CDC) recommends CD4+T testing every three to six months in all HIV-1infected patients, although the need may vary from individual to individual.

1.2 Mucosal Associated Lymphoid Tissue and HIV-1 Infection:

The mucosal immune system, including the lymphoid tissues in nasal, bronchial, gastrointestinal and urogenital systems, is an integral component of our body to protect us from microbial infections. The mucosal immune system provides the first line of defense against the invading pathogens through a cluster of uniquely organized lymphoid tissues known as Mucosa-associated lymphoid tissue (MALT) (Figure 4). Natural transmission of HIV-1 occurs through mucosal surfaces and thus HIV-1 is considered a mucosal pathogen. Mucosal transmission of Simian Immunodeficiency Virus(SIV) has been shown experimentally in non-human primates through oral, rectal and vaginal mucosa [8].

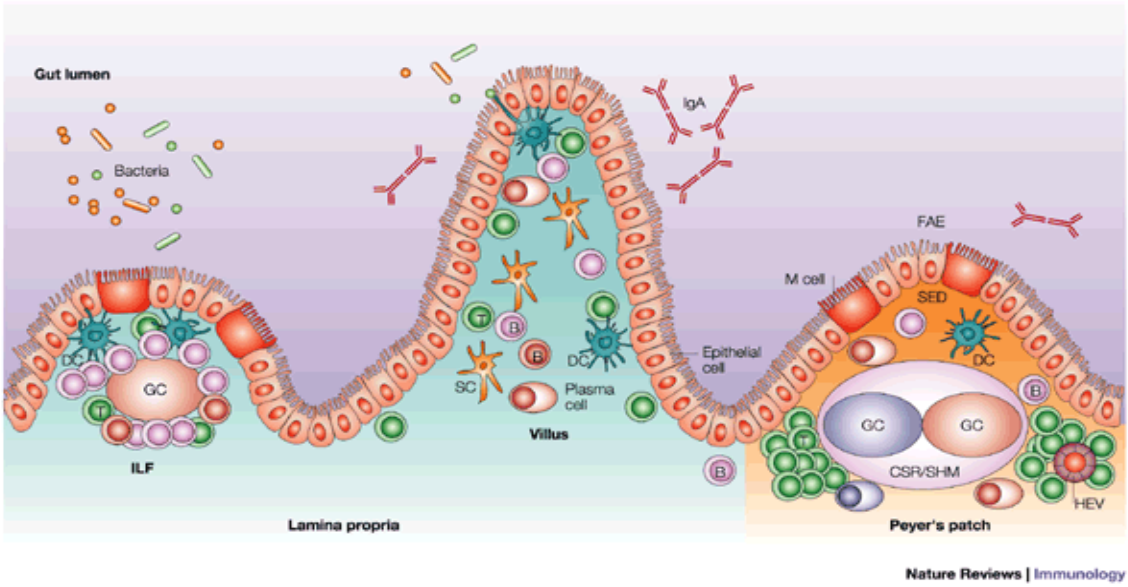


Figure 4: Schematic representation of gut-associated lymphoid tissue (GALT), with organized lymphoid structures.

Source: Honjo et al, Nature Reviews Immunology, 3, 63-72, Jan 2003

1.2.1 The mucosa Associated Lymphoid Tissues: structure and functions

The largest component of the mucosal immune system is the Gut Associated Lymphoid Tissue (GALT), which resides in the Gastrointestinal tract (GI tract) [9]. GALT can be functionally divided into inductive and effector sites. The inductive sites include mesenteric lymph nodes, Peyer's Patches (PP) and lymphoid follicles, where the antigen specific immune responses are initiated. The effector sites include lamina propria (LP) and the epithelium, where the antigen specific immune responses are implemented. The surface of PP are covered by a unique epithelial layer known as the follicle-associated epithelium (FAE) which contains specialized antigen-sampling cells-microfold cells(M cells). M cells can deliver intact antigens from intestinal lumen to antigen presenting cells (APCs) residing in the basal membrane side of the M cells[10] . Following acquiring and processing of the

antigens, APCs migrate to the B cell (germinal centers) and T-cell zones in the PP to present the antigens to T and B cells. After the recognition and activation of immune cells, the antigen specific T cells and B cells emigrate from the inductive sites via lymphatic drainage, circulate through the bloodstream and reach the effector sites of GALT. The effector sites, mainly LP regions, contain predominantly memory T cells, natural killer cells and B cells (Figure 5)[11, 12]. When the immune cells are re-exposed to the specific antigen, strong cellular and humoral immune responses occur in the effector sites.

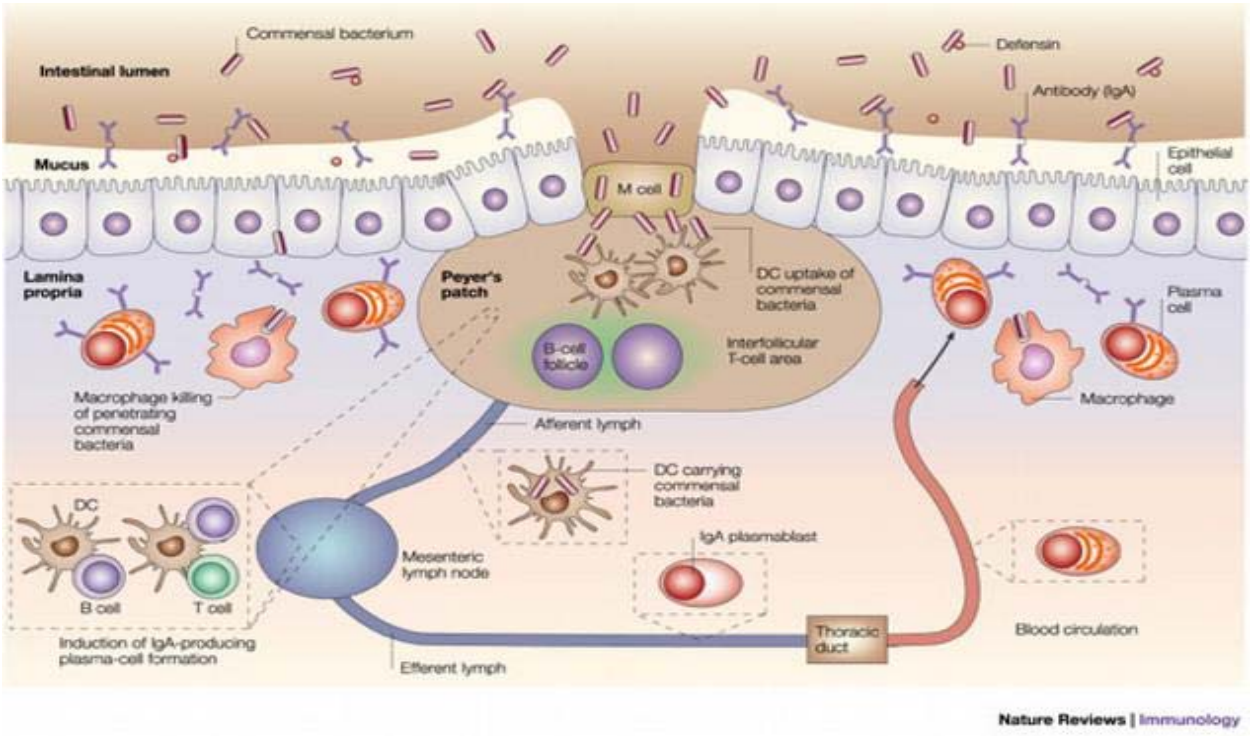


Figure 5: Depiction of the human mucosal immune system

Source: Russell M, Nature Reviews immunology, 2008, 2,31

1.2.2 Dysfunction of Mucosal immune system during acute HIV-1 and SIV infections:

HIV-1 and SIV target the lower gastrointestinal tract as an initial site of infection showed by the studies performed on human and Simian models [13]. The lamina propria in rectal mucosa contains an abundant amount of lymphoid cells, especially CD4+ memory T cells expressing CCR5, the most commonly infected cells following HIV-1 infection. In experimental SIV infection of macaque models, the early accumulation of SIV-infected lymphocytes and high viral load in GI mucosa were observed compared to blood and lymph nodes, which indicated that the GI mucosa is the initial and predominant site of SIV infection [13]. A series of studies have revealed the early immunologic events of HIV-1 and SIV infection at the mucosal tissue level[14]. These studies concluded that early HIV-1/SIV infection is consistently associated with a dramatic and largely irreversible depletion of mucosal CD4+memory T cells, particularly those expressing HIV-1/SIV coreceptor CCR5[15]. In contrast, CD4+ T cells residing in the peripheral blood and lymph nodes are relatively preserved. The possible explanation for depletion of CD4+ T cells from the GI tract may involve several factors: 1) a direct infection accounting for the earliest loss and 2) activation induced death mediated by host cytotoxic cellular responses. Similar studies with human models to evaluate the events occurring in the mucosal system following HIV-1 infection are extremely difficult to perform for ethical reasons. Though few groups conducted these kind of studies, and their data clearly indicate that HIV-1 infection also leads to an early, rapid depletion of mucosal CD4+CCR5 memory T cells[16]. Thus based on these studies it can be concluded that a selective depletion of memory CD4+ T cells from mucosal tissues during acute HIV or SIV infection is a key determinant of the disease progression. This early loss of CD4+ T cells from mucosal tissues leads to an impairment of mucosal immunity that may lead to the downstream chronic

infection. It should also be noted that the exact hypothesis for the viral pathogenicity for this early depletion of CD4+ T cells in gut is still unclear and needs further investigation.

1.2.3 Dysfunction of Mucosal immune system during chronic HIV-1 and SIV infection:

In the chronic phase of infection the immune system exerts some control over a steady state viral replication. However, the overall state of chronic and general immune activation plays a central role for a progressively dysfunctional immune system [17].During chronic HIV infection the extent of GALT CD4+ T cell depletion remains more severe, mainly involving CD4+ CCR5 + T cells residing in the lamina propria as observed in tissue specimens collected from the terminal ileum[18].A subsequent study on SIV mac239-infected rhesus macaques revealed that after the acute depletion of CD4+T cells, a consistent and progressive decline in the CD4+ T cells is observed during chronic infection which coincides with a similarly progressive exhaustion of CD4+ central memory T cells at the level of blood and lymph node[19]. In addition, this study showed a progressive, systemic decline of CD4+ central memory T cells that resulted in insufficient mucosal CD4+ effector memory T cells that are associated with the progression of AIDS. This systemic loss of CD4+ central memory T-cells during chronic $SIVmac_{239}$ infection of macaques was more closely associated with the immune activation than viral load, thus consistent with the chronic immune activation in HIV-1 infection[9]. In HIV-1 infected individuals low levels of mucosal CD4+ T cell and increased mucosal immune response are associated with increased collagen deposition in the lymph nodes, thus suggesting a parallel loss of effector memory and central memory T-cell function in mucosal and systemic lymphoid tissues respectively during chronic progressive HIV-1 disease [20].The target cell

distribution and HIV-1 replication in the intestinal tract has been shown below in Figure 6.

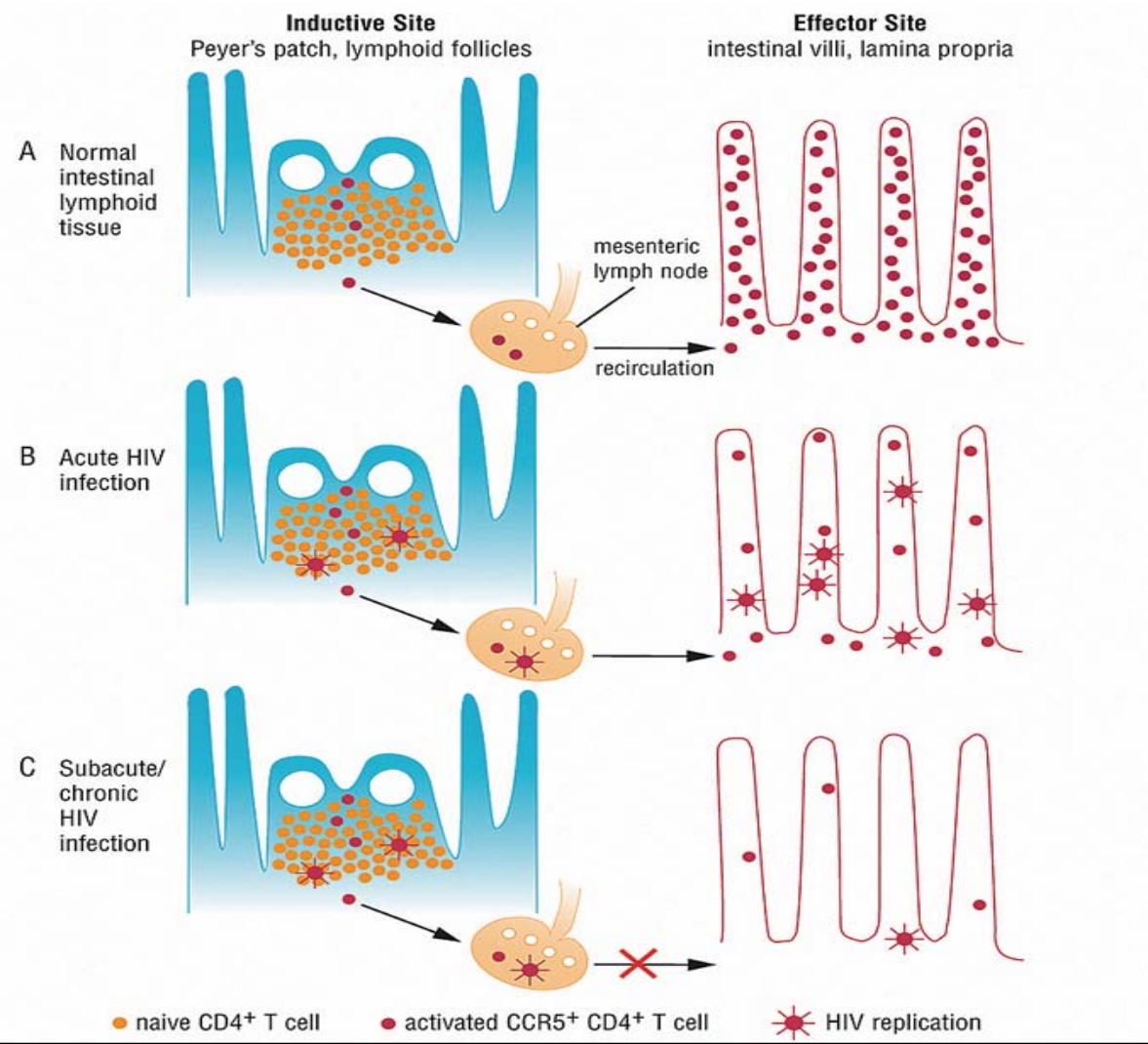


Figure 1. *Target cell distribution and HIV replication in the intestinal tract. (A) In normal intestinal lymphoid tissue, naïve CCR5⁻CD4⁺ T cells mostly reside in inductive sites. As they are exposed to the variety of antigens normally found in the intestinal tract (dietary antigens or intestinal microbes) these cells are activated, causing them to coexpress CCR5 and then rapidly recirculate and home to effector sites. Large numbers of these activated CD4⁺CCR5⁺ T cells then reside there. (B) In acute HIV infection the activated CD4⁺CCR5⁺ T cells are primary targets for HIV replication and are therefore soon destroyed. This coincides with the peak plasma viremia in early infection, and the subsequent decline in viremia may be due to the loss of this subset of cells. (C) During subacute/chronic HIV infection the extensive loss of CD4⁺CCR5⁺ T cells in the lamina propria means fewer targets and so lower levels of viral replication at effector sites. Steady-state T-cell activation due to antigen encounter in inductive sites maintains viral replication there, providing an inherent mechanism for viral persistence and ongoing replication. The majority of infected cells may be eliminated before homing to effector sites so CD4⁺CCR5⁺ T cells are never restored in chronically-infected patients, even in those given HAART. (Adapted from Veazey and Leckner,* J. Exp. Med. *200, 697, 2004)*

Figure 6: Target cell distribution and HIV-1 replication in the intestinal tract

Source: Leckner et al, J. Exp.Med, 200, 697, 2004

1.3 Detection of HIV-1 in feces sample:

Since HIV infects GALT in the early phase of infection and GALT serves as a viral reservoir throughout the entire course of infection, a patient's feces may provide a convenient specimen for monitoring HIV-1 related pathogenesis.

1.3.1 Human feces: composition and prevalence of viral communities:

Human feces also known as stool are the waste product of the human digestive system and it varies significantly in appearance, composition and pathology depending upon the state of human health and diet. About 3/4 portion of normal feces is made of water. The remaining portion is a mixture of dead bacteria, fats such as cholesterol, inorganic salts like phosphates, live bacteria, dead cells and mucus from the lining of the intestine, and proteins [21]. Clinically, fecal samples have been used for laboratory tests of micropathogens or parasites for diagnosis and monitoring disease progression. Three main types of laboratory tests are commonly performed with fecal samples to detect pathogens, Enzyme linked ImmunoSorbent assay (ELISA), microscopic examination for intestinal parasites and routine culture to find bacterial pathogens.

The human gastrointestinal tract is known to be a natural habitat for a large microbial community including bacteria, archaea, eukarya and viral pathogens. Many viral pathogens have been detected from the feces of gastroenteritis patients including astrovirus, rotavirus, coronavirus, calcivirus and adenovirus[12, 22]. A comparative metagenomic analysis of the RNA viruses in fecal samples revealed that plant viruses are prevalent in the normal human feces. Further study revealed that the most common animal virus identified in human feces was a picornavirus(PBV-733)[23].The other viruses identified in human feces in

abnormal gastrointestinal conditional are the Hepatitis-C virus[24] and the Human immnuodeficiency Virus-1(HIV-1) and other enteric RNA viruses.[25].

1.3.2 Detection of Human Immunodeficiency Virus Type-1 from Feces samples:

HIV-1 infects and depletes CD4+ T cells in GALT of GI tract at very early stages of infection and the HIV associated GALT pathogenesis is maintained throughout the entire course of infection[26]. Detection of HIV-1 has been reported[25] in fecal specimens from HIV-1 infected individuals in acute phase of infection and without any therapeutic intervention. Although no HIV-1 proviral DNA was detected in feces, HIV-1 RNA was detected in 67% of patients' fecal samples by RT-PCR. Direct sequencing revealed differences in sequence heterogeneity of the PCR amplimers obtained from paired fecal and serum specimens. The HIV-1 RNA detected in feces could be either from infected cells or free virus shed from the lamina propria, which pass through the epithelial barrier because of the increased permeability of the epithelial cell layer [27].It is not clear whether the HIV-1 contained in feces could pose a threat to the public health. However one report showed that a full-length SIVcpz sequence (TAN1) was isolated from the feces of an infected chimpanzee from Gombe National Park (Tanzania)[28-30].

1.4 Detection of HIV in Urine Sample:

1.4.1: Structure and function of urinary system:

The urinary system is the organ system that produces, stores, and eliminates urine. In humans it includes two kidneys, two ureters, the bladder, and the urethra as shown below in Figure 7. The urinary system keeps chemicals, such as potassium

and sodium, and water in balance, urea and other excretory product from the body. Glomerulus is a very important structure of the urinary/excretory system which performs the role of the basic filtration system of the kidney. The resistance of the glomerular- arterioles results in high pressure in the glomerulus aiding the process of ultrafiltration where fluids and soluble materials in the blood are forced out of the capillaries and into Bowman's capsule[31]. The structures of the glomerulus determine their permeability-selectivity, called as permselectivity. The factors that influence permselectivity are the negative charge of the basement membrane and the podocytic epithelium, and the effective pore size of the glomerular wall (8 nm). As a result, large and/or negatively charged molecules pass through far less frequently than small and/or positively charged ones. For instance, small ions such as sodium and potassium pass freely, while larger proteins, such as hemoglobin and albumin have practically no permeability at all[32, 33].

Now in abnormal conditions such as during viral infection, inflammation occurs in glomerular walls and the permeability changes due to disruption of the selective pressure that keeps the larger particles like protein, virus and cells going through the glomerulus. We hypothesize that the glomerular walls undergoes inflammation and causes disruption of the selective pressure during HIV-1 infection. As a result it creates a possibility that HIV-1 RNA/DNA could shed from urinary tract to urine through the glomerulus. Pathogenic changes in uro-genital system are very common in HIV-1 infected patients. Monitoring urine samples may provide important information for HIV infection and its related disease progression.

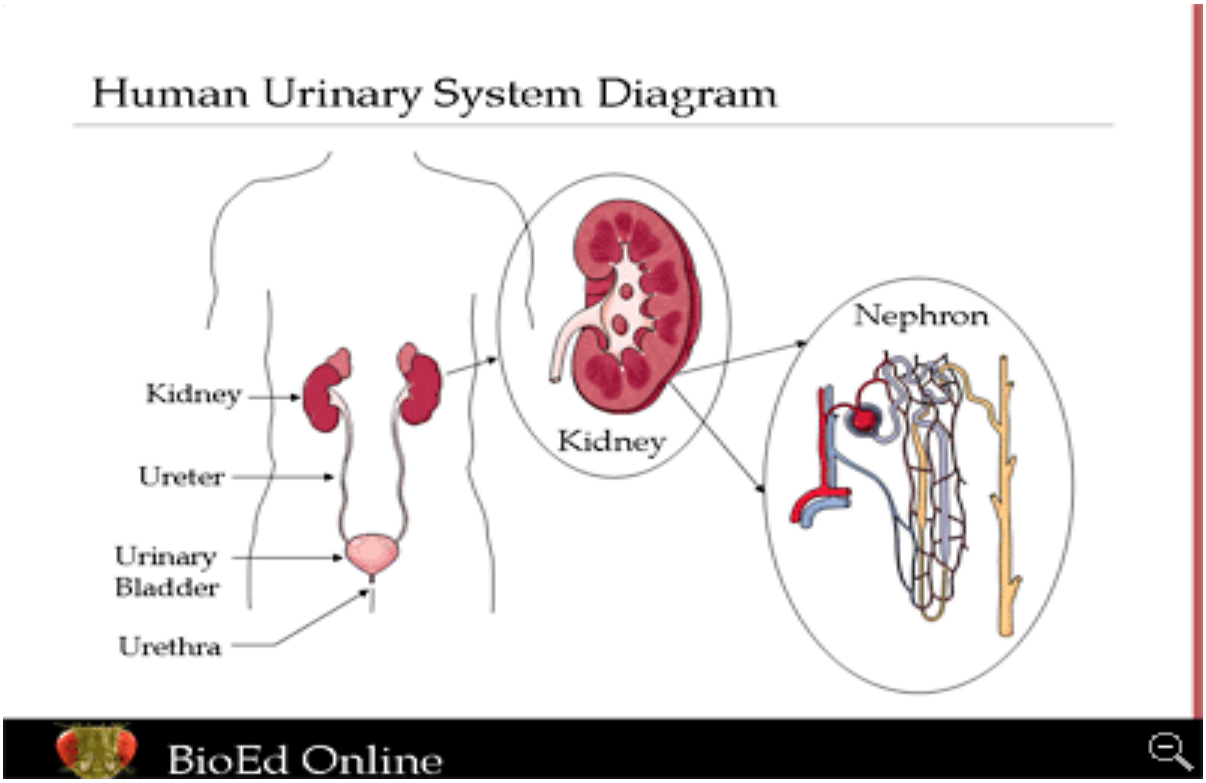


Figure 7: Structural organization of Human urinary system

Source: www.bioedonline.com

1.4.2 Human Urine: composition and prevalence of invading pathogens:

The urine is composed of mainly water, dissolved salts and organic compounds. Urine is generally colorless and sterile. However, in pathogenic conditions, micropathogens can be detected in urine. The most commonly identified bacterial pathogens in urine are salmonella typhi, salmonella paratyphi, Leptospira, Yersinia and E.coli[34]. In patients with acute viral infections, the virus is commonly present in the blood, occasionally in the cerebrospinal fluid and the urine. Detection of cytomegalovirus, human papillomavirus in human urine has been reported previously[35, 36]

1.4.3 Detection of Human Immunodeficiency Virus Type-1 from Urine samples:

HIV-1 has been isolated from various body fluids from HIV-1 seropositive individuals, including blood, semen, tears, saliva, cerebrospinal fluid, breast milk and cervical secretions[37, 38]. With the increasing prevalence of HIV-1 infection, assessment of HIV-1 contained in urine from infected individuals can be extremely important since urine could serve as a vehicle for HIV-1 transmission. It has been reported that anti-HIV-1 antibodies were detected in urine by ELISA and Western blot [39]and that HIV-1 DNA were detected in urine pellets from HIV-1-infected individuals[27]. Moreover, HIV-1 p24 core antigen and HIV-1 RNA were detected in the cells contained in urine from HIV-1-seropositive individuals by immunohistochemical staining and in situ hybridization [33, 40]. A broad spectrum of renal diseases has been described in HIV infected patients with symptomatic HIV-1 disease [40]. Studies in a nephropathy revealed that rapidly advancing renal insufficiency, nephritic disorders and glomerular sclerosis occur in HIV-1 infected patients[41]. It is highly possible that monitoring HIV-1 amount in urine and correlating the detected HIV-1 quantities with the disease progression may become extremely significant for future diagnosis and preventive approaches.

2.0 STATEMENT OF THE PROBLEM AND AIMS OF STUDY

HIV-1 replication results in massive tissue damage and CD4+ T cell depletion in GALT. In the entire course of HIV infection, the HIV-1 constantly sheds from GALT into the intestinal lumen in the form of free virus and/or cell associated virus particles. In addition, increased numbers of infected and uninfected CD4+ T cells are also shed from GALT into the intestinal lumen. So far no comprehensive studies have been performed to correlate the detection of HIV RNA/DNA and CD4 mRNA in feces and urine with disease progression, which is important for possible future development of a non-invasive clinical diagnostic method and therapeutic interventions. Urinary complications are common in HIV-1 infected patients with several clinical manifestations, but whether it can serve as a vehicle for HIV-1 transmission is poorly understood. We hypothesize that the HIV-1RNA/DNA and CD4+ T cell quantities identified in feces and urine are linked to disease progression and can be used to predict disease prognosis. To address these questions three specific aims will be emphasized in this study.

SPECIFIC AIMS:

- **SPECIFIC AIM #1:** Standardization of sensitive methods for the detection of HIV-1 DNA/RNA and CD4 mRNA in human feces.

- **SPECIFIC AIM #2:** Detection of HIV-1 DNA/RNA and human CD4 mRNA in fecal specimens of the Multicenter AIDS Cohort Study volunteers and correlation of the findings with disease stages.

- **SPECIFIC AIM #3:** Detection of HIV-1 DNA/RNA in urine specimens of Multicenter AIDS Cohort Study volunteers and correlation of the outcome with results from the blood and fecal specimens.

3.0 MATERIALS AND METHODS

3.1 Study Participants:

The normal and HIV-1 infected population used in this study is part of the Multicenter AIDS Cohort Study in Pittsburgh. The Multicenter AIDS Cohort Study (MACS) is an ongoing prospective study of the natural and treated histories of HIV-1 infection in homosexual and bisexual men conducted by sites located in Baltimore, Chicago, Pittsburgh and Los Angeles. The MACS was designed to elucidate the natural history of the infection causing acquired immunodeficiency syndrome (AIDS), identify risk factors for occurrence and clinical history of the infection, and establish a repository of biologic specimens for future study. The study participants for our project are from the Pittsburgh site of MACS who signed the consent forms and completed a survey questionnaire following all Institutional Review Board (IRB) guidelines before donating the biologic specimens. The participation in this research study was completely voluntary and the donors had the right to withdraw from participation at any time. Fecal specimens were collected from 11 consented HIV-1 seropositive MACS donors in year 2007. Later in the year 2008, biologic specimens were collected from the subjects in four different groups: Group A: HIV-1 negative; Group B: HIV-1 positive but not on Highly Active Antiretroviral Therapy (HAART); Group C: HIV-1 positive on HAART with non-detectable viral load; Group D: HIV-1 positive on HAART with detectable viral load (Table 3).

3.2 Collection and storage of biological specimens:

All participants were contacted before the visit to our clinic with instructions to collect stool samples at home within 24 hours before their visit to the clinic. They were provided with special stool collection tube (SARSTEDT) and urine collection

cups (Falcon) to store the samples. Within six hours after the samples arrived in clinic, feces samples were stored in RNAlater solution (AMBION) or Cell-Lysis buffer in -80C freezer. Urine samples were collected at the clinic and stored in 4C temporarily and processed within 6 hours after collection. In addition, all the study participants also donated blood samples at the same time of feces and urines collection. Plasma, serum and PBMC were isolated from these blood samples and used for CD4 count and viral load measurement.

3.3 HIV-1 infected cell line:

The 8E5 cell line, derived from HIV-1-infected CD4+ CEM cells, carries a single, integrated and RT-defective HIV-1 genome. The lack of functional RT production by this genome is the consequence of a frame-shift mutation in the pol gene, due to a single base addition at position 3241. These CD4+ T cells express gag and env proteins and produce HIV particles which are non-infectious[42].

3.4 HIV-1 positive plasma and virus culture:

The HIV-1 positive plasma with known copy number used for spiking normal feces before nucleic acid isolation was obtained from a HIV-1 infected Brazilian blood donor (subtype B) with plasma. Viral load in plasma for this sample was 170,000copies/ml. Virus culture supernatant containing 270ng/ml p24 of a Chinese subtype B' HIV-1 isolate was used for spiking normal donor feces for the HIV-1 p24 measurement by ELISA.

3.5 Extraction of RNA/DNA from feces samples:

200mg of feces sample were used to isolate RNA/DNA using nucleic acid isolation kit from BIOMERIEUX [25]. Fecal specimens stored in Cell- Lysis buffer after collection at -80C were thawed and mixed completely by vortexing or inverting.

50ul of silica bead suspension was immediately added to the sample and the sample mixture was incubated at room temperature for 10 min. with vortexing every 2 min. to allow the nucleic acids to bind to the beads. Then, the sample was vortexed again and centrifuged for 3 min. at 1500g to pellet the silica. The supernatant was carefully removed to avoid disturbing the silica pellet and the pellet was washed five times: 2 times with wash buffer, 2 times with 70% ACS grade ethanol and 1 time with analytical grade acetone. The silica-nucleic acid complexes were dried on a heat block at 56C for 10 min. and nucleic acids were eluted using 100ul of elution buffer. Eluted nucleic acids were immediately stored at -70C for further use. Under these conditions, the nucleic acids are stable and can be used for a wide variety of applications.

3.6 Extraction of RNA/DNA from urine samples:

26-83ml of urine samples were collected from consented MACS donors and processed within a 6 hour time frame. These urine samples were centrifuged at 3000rpm for 10 min. at 4C. The urine pellets were saved in -80C for DNA isolation. The urine supernatant was concentrated by a Centricon plus-70 filter with molecular weight cutoff 100kDa (Amicon centricon plus-70, Ultracel PL-100, MWCO: 100kDa, Millipore) according to manufacture's instruction. Briefly, urine supernatant was centrifuged in a pre-wet Centricon at 500g for 1.5hrs and the concentrated supernatant was collected by inverted spinning and further concentrated by ultracentrifugation at 22,000rpm for one hour at 4C. Finally 50ul of concentrated supernatant was saved at -80C for further RNA isolation.

RNA was purified from the concentrated urine supernatant using RNA-Bee RNA Isolation kit (TEL-TEST, INC) according to manufacture's instruction. Briefly, 1ml of RNA-Bee solution and 200ul of chloroform were added to the

concentrated urine supernatant and shaken vigorously for 30 seconds at room temperature. Then, the sample was incubated in 4C for 5 minutes and centrifuged at 12,000g for 15 minutes at 4C. After centrifugation, the aqueous phase containing RNA was carefully recovered. The RNA was precipitated using isopropanol, washed with 75% Ethanol, air dried and stored in nuclease free water at -80C for future RT-PCR work.

DNA was purified from the urine pellet using PUREGENE DNA Purification Kit (GENTRA SYSTEMS) according to manufacture's instruction. Briefly, urine pellet was resuspended in 900ul of Cell Lysis Solution and incubated at 65C for 30 min. After incubation, 5ul of RNase A Solution was added to the mixture and incubated at 37C for 30min. Then, 300ul of Protein Precipitation Solution was added to the mixture, which was vortexed and centrifuged at 2000g for 10 minutes. DNA was precipitated from the supernatant by isopropanol, washed by 70% ethanol and air dried. The DNA was re-hydrated in nuclease free water and stored at -20C for subsequent PCR work.

3.7 Nested PCR and RT-PCR:

Nested PCR and RT-PCR were performed on isolated RNA/DNA samples from Feces and Urine to quantitate HIV-1 and CD4 mRNA. Specific primers listed in Table 1 were used for optimal detection of HIV-1 subtype B env and gag regions, human beta-globin DNA, human beta-actin mRNA and CD4 mRNA.

First a cDNA strand was generated using Superscript II RT (Invitrogen, Carlsbad, CA). A 10ul reaction consisting of 10ug of RNA, 2uM of primer, 10mM dNTP mix, and H2O was incubated at 70°C for 10 minutes. Following incubation, 5x RT buffer, 0.1 M DTT, RNA guard (RNase) (40U/ul), and Superscript II RT (200U/ul)

was added respectively. The reaction was incubated in H20 bath at 42°C for 50 minutes followed by a second incubation in dry bath at 70C for 10 minutes. Amplification of specific target sequences in the cDNA was performed using 10µl cDNA, forward and reverse primer pairs, dNTPs, Taq polymerase buffer and Taq-polymerase enzyme in Thermocycler (Applied Biosystems) with cycling conditions of 94º C, 10 min followed by 35 cycles of 94º C, 1min, 55º C, 1min, 72º C, 1 min. Presence of the amplicon was analyzed on a 1%-2.5% agarose gel in 1X TAE buffer. The primers used for PCR/RT-PCR and Nested PCR/RT-PCR amplification are listed in Table 1.

Table 1: The primers used for PCR/RT-PCR and Nested PCR/RT-PCR amplification

Name	Sequences (5'⟶3')	Description
ED31	CCT CAG CCA TTA CAC AGG CCT GTC CAA AG	1st round PCR forward primer for HIV env gp120
BH2	CCT TGG TGG GTG CTA CTC CTA ATG GTT CA	1st round PCR reverse primer for HIV env gp120
DR7	TCA ACT CAA CTG CTG TTA AAT GGC AGT CTA GC	2nd round PCR forward primer for HIV env gp120
DR8	CAC TTC TCC AAT TGT CCC TCA TAT CTC CTC C	2nd round PCR reverse primer for HIV env gp120
Hu-CD4 Inside F	CTC CCG CTC CAC CTC ACC CTG	2nd round PCR forward primer for Human CD4 mRNA
Hu-CD4 Inside R	CAT GTG GGC AGA ACC TTG ATG TTG G	2nd round PCR reverse primer for Human CD4 mRNA
Hu-CD4 outside F	CCA AGT CTT GGA TCA CCT TTG ACC TGA AG	1st round PCR forward primer for Human CD4 mRNA
Hu-CD4 outside R	AGA AGA AGA TGC CTA GCC CAA TGA AAA GC	1st round PCR reverse primer for Human CD4 mRNA
B-globin inside F	GGT TCT TTG AGT CCT TTG GGG ATC	2nd round PCR forward primer for Human Beta globin DNA
B-globin	GTC ACA GTG CAG CTC ACT CAG TGT G	2nd round PCR reverse primer for

(Table 1 continued)

inside R		**Human Beta globin DNA**
B-globin outside F	**CTG CTG GTG GTC TAC CCT TGG AC**	**1st round PCR primer for Human Beta globin DNA**
B-globin outside R	**CTC AAG TTC TCA GGA TCC A**	**1st round PCR primer for Human Beta globin DNA**
B-actin inside F	**TAC CAC TGG CAT CGT GAT GGA CTC**	**2nd round PCR primer for Human Beta actin RNA**
B-actin inside R	**CGC TCA TTG CCA ATG GTG ATG AC**	**2nd round PCR primer for Human Beta actin RNA**
B-actin outside F	**GCA CCA CAC CTT CTA CAA TG**	**1st round PCR primer for Human Beta actin RNA**
B-actin outside R	**TGC TTG CTG ATC CAC ATC TG**	**1st round PCR primer for Human Beta actin RNA**
Gag inside F	**GGC ACA TCA AGC AGC CAT GCA AAT G**	**2nd round PCR primer for HIV Gag**
Gag inside R	**TAG TTC CTG CTA TGT CAC TTC CCC TTG G**	**2nd round PCR primer for HIV Gag**
Gag outside F	**GGC CAT ATC ACC TAG AAC TTT AAA TGC ATG G**	**1st round PCR primer for HIV Gag**
Gag outside R	**CCT ACT GGG ATA GGT GGA TTA TTT GTC ATC CA**	**1st round PCR primer for HIV Gag**
Hu-CD4 RT primer	**ATG TCT TCT GAA ACC GGT GAG GAC ACT G**	**RT primer**
ED12	**AGT GCT TCC TGC TGC TCC CAA GAA CCC AAG**	**RT primer**

3.8 Measurement of HIV-1 p24 from feces sample by ELISA:

Supernatant derived from feces sample was measured for p24 concentration by a commercial available HIV p24 ELISA kit (PerkinElmer Life Sciences, Inc., MA, USA).

In order to standardize the HIV-1 p24 detection limit, normal donor feces sample were spiked with HIV-1 virus (Chinese isolate, subtype B') in serial dilutions and then treated with protease inhibitors. These samples were then mixed completely

by vortexing and incubated for 10min at room temperature. These inhibitors are capable of inhibiting a broad spectrum of serine, cysteine and metalloproteases as well as calpains and thus suitable for the protection of protein degradation. Subsequently, the samples were vortexed again and centrifuged for 3 min. at 1500g. Supernatant was saved for measurement of HIV-1 p24 antigen by HIV-1 p24 ELISA kit.

200mg of fecal specimens from MACS participants were resuspended in 800ul of PBS containing the protease inhibitor cocktail (Roche Applied Science, Catalog No. 11836153001), mixed completely by vortexing and incubated for 10 min. at room temperature. These inhibitors are capable of inhibiting a broad spectrum of serine, cysteine and metalloproteases and are suitable for the protection of proteins [43]. Subsequently, the samples were vortexed again and centrifuged for 3 min. at 1500g. Supernatant was saved for measurement of HIV-1 p24 antigen by HIV-1 p24 ELISA kit.

3.9 Detection of fecal occult blood from feces samples:

Feces samples from MACS donors were tested for presence of occult blood by a Hemoccult II SENSA kit (BECKMAN COULTER, Catalog no. 395035). Trace amount of the fecal sample was smeared onto an absorbent paper that has been treated with a chemical guaiac. Hydrogen peroxide was dropped onto the fecal smear. If trace amounts of blood was present, blue color development would occur. A total of 39 fecal specimens from four different groups of 2008 MACS donors (Table 2) were tested. In addition, one normal donor fecal sample was included as negative control and normal donor fecal sample mixed with blood as positive control.

4.0 RESULTS

SPECIFIC AIM #1: Establishment of sensitive methods for detection of HIV-1 DNA/RNA and human CD4 mRNA in feces.

4.1 Exploration of the most suitable technique for isolating nucleic acid from feces samples:

Feces components are very complex and contain nucleases proteases, proteins and nucleic acids from human as well as animals, plants and microbes. Since quality and quantity of isolated nucleic acids from feces are the keys for successful PCR amplification, column based isolation (QIAGEN) and silica based isolation (NUCLISENS) and elution methods have been compared and we found that the NUCLISENS (BIOMERIEUX INC) method is the most effective one in terms of DNA/RNA quality and quantity, which has been used in all of our subsequent experiments.

4.2 Evaluation of the sensitivity of the detection assay for HIV-1 DNA/RNA in human feces:

4.2.1 The nested PCR sensitivity for detection of HIV-1 DNA:

We have used nested PCR to detect HIV-1 DNA/RNA in feces. In order to test the sensitivity, 200mg of normal donor fecal sample was spiked with different concentrations of HIV-1 positive 8E5 cells. DNA was isolated using NUCLISENS (BIOMERIEUX) nucleic acid isolation kit. For every DNA sample, the human beta-globin gene was PCR amplified to ensure that the isolated DNA was amplifiable and contained the comparable amount of human DNA. Figure 8B shows the representative results of PCR amplification of beta globin DNA from

three feces samples. Subsequently, a nested PCR reaction was performed to detect HIV-1 DNA from the isolated DNA using HIV-1 env specific primers. (Figure 8A) which was visualized by electrophoresis in 1% agarose gel. As shown in Figure 8C, HIV-1 DNA was detected from the DNA isolated from normal donor feces spiked with 8E5 cells. The detection limit was as low as 2.5 copies/ reaction.

A.

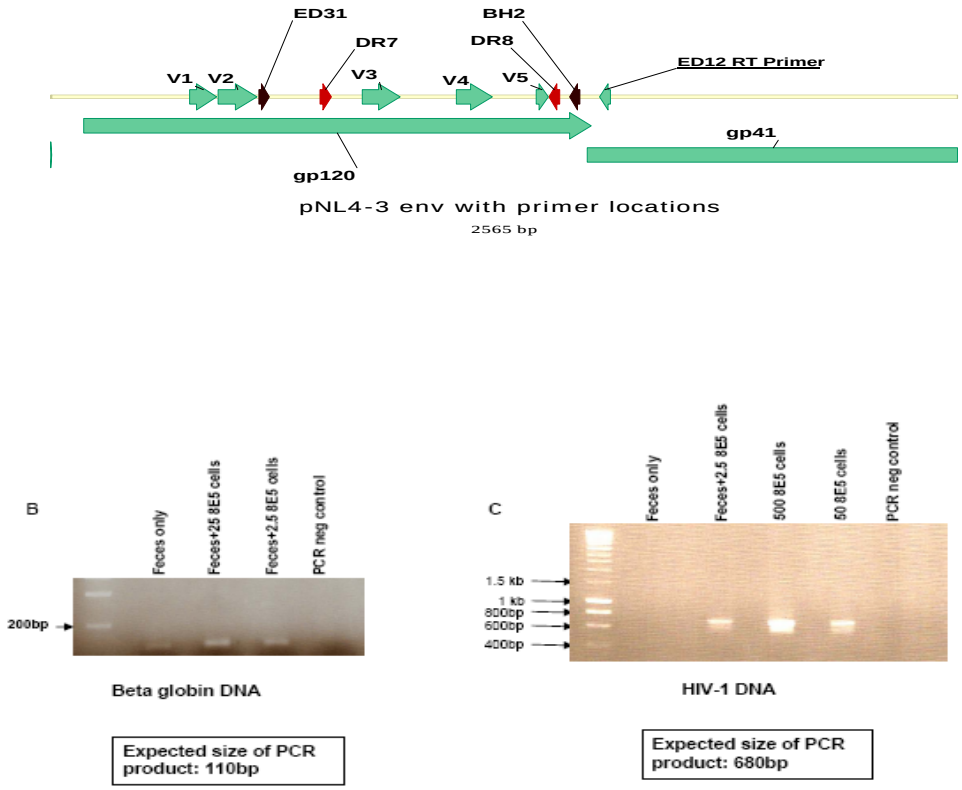


Figure 8A: Schematic representation of HIV-1 env primer.

8B. PCR was performed in isolated DNA using beta globin specific primers: lane 1 from normal stool, lane 2-3 from normal feces spiked with 25 and 2.5 8E5 cells respectively, lane 4 PCR negative controls

8C. Nested PCR was performed in isolated DNA using HIV-1 env specific primers: lane 1-3 from normal feces only, lane 4 from normal feces spiked with 2.5
8E5 cells, 5-6 from 500 and 50 8E5 cells as positive control, lane 7 PCR negative controls.

4.2.2 The nested RT-PCR sensitivity for detection of HIV-1 RNA:

Two hundred milligrams normal donor feces were spiked with different concentrations of HIV-1 positive plasma (with known HIV-1 RNA copies) and nucleic acid was isolated using NUCLISENS nucleic acid isolation kit. Initially, HIV-1 env specific RT primer, ED12, was used to synthesize cDNA from isolated RNA followed by nested-PCR reaction using the HIV-1 env specific primer that was used for HIV-1 DNA amplification. As the human RNA input control in each sample, cDNA was synthesized by random hexamer followed by nested PCR amplification using human beta-actin mRNA specific primers. As shown in Figure 9, HIV-1 RNA was detected in isolated RNA from normal feces spiked with HIV-1 positive plasma. To evaluate the influence of other non-human nucleic acid contained in the feces on the RNA isolation and PCR amplification procedures, RNA was isolated and amplified in parallel from the same HIV-1 positive plasma alone. A similar detection limit was observed with HIV-1 RNA as low as 40copies/reaction in the plasma sample with or without the presence of feces.

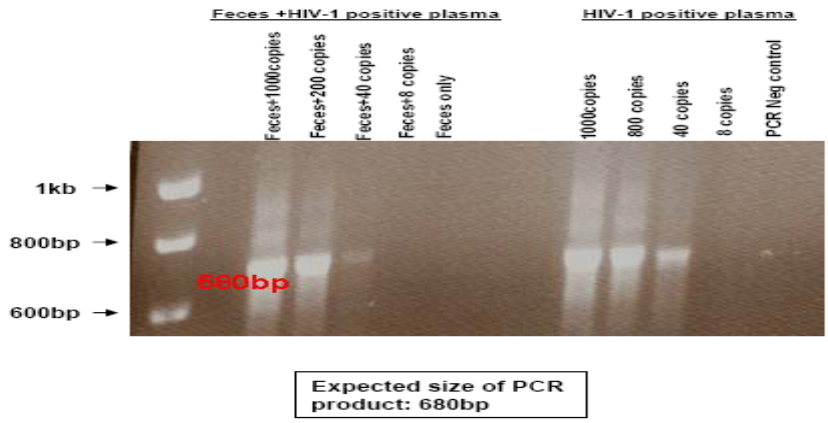
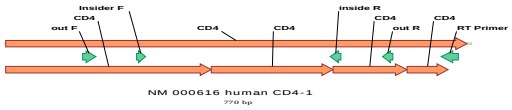


Figure 9. Quantitation of HIV-1 RNA in normal donor feces spiked w/HIV positive Plasma: Nested RT-PCR was performed using HIV-1 env specific primers in RNA isolated from normal feces only (lane 5) or spiked with HIV-1 positive plasma containing 1000 (lane 1), 200 (lane 2), 40 (lane 3) or 8 copies (lane 4) of HIV-1. The PCR run for same HIV-1 positive plasma as control (lane 6-10).

4.3 Detection of Human CD4 mRNA from human feces:

To monitor the evidence of human CD4+T cells shedding from GALT to feces, human CD4 mRNA specific RT and nested PCR primers were designed (Table 1) and used to detect CD4 mRNA from feces. Two hundred milligrams normal donor feces sample were spiked with different concentrations of CD4+ 8E5 cells and then nucleic acids were isolated. Human CD4 specific cDNA was synthesized using CD4 RT primer followed by nested-PCR amplification with CD4 specific primer set (Figure 10A). The final PCR product of 341bp was visualized in 2.5% agarose gel. Figure 10B, shows that CD4 mRNA was not detected from normal donor feces, but was detected in feces sample spiked with 50 8E5 cells.

A.



B.

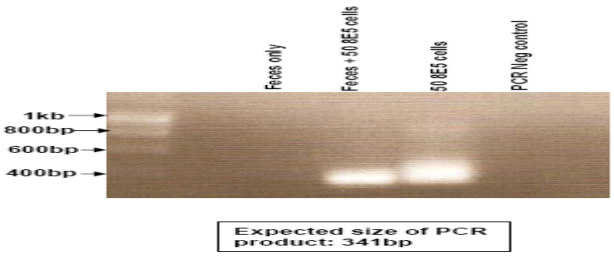


Figure 10A. Schematic representation of Human CD4mRNA Primer.

10B. Detection of Human CD4 mRNA from normal feces with or without 8E5 cells. Nested RT-PCR was performed in RNA from normal feces using human CD4 mRNA specific primers: lane 1—normal feces only, lane 2—normal feces spiked with 50 8E5 cells, lane 3---50 8E5 cell only as positive control, and lane 4—PCR negative control.

4.4 Detection of HIV-1 p24 antigen from fecal sample:

To detect HIV-1 protein from human fecal specimen, HIV-1 p24 ELISA was performed since p24 antigen is the most abundant structural component of the virus. Two hundred milligrams of normal donor feces sample were spiked with the virus culture supernatant after a serial dilutions ranging from 1:45 to 1:295245 (Chinese HIV-1 subtype B' isolate). Then these fecal extracts were tested for HIV-1 p24 antigen. To evaluate the influence of common fecal components, such as proteases (bacterial, microbial, plant) on the protein processing and detection, the same series of supernatant dilutions were also tested for HIV-1 p24. ELISA data in figure 11 shows that the detection limit is 15pg/ml and similar amount of HIV-1

p24 in the fecal extracts spiked with HIV-1 positive culture supernatant was also detected as compared to that of the same concentration of HIV-1 positive culture supernatant samples alone.

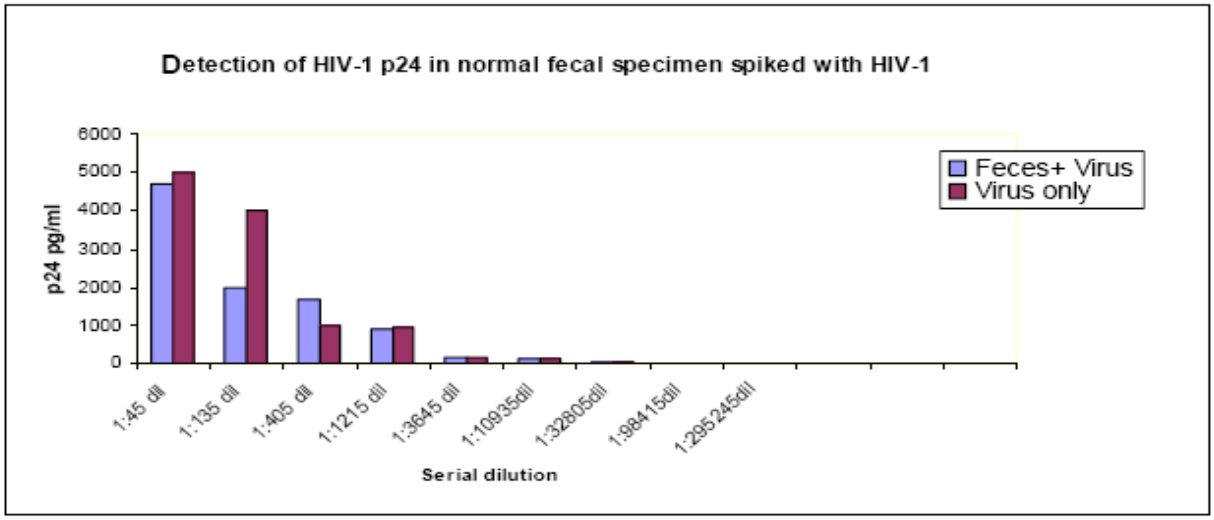


Figure 11: HIV-1 p24 was detected from normal donor feces sample spiked with HIV-1 virus. HIV-1p24 was not detected from normal donor feces as negative control.

SPECIFIC AIM #2: Detection of HIV-1 DNA/RNA and human CD4 mRNA in fecal specimens of the Multicenter AIDS Cohort Study volunteers and correlation of the findings with disease stages.

4.5: Detection of HIV-1 RNA/DNA from 2007 MACS volunteers fecal specimens:

4.5.1: Frozen fecal specimens from 2007 MACS Volunteers:

A total of 10 fecal specimens were collected from MACS volunteers and frozen in RNA later solution in 2007. Among these 10 volunteers, five subjects were on antiretroviral therapy (HAART) without detectable viral load in blood. Five

subjects were without antiretroviral therapy (HAART) and with detectable viral load ranging from 14,690 to 58,768 (Table 2)

Table 2: Clinical information of 2007 Multicenter AIDS Cohort Study (MACS) Volunteers.

Sample ID #	Viral Load(copies/ml of blood plasma)	CD4 count(mm3)	HAART
XX523	<50	752	Y
XX690	<50	517	Y
XX330	<50	469	Y
XX163	<50	600	Y
XX245	<50	454	Y
XX289	14690	473	N
XX293	23258	384	N
XX053	32778	501	N
XX274	55396	314	N
XX090	58768	575	N

4.5.2: Detection of HIV-1 DNA/RNA from 2007 MACS Volunteer fecal specimens:

Whole nucleic acid was isolated using NUCLISENS method from 200mg fecal sample followed by nested DNA PCR and nested RT -PCR using the same primer set described previously. HIV-1 DNA was not detected in any of the 10 fecal specimens, however as shown in Figure 5, HIV-1 RNA was detected in 2 out of 5 patients with detectable viral load.

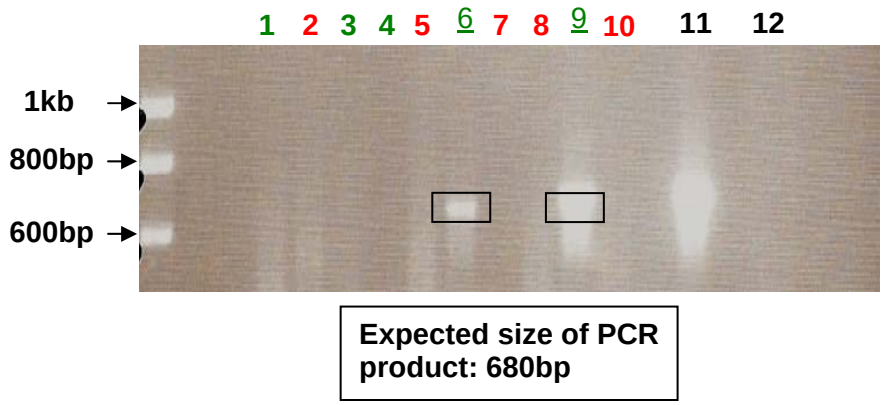


Figure12: Detection of HIV-1 RNA from 2007 frozen feces from HIV-1 infected donors Lane 1,3,4,6,9(Green): MACS donor without HAART, Lane 2,5,7,8,10(Red): MACS donor with HAART. Lane 11: RT control, Lane12: PCR negative control. Lane 6: XX274, viral load 55396, Lane 9: XX289, viral load 14690 were both detected in HIV-1 RNA.

4.6: Detection of HIV-1 RNA/DNA and CD4mRNA from 2008 MACS volunteer fecal specimens:

4.6.1 Collection of biological specimens from MACS volunteers in 2008:

Four groups of MACS volunteers were recruited. **Group A:** composed of 10 MACS volunteers who were HIV-1 negative; **Group B:** composed of 11 HIV-1 infected MACS volunteers with detectable viral load in plasma ranging from 428 to 78,636 and CD4+ T cell count ranging from 149 to 923. All these volunteers from this group were without any antiretroviral therapy; **Group C:** composed of 13 HIV-1 infected MACS volunteers with non-detectable viral load in plasma and CD4+ T cell count ranging from 385 to 922. All the volunteers from this group were under antiretroviral therapy; **Group D:** composed of 5 HIV-1 infected MACS volunteers with detectable viral load in plasma ranging from 186 to 33751 and CD4+ T cell count ranging from 152 to 540. All the volunteers from this group

were receiving antiretroviral therapy (Table 3). At the time of feces collection, patient's blood and urine samples were also collected.

Table 3: Clinical information of 2008 Multicenter AIDS Cohort Study (MACS) Volunteers

	ID	Sample Date	Viral Load(copies/ml)	CD4/mm3
GroupA=HIV-1 Negative N= 10	XX110	8/27/2008	N/A	642
	XX712	8/16/2008	N/A	1317
	XX163	8/19/2008	N/A	899
	XX983	8/19/2008	N/A	1153
	XX271	9/5/2008	N/A	828
	XX003	9/16/2008	N/A	547
	XX744	9/25/2008	N/A	1520
	XX186	9/24/2008	N/A	839
	XX148	9/17/2008	N/A	724
	XX021	8/19/2008	N/A	845
GroupB=HIV-1 Positive/No antiretroviral treatment(HAART) N = 11	XX280	8/26/2008	1003	923
	XX495	8/16/2008	35471	238
	XX326	8/23/2008	20149	392
	XX286	9/17/2008	2974	320
	XX119	8/26/2008	18231	301
	XX200	8/26/2008	58200	149
	XX305	9/4/2008	582	388
	XX053	9/26/2008	78636	406
	XX109	9/25/2008	428	358
	XX013	9/12/2008	20441	457
	XX634	9/18/2008	6779	352
GroupC=HIV-1 Positive/ HAART/ Non-	XX484	8/19/2008	<50	440
	XX008	8/20/2008	<50	890
	XX523	9/5/2008	<50	696
	XX245	9/23/2008	<50	566
	XX163	9/10/2008	<50	549
	XX690	9/9/2008	<50	385

(Table 3 Continued)

detectable viral load **N = 13**	**XX005**	**8/28/2008**	**<50**	**777**
	XX144	**8/28/2008**	**<50**	**583**
	XX154	**8/20/2008**	**<50**	**922**
	XX327	**9/30/2008**	**<50**	**426**
	XX350	**9/3/2008**	**<50**	**466**
	XX263	**9/17/2008**	**<50**	**697**
	XX265	**9/25/2008**	**<50**	**478**
GroupD=HIV-1 Positive/HAART/ detectable viral load N=5	**XX127**	**9/3/2008**	**694**	**161**
	XX229	**8/28/2008**	**186**	**540**
	XX371	**9/10/2008**	**33751**	**152**
	XX099	**9/23/2008**	**842**	**153**
	XX274	**9/9/2008**	**16842**	**279**

4.6.2 Detection of HIV-1 DNA in feces from 2008 MACS volunteers fecal specimens

DNA was isolated using NUCLISENS method from 200mg fecal specimen followed by nested-PCR using the same primer set described previously. For every DNA sample, human beta-globin gene was PCR amplified to ensure the comparable amount of human DNA contained in the isolated DNA sample. Beta globin gene was detected in 17 out of 39 fecal specimens. Among these 17 beta globin positive samples were 1of 10 from Group A, 8 of 11 from Group B, 3 of 13 from Group C and 5 of 5 from Group D. The representative gel picture of this beta globin detection is shown below in Figure 13A. However, HIV-1 DNA was detected only in one fecal specimen from a patient with detectable viral load (XX326, viral load 20,149, Group B) as shown below in Figure 13B.

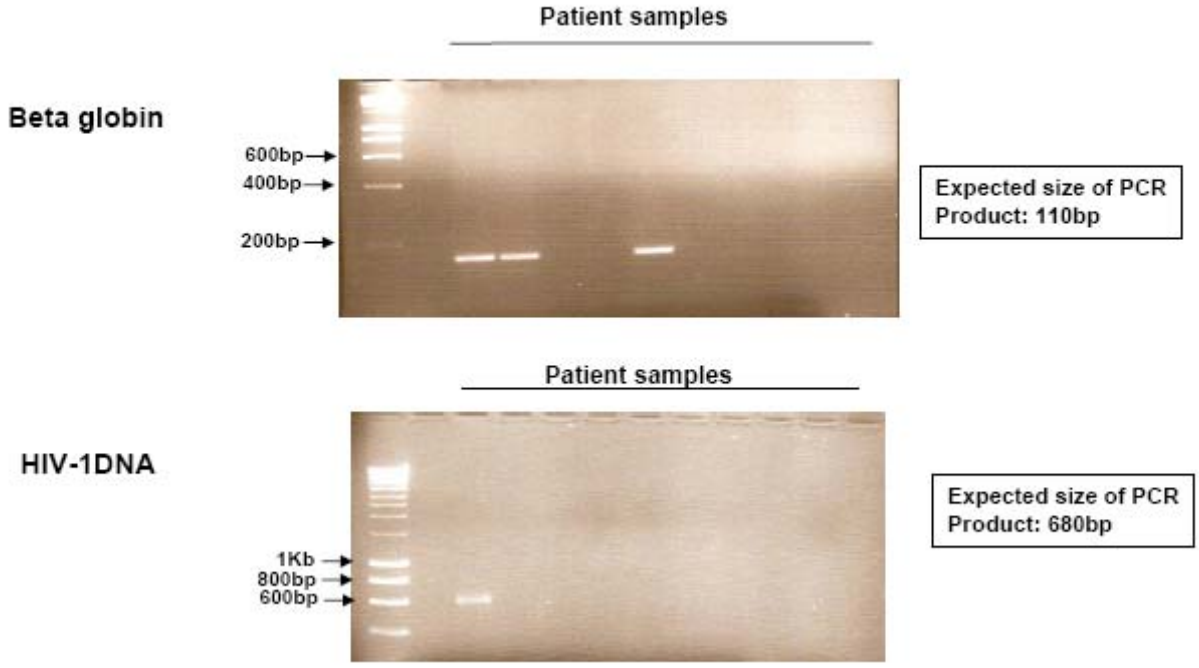


Figure 13A: Representative figure of Detection of human beta-globin from 2008 MACS donor feces sample.Lane1, 2: human beta-globin from MACS donors, 3, 4: human beta-globin not detected, Lane 5: PCR control.

13B: Representative figure of Detection of HIV-1 DNA from 2008 MACS donor feces sample. Lane1: Subject XX495; HIV-1 DNA detected.

4.6.3 Detection of HIV-1 RNA in feces from 2008 MACS volunteers fecal specimens:

RNA was isolated using NUCLISENS method from 200mg of fecal specimen, followed by RT nested-PCR. Initially, HIV-1 env specific RT primer, ED12, was used to synthesize cDNA from isolated RNA samples followed by nested-PCR using the same primer set for HIV-1 DNA amplification. To serve as the human RNA input control in each sample, cDNA was synthesized from the RNA sample using random hexamer followed by nested PCR amplification with human beta-

actin mRNA specific primers. As demonstrated in Figure 14, relatively equal amounts of beta actin mRNA were detected from all isolated fecal RNA, whereas HIV-1 RNA was detected in 3 out of 16 (19%) patients with detectable viral load in blood.

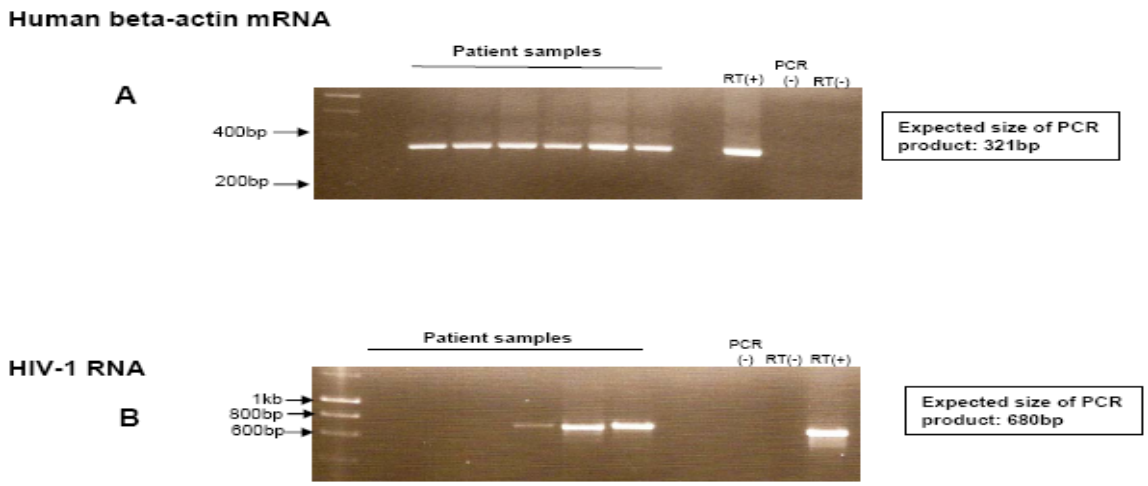


Figure 14A: Representative picture of detection of Human beta actin mRNA from 2008 MACS donor feces sample. Lane 1-6: MACS donor feces samples, Lane 7: PCR negative control, Lane 8: RT negative control, Lane 9: RT control

14B: Representative picture of detection of HIV-1 RNA from 2008 MACS donor feces sample. Lane 1-7: MACS donor feces samples, Lane 8: PCR negative control, Lane 9: RT negative control, Lane 10: RT control

4.6.4: Confirmation of PCR specificity by using primers for another region (Gag) of HIV-1 genome:

To confirm the PCR amplification specificity of HIV env target, another primer pair, binding to HIV gag region, was designed and used for RT-nested PCR as described previously. Two out of three env positive and two out of two envelope

negative fecal RNA samples maintained the identical outcome using Gag specific primers (Figure 15).

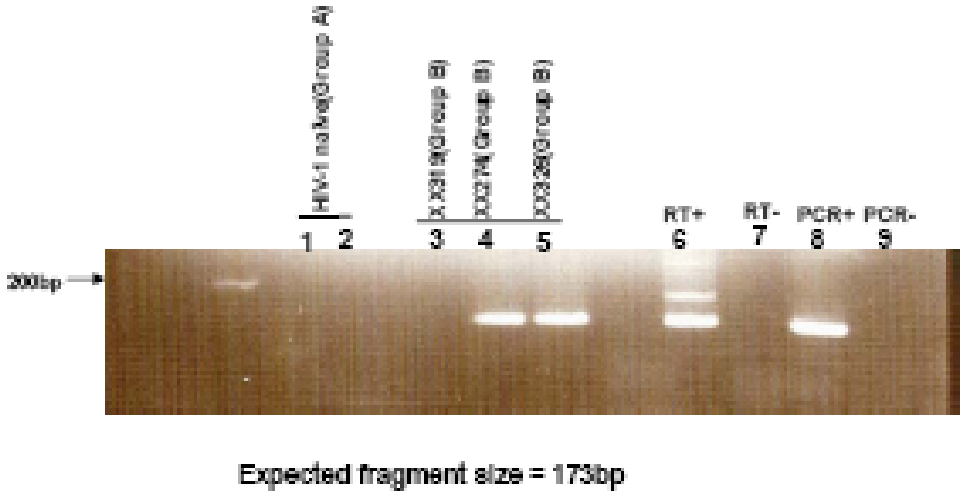


Figure 15: Confirmation of PCR specificity by using primers from another region (Gag) of HIV-1 genome. Lane 1-5: MACS donor feces samples, Lane 6: RT control, Lane 7: RT negative control, Lane8: PCR positive control, Lane 9: PCR negative control.

4.6.5 Detection of Human CD4 mRNA from MACS volunteer fecal specimens:

To monitor CD4 mRNA contained in isolated fecal RNA samples from MACS donors, human CD4 mRNA specific RT and nested PCR primers were designed to amplify 110 bp PCR products. Human CD4 mRNA was detected in 5 out of 16 (32%) patients with detectable viral load in blood.Whereas, no CD4 mRNA was detected in any HIV-1 uninfected donor's fecal specimens. The representative result of human CD4mRNA detection by nested RT-PCR has been shown below in figure 16.

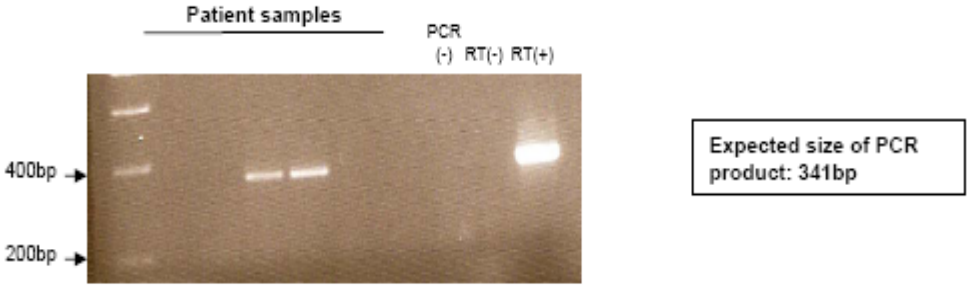


Figure 16: Representative picture of detection of human CD4mRNA from 2008 MACS donor feces. Lane 1-5: MACS donor feces samples, Lane 7: PCR negative control, Lane 8: RT negative control, Lane 9: RT control

All the results from 2008 MACS donor fecal samples are summarized below in Table 4.

Table 4: Summary of detection of HIV-1RNA/DNA in fecal specimens of 2008 Multicenter AIDS Cohort Study (MACS) volunteers.

	Group A (HIV Negative)	**Group B (HIV+/HAART Naive)**	**Group C (HIV+/HAART/ Non-detectable viral load)**	**Group D (HIV+/HAART/ detectable viral load)**
HIV-1 RNA	**0/10**	**2/11**	**0/13**	**1/5**
HIV-1 DNA	**0/1**	**1/8**	**0/3**	**0/5**
CD4mRNA	**0/10**	**3/11**	**0/13**	**2/5**

Numerator = Number of subjects detected with HIV-1 RNA/DNA and CD4mRNA.

Denominator = Total number of subjects whose housekeeping genes (beta actin/ beta globin) were detected

4.7: Detection of HIV-1 p24 antigen from MACS donor feces samples:

To detect HIV protein from MACS donor feces samples, HIV-1 p24 ELISA was performed in all fecal specimens. Two hundred milligram of MACS donor feces sample were treated with protease inhibitor cocktail (Roche) and tested for HIV-1 p24 antigen. No HIV-1 p24 in the fecal extracts from these donors were detected in ELISA. HIV-1 p24 was detected in normal donor feces sample spiked with HIV-1 positive culture supernatant.

4.8: Detection of fecal occult blood from 2008 MACS donor feces samples:

To detect the possible blood content in the fecal samples, an occult blood test was performed in all 2008 MACS fecal specimens as described in Materials and Methods section. As shown in Table 4, fecal blood was detected in 7 out of 39 MACS specimens: 2 from Group A(HIV-1 Negative), 1 from Group B (HIV-1 infected/ Not under HAART), 3 from Group C (HIV-1 infected/under HAART/ non-detectable viral load) and 1 from Group D (HIV-1 infected/under HAART/ detectable viral load)(Table 5). Fecal occult blood test was negative for the fecal samples which were HIV-1 RNA or DNA positive. One of the five CD4mRNA positive fecal specimens was positive for occult blood; the remaining samples were negative.

Table 5: Summary of detection of Fecal Occult blood in 2008 Multicenter AIDS Cohort Study(MACS)volunteers.

Group	ID	Sample Date	Viral Load(copies/ml)	CD4/mm3	Fecal Occult Blood detection(Yes/No)
Group A= HIV negative	XX271	9/5/2008	N/A	828	Yes
	XX744	9/25/2008	N/A	1520	Yes
Group B = HIV-1 Positive/ Noantiretroviral treatment(HAART)	XX053	9/26/2008	78636	406	Yes
Group C = HIV -1 Positive/ HAART/Non-detectable viral load	XX245	9/23/2008	<50	566	Yes
	XX690	9/9/2008	<50	385	Yes
	XX265	9/25/2008	<50	478	Yes
Group D = HIV-1 Positive/ HAART/ detectable viral load	XX099	9/23/2008	842	153	Yes

SPECIFIC AIM #3: Detection of HIV-1 DNA/RNA in urine specimens of Multicenter AIDS Cohort Study volunteers and correlation of the outcome with results from the blood and fecal specimens.

4.9 Measurement of HIV-1 RNA/DNA from 2008 MACS Volunteers :

Urine samples, volume ranging from 19ml-80ml, were processed to acquire urine concentrated supernatant and pellet as described in the Materials and Methods section. DNA and RNA were isolated from the urine pellet and concentrated supernatant, respectively.

4.9.1: Detection of HIV-1 RNA/DNA from 2008 MACS volunteer urine specimen:

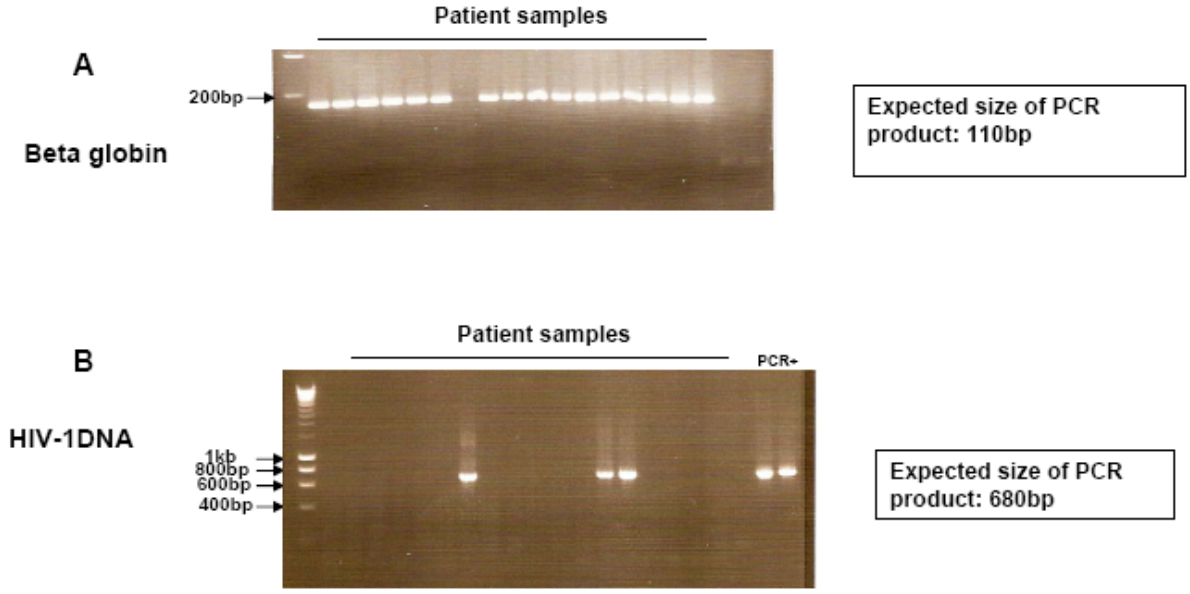


Figure 17A: Representative picture of detection of human beta globin from Urine sample. Lane 1-9: beta globin detected **17B:** Representative picture of detection of HIV-1 DNA from Urine sample.Lane 1-4: HIV-1 DNA detected, Lane 5: PCR control

HIV env region was amplified from the DNA purified from the MACS donor urine pellet in nested-PCR reaction using the same env primer set described above. For each DNA sample, human beta-globin gene was PCR amplified to ensure the comparable amount of human DNA contained in the isolated DNA sample.Table 6, shows that all urine pellet samples were positive for beta-globin amplification. HIV-1 DNA was detected in 5 urine pellet samples from HIV infected patients, 3 of 5 with detectable viral load, and 2 of 6 with undetectable viral load.

4.9.2: Detection of HIV-1 RNA from MACS Volunteer urine specimen:

HIV env region was amplified from RNA purified from the urine supernatant by RT nested-PCR. Initially, HIV env specific RT primer, ED12, was used to synthesize cDNA from isolated RNA samples followed by nested-PCR using the same primer set for HIV DNA amplification. As shown in Figure 18, HIV-1 RNA was detected in one concentrated urine sample from a patient with detectable viral load.

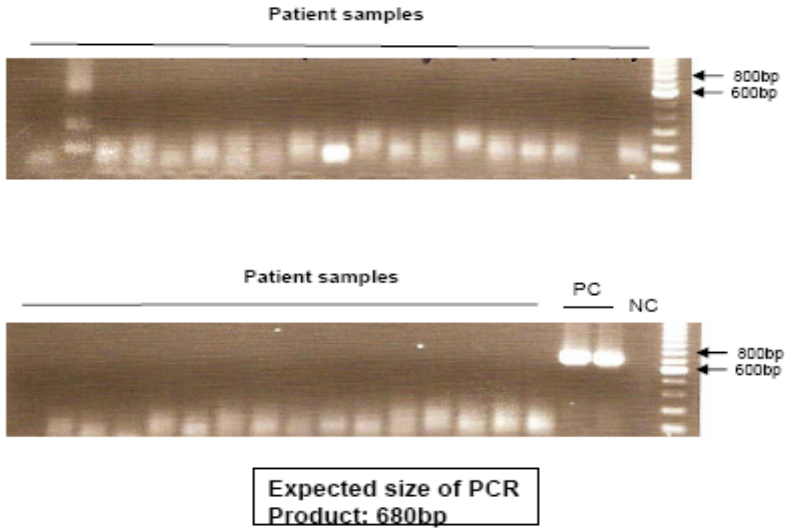


Figure 18: Detection of HIV-1RNA in urine specimens of 2008 Multicenter AIDS Cohort Study (MACS) volunteers.

Summary of detection of HIV-1RNA/DNA in Urine specimens of 2008 Multicenter AIDS Cohort Study (MACS) donors are shown below in Table 6.

Table 6: Summary of detection of HIV-1RNA/DNA in Urine specimens of 2008 Multicenter AIDS Cohort Study (MACS) volunteers.

	Group A= HIV Negative	**Group B = HIV-1 Positive/ Noantiretroviral treatment(HAART)**	**GroupC=HIV-1 Positive/ HAART/ Non-detectable viral load**	**GroupD=HIV-1 Positive/HAART/ detectable viral load**
HIV-1 RNA	**0/9**	**1/10**	**0/8**	**0/9**
HIV-1 DNA	**0/9**	**2/10**	**3/9**	**2/8**

Now overall summary of detection of HIV-1RNA/DNA/ human CD4mRNA /fecal occult blood from 2008 Multicenter AIDS Cohort Study (MACS) volunteers are shown below in Table 7.

Table 7: Summary of detection of HIV-1RNA/DNA/ human CD4mRNA /fecal occult blood from 2008 Multicenter AIDS Cohort Study (MACS) volunteers.
Group A: total numbers of subjects detected= 2, Group B: total numbers of subjects detected= 8, Group C: total numbers of subjects detected= 5, Group D total numbers of subjects detected= 3

Group	ID	Viral Load(copies /ml)	CD/mm3	URINE		FECES			
				HIV-1DNA	HIV-1 RNA	HIV-1 DNA	HIV-1 RNA	CD4 mRNA	Fecal occult blood
Group A (N=10)	XX271	N/A							+
	XX744	N/A							+
Group B (N=11)	XX053	78636	406						+
	XX280	10003	923					+	-
	XX495	35471	238			+			-
	XX326	20149	392				+		-
	XX286	2974	320					+	-
	XX119	18231	901		+		+	+	-
	XX200	85820	149	+					-
	XX013	20441	457	+					-
Group C (N=13)	XX245	<50	566						+
	XX690	<50	385						+
	XX265	<50	478	+					+
	XX523	<50	696	+					-
	XX327	<50	426	+					-
Group D (N=5)	XX099	842	153					+	+
	XX274	16862	279	+			+	+	-
	XX371	33751	152	+					-

5.0 DISCUSSION

In this study, we have developed sensitive methods for detection of HIV-1 RNA/DNA and CD4 mRNA in feces. Using these methods, we have measured the amount of HIV-1 RNA/DNA and CD4 mRNA in feces samples from HIV-1 uninfected as well as HIV-1 infected patients with or without antiretroviral therapy. We also determined if this detection correlates with the patient's viral load or not.

The components of human feces are very complex, containing DNA/RNA and proteins from different species, proteases, various inhibitors[44]. However, fecal samples, which can be obtained easily by non-invasive technique, have been used in laboratory tests for intestinal pathogens or other diseases, such as: antibody-antigen type test to look for a specific virus, microscopic examination for intestinal parasites, laboratory culture to find bacterial and viral pathogens and PCR/RT-PCR to detect viral nucleic acid. Many viral pathogens have been detected from the feces, such as astrovirus, rotavirus, coronavirus, calcivirus, adenovirus and hepatitis-C virus [45]. However, no comprehensive studies have been performed so far on the fecal samples from HIV-1 infected patients. A few available reports in the literature showed that HIV-1 RNA has been detected in feces from HIV-1 infected children and adults [25] at acute stage of infection with high viral load in plasma. Furthermore, no reports have been found so far to evaluate HIV and CD4 mRNA level in feces from patients at chronic stages of disease with or without Highly Active Anti retroviral Therapy (HAART).

Since the components of human feces are very complex and so far no sensitive methods for detection of viral and human RNA/DNA in human feces have been established, we have developed the sensitive methods for isolation and detection of HIV-1 DNA/RNA and human CD4 mRNA in feces. Our data showed that HIV-1 DNA was detected from normal donor feces spiked with 8E5 cells with the detection limit of 2.5 copies of HIV DNA /reaction. HIV-1 RNA was detected from normal feces spiked with HIV positive plasma with detection limit of 40 copies of HIV RNA/reaction. There is no influence of complex components contained in the feces on the isolation and PCR amplification process since HIV RNA from the same HIV-1 positive plasma (without feces) showed a similar level of detection .

GALT contains an abundant amount of CD4+ T cells to maintain the mucosal immunity. HIV-1 infection causes a massive CD4+ T cell depletion in GALT and compromised mucosal immunity[16]. Ideally to monitor HIV-1 related GALT pathogenesis, a GI biopsy should be performed regularly to evaluate the dynamic changes of CD4 T cells in GALT, however such studies are very difficult to implement. We hypothesize that during the massive CD4+ T cell depletion, CD4+ T cells shed into intestinal lumen and the amount of CD4+ T cells in the feces would associate with the pathogenic changes in GALT. To date no comprehensive studies have been performed to monitor the dynamic changes of CD4+ T cell levels in feces. In the harsh environment of GI tract, it is very difficult to detect and quantitate the intact CD4+ T cells and CD4 proteins in fecal specimens. Therefore, we have developed PCR methods to detect CD4 mRNA from feces. As expected, CD4 mRNA was not detected from normal donor feces, but detected from normal feces spiked with 50 HIV-1 infected CD4+ 8E5 cells.

In order to monitor HIV-1 p24 protein in stool samples from HIV-1 infected donors, we have standardized the detection method using normal donor feces spiked in vitro with serial dilutions of HIV-1 virus treated with protease inhibitors and then detected HIV-1 p24 using a commercially available ELISA kit. ELISA data (shown in figure 11), revealed that the detection limit is 15pg/ml, which was similar to detection limit observed in HIV-1 virus alone without feces. These data showed that the normal fecal constituents are not interfering with the HIV-1 p24 detection in our currently used method, which have been used in subsequent study for HIV-1 p24 detection in MACS donor feces samples.

To evaluate the significance of using fecal specimens to monitor HIV-1 associated GALT pathogenesis, we have collected the feces samples from HIV-1 negative and HIV-1 infected donors of the Multicenter AIDS Cohort Study (MACS) with or without HAART treatment. Among samples collected and frozen for one year,we have detected HIV-1 RNA in 2 feces out of 6 with detectable viral load and without any anti- retroviral therapy. HIV-1 DNA was not detected from any of the fecal specimens. One reason for this lower detection limit could be that the 1 year frozen condition reduced the detectable HIV-1 DNA.

DNA/RNA was isolated from 39 freshly frozen fecal samples collected in 2008 and the amount of human DNA/RNA contained in each sample was quantified. Subsequently, HIV-1 DNA/RNA and CD4 mRNA in the samples were tested. Our results showed that HIV-1 DNA was detected in 1 subject from Group B (HIV-1 infected but not on HAART) with detectable viral load of 35,471copies/ml in plasma. HIV-1 RNA was detected in two subjects from Group B (HIV-1 infected but not on HAART) and in one subject from Group C (HIV-1 infected on HAART). The HIV-1 detection rate is lower in feces samples from patients with

detectable plasma viral loads and is lower compared to the 67% detection level reported in the paper by Boom et al. However, in the Boom et al. study, all feces samples were collected from patients in acute stage of infection, whereas in our current study, all feces samples were collected from patients in the chronic stage of infection. Also in Boom et al. paper those subjects tested were not receiving antiretroviral treatment but all the subjects (in Group C and Group D) were under HAART therapy. However the subjects from Group B were not under any HAART treatment. So far no comprehensive study has been performed for monitoring the HIV-1 status in feces from patients in chronic stage of infection. It is likely that levels of HIV shedding from GALT to intestinal lumen is higher in the acute phase of infection compared to the later phase of infection because of the rapid replication of HIV-1 and destruction of lymphoid tissues that occurs in GALT during early phase of infection.

All feces samples were screened for CD4 mRNA, a surrogate marker for CD4+ T cells. No CD4 mRNA was detected in any feces samples from the HIV-1 negative donors, but CD4 mRNA were detected from 4 out of 5 feces samples from the subject with detectable viral load, three from group B (HIV-1 infected but not under any HAART) and two from group D (HIV-1 infected on HAART but with detectable viral load) .These results suggest that CD4+ T cell shedding from GALT to intestinal lumen can be associated with high virus shedding in feces.

Due to the complexity of fecal DNA/RNA contents, it is necessary to validate the PCR specificity in the PCR amplification reaction. For this purpose, we designed another primer pair hybridizing to the gag region of HIV-1 genome and tested them in two HIV negative and three HIV-1 positive feces samples determined previously by PCR reaction with env primers. In these selected five samples, the

identical results were observed in four samples with the gag primers. This result strongly indicated that the env primers were specifically amplifying HIV-1 env regions. However, one HIV positive feces sample determined with env primer turned out negative with gag primers. This might be due to the extremely low copy number of HIV genomes contained in the sample, which could lead to limited PCR positive detection in the PCR amplifications.

Measurement of HIV proteins is another commonly used method for quantitation of HIV concentration in a biological specimen. To measure HIV-1 proteins contained in feces samples from MACS patients, the HIV-1 gag protein p24, was measured by ELISA in the supernatant derived from feces treated with protease inhibitors. However, HIV-1 p24 was not detected in any of the feces samples from MACS donors including some feces samples with positive HIV-1 RNA result (showed in Figure 5). Lack of detection of HIV-1 protein in feces could be due to: 1) low level of p24 protein present in the sample, which was below the p24 ELISA detection limit; 2) HIV-1 p24 proteins might be degraded by proteases in the intestinal lumen before voiding out of the GI tract. 3) Sensitivity of detection of p24 is much lower than RNA detection as 1pg of HIV-1 p24 is equivalent to 10,000 copies of RNA. Further experiments are needed to explore these possibilities.

Fecal occult blood is a term for blood present in the feces that is not visibly apparent. Detection of blood in feces has long been regarded as an indicator of patient's state of health[46].

HIV-1 RNA/DNA and CD4 mRNA detected in the fecal specimens of HIV-1 infected patients could be the result either from internal bleeding in the

gastrointestinal tract or from shedding of HIV-1 infected cell and free virus from GALT. To dissect these two possibilities, fecal blood test was performed in all 2008 samples. As listed in Table 4, a total of 7 fecal samples were positive in the test: 2 from Group A, 1 from Group B, 3 from Group C and 1 from Group D. No fecal samples positive for HIV-1 RNA/DNA were positive in the occult blood test. This result strongly suggests that the HIV-1 RNA/DNA detected in the fecal samples were from the shedding of HIV-1 infected cells or virus from GALT into intestinal lumen.

To accurately assess the occult blood in fecal sample, certain diet restriction should be imposed in a few days before collecting fecal samples. Unfortunately, in this study, patients were not informed about the diet restriction before sample collection and unrestricted food intake might lead to false positive result in the occult blood test. One specimen from Group D (XX099) was positive in occult blood test and also positive for CD4 mRNA. Therefore, the CD4 mRNA detected in the fecal sample of patient XX099 could be due to the blood contained in the feces or CD4+ T cells shed from GALT due to HIV-1 associated pathogenesis. Further study is needed to dissect the two possibilities.

Detection of HIV-1 DNA/RNA in HIV-1 infected donor urine was also examined as urine could serve as an important vehicle for HIV-1 transmission. A wide range of renal diseases have been described in HIV-1 infected patients [47]. Li et al. in 1992 reported the presence of HIV-1 genomic sequence and gene products from fresh urine pellets from HIV-1 seropositive individuals. By using the PCR based method HIV-1 DNA proviral sequences were detected in 53 of 80 (66.25%) fresh urine pellets from HIV-1-seropositive individuals [27] . But so far no cross sectional studies have been shown to detect HIV-1 RNA/DNA from infected

donors urine samples in the presence or absence of anti-retroviral therapy. Our data showed that it is possible to detect HIV-1 DNA and RNA from an infected donors urine pellet and concentrated supernatant respectively regardless of the corresponding plasma viral load. A nested RT-PCR and nested DNA PCR were chosen for the detection of HIV-1RNA/DNA and CD4mRNA because it is more rapid and sensitive for detection.

HIV-1 DNA was not detected in any of urine samples from the HIV negative donors, but were detected from 2 out of 10 urine pellets from group B (HIV-1 infected but not on HAART) and both of them with high viral load, 3 out of 13 urine pellets from Group C(HIV-1 infected on HAART/ non-detectable plasma viral load) and 2 out of 5 urine pellets from group D (HIV-1 infected on HAART but with detectable viral load. Thus our data from Table 6 demonstrates that HIV-1 DNA from urine pellet was detected in patients across a range of plasma viral load. This result suggested that HIV-1 DNA detected in urine not always accompanied with the shedding of virus in high quantity. We have detected only one HIV-1 RNA from subject XX119 from group B (HIV-1 infected but not on HAART).

HIV can be present in a variety of body fluids and secretions like blood, semen, vaginal fluids and breast milk, but the presence of HIV-1 in urine in chronic infected subjects with or without antiretroviral therapy (HAART) has not been addressed yet. On the basis of this inquisition, the question of whether urine is potentially an infectious body fluid in HIV-1 infected individual is raised. So far attempts of isolating the infectious HIV-1 from urine of HIV-1 seropositive donors were largely unsuccessful[48]. Detection of HIV-1 DNA/ RNA from urine of the HIV infected individual can be extremely important as this might lead to future

work to evaluate whether urine could potentially serve as a vehicle for HIV-1 transmission.

So far, fecal and urine specimens have not been studied extensively, most likely because of the challenges with recovery and inhibition of amplification of viral DNA and RNA. Our cross sectional study data based on a simple PCR based detection method showed that it is possible to isolate and detect HIV-1 RNA/DNA and CD4 mRNA from feces and urine samples. This study indicates that feces and urine may therefore be two suitable specimens for diagnosis of HIV-1 infection without the need for invasive sampling techniques. In addition the detection of CD4mRNA from feces samples could lead to the evaluation of the immune profile of Gut Associated Lymphoid Tissue (GALT) of HIV-1 infected individual. Knowledge of this detection is of importance in public health for the possible future development of therapeutic intervention against HIV-1. Future longitudinal studies will reveal whether there is a correlation between onset of gastrointestinal symptoms and detection of HIV-1 RNA/DNA and CD4mRNA in feces and urine samples of the HIV-1 infected individual.

6.0 FUTURE DIRECTIONS

Future studies using this simple non-invasive technique will further explore whether it is possible to detect various human gut cytokine mRNA from HIV-1 infected donor feces. Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They must be produced de novo in response to an immune stimulus. Secretion of various gut cytokines in fecal specimens in response to HIV-1 infection may reveal the immune activation status of the infected individual for future therapeutics development[49]. Chemokines like RANTES, MCP-1, MIP-1a, and MIP-1b could be evaluated if they could be detected in feces samples.

Future longitudinal study is essential to evaluate the detection of HIV-1 RNA/DNA and CD4 mRNA in feces and urine samples of the Multicenter AIDS Cohort Study Donors. Ideally feces and urine samples need to be tested on different time points like 0 month, 3 month, 6 month, 1 year and 2 years in the same cohort from MACS donors. This further longitudinal study is necessary to correlate the detection of HIV-1 RNA/DNA and CD4mRNA with the disease progression and prognosis.

7.0 PUBLIC HEALTH IMPLICATION

The study of disease progression and prognosis in AIDS patients with respect to immunopathological changes in GALT is challenging because of the limited availability of gut tissue from infected donors. Depletion of CD4+ T cells during the course of HIV-1 infection is a hallmark of AIDS pathogenesis, but so far the specific mechanisms are not well established. Therefore a detection and diagnosis approach based on simple noninvasive technique is essential in this aspect.

The biggest advantage of detecting HIV-1 from urine and fecal samples is that the patient does not need to have blood drawn with a needle. This makes it easier and less painful to obtain a sample, particularly with very young children. In addition, this method of collection is safer for the health-care worker because there is very little risk that the worker will be exposed to the virus, something that can occur if he or she is accidentally stuck with the same needle used to draw the patient's blood. A urine and feces test could become a significant weapon in the battle to slow the AIDS epidemic because it makes testing safer, easier and more accessible compared with the standard blood test. Thus our cross sectional study has major implications for public health, since through this simple non-invasive method of obtaining feces and urine samples we can assess HIV-1 profile and immune status in the gastrointestinal tract of an HIV-1 infected patient.

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