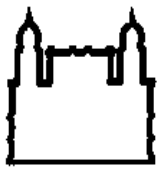

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FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa

TESE DE DOUTORADO

FATORES GENÉTICOS E CLÍNICOS RELACIONADOS À INFECÇÕES PELO HIV-1 E HTLV-1

FILIPE FERREIRA DE ALMEIDA REGO

Salvador - Bahia

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Tese apresentada ao Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa para obtenção do título de Doutor.

Orientador: Prof. Dr. Luiz Carlos Júnior Alcântara

Co-orientador: Prof. Dr. Tulio de Oliveira

Salvador - Bahia

2014

Ficha Catalográfica elaborada pela Biblioteca do
Centro de Pesquisas Gonçalo Moniz / FIOCRUZ - Salvador - Bahia.

Rego, Filipe Ferreira de Almeida
R343f Fatores genéticos e clínicos relacionados à infecções pelo HIV-1 e HTLV-1. / Filipe Ferreira de Almeida Rego. - 2014.
109 f. : il. ; 30 cm.

Orientador: Prof. Dr. Luiz Carlos Júnior Alcântara, Laboratório de Hematologia, Genética e Biologia Computacional**.**
Tese (Doutorado em Biotecnologia em Saúde e Medicina Investigativa) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2014.

1. HTLV-1. 2. HIV-1. 3. LTR. 4. Antirretrovirais. 5. Coinfecção. I. Título.

CDU 616.98

Quando tudo está perdido

sempre existe um caminho

Renato Russo

Dedico este aos que gostariam de estar presente nesta conquista mas que por razões divinas passaram a me ajudar de um outro lugar e a minha mãe, que me guiou pelos caminhos da vida e por demonstrar que mesmo os momentos mais difíceis podem ser superados com serenidade e bom humor.

AGRADECIMENTOS

A minha mãe, Maria das Graças Ferreira Rego, que mesmo com todas as dificuldades e limitações, não poupou esforços para me educar e oferecer as condições necessárias para que eu concluísse mais uma etapa de minha vida e também por todo o amor e carinho doado a mim durante toda a minha existência.

Ao meu pai, José Fernando de Almeida Rego (*in memorian*), pelo exemplo de caráter inigualável, amor e pelo papel fundamental em minha educação.

As minhas avós (*in memorian*) pelo exemplo de vida e amor que guardarei comigo para todo o sempre.

A minha família e amigos que conferem a base da minha existência e que pude sempre contar com o apoio mesmo em momentos em que as rosas não pareciam mais rosas.

Ao professor e orientador Dr. Luiz Carlos Junior Alcântara que me transmitiu conhecimentos valiosos, sempre me dando apoio e motivação que foi de grande importância para a conclusão deste trabalho.

Ao professor Dr. Tulio de Oliveira por seu apoio e inspiração no amadurecimento dos meus conhecimentos e conceitos que me levaram a execução e conclusão desta tese, além de seu espírito empreendedor na tarefa de multiplicar os conhecimentos.

A todos os professores do Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa com os quais tive a oportunidade e o prazer de aprender.

A todos os colegas de trabalho do LHGB pelos agradáveis momentos vividos e apoio

A Biblioteca de Ciências Biomédicas Eurydice Pires de Sant'Anna pelo auxilio na formatação da tese.

A todos os coautores dos trabalhos pela confiança e colaboração.

REGO, Filipe Ferreira de Almeida. Fatores Genéticos e clínicos relacionados à infecções por retrovírus humanos. 109 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2014.

RESUMO

Nesta tese foram realizados três trabalhos distintos sendo que todos envolvem identificar possíveis fatores genéticos ou clínicos relacionados com a infecção pelo HIV-1 ou pelo HTLV-1 ou por ambos. No primeiro trabalho nós objetivamos identificar mutações que poderiam estar relacionadas com o desenvolvimento da TSP/HAM ou carga proviral. Para isto sequenciamos a região LTR5' do HTLV-1 por Ion Torrent para verificar mutações com baixa frequência. Nós encontramos mutações em 52 posições que estavam presentes em mais de um indivíduo, porém apenas 11 destas estavam presentes em TFBS previamente descritos. Três mutações que não estavam presentes em TFBS previamente descritos foram estatisticamente significantes quando comparadas entre os grupos, sendo que estes sítios podem ser importantes para a mediação da transcrição viral. No segundo estudo nós objetivamos determinar a prevalência do genótipo selvagem em Hlabisa, Kwazulu-Natal na África do Sul além de identificar possíveis fatores associados a presença deste genótipo em 220 pacientes submetidos a ART. O genótipo selvagem foi detectado em 28 amostras (12,7%). Selecionamos 11 pacientes para realizar o sequenciamento pelo Ion Torrent, nove confirmaram não ter mutações de resistência aos antirretrovirais em alta frequência. Foi encontrada uma alta contagem de CD4+ no início da terapia associado a falha terapêutica assim como uma alta carga viral antes da genotipagem e não foi encontrado associação entre aderência a terapia auto-reportada e a presença do genótipo selvagem. Aproximadamente um em cada oito adultos que falham a terapia possuem o genótipo selvagem sendo este dado confirmado através de sequenciamento de nova geração. Devido ao alto número de genótipos selvagem encontrados, o teste de resistência genotípica deve ser solicitado para se obter um melhor desfecho clinico em níveis individuais e populacionais. No terceiro estudo nós analisamos as diferenças na contagem de linfócitos T CD4+ entre indivíduos infectados apenas com o HIV-1 e indivíduos coinfectados HIV-1/HTLV-1 com falha terapêutica, além de analisarmos a soroprevalência do HTLV-1 em indivíduos infectados pelo HIV-1. Foram encontrados oito pacientes coinfectados (2,1%) dos 381 pacientes analisados. Nós não observamos nenhuma diferença estatística quando analisamos transversalmente os dados clínicos dos pacientes, exceto na primeira contagem de linfócitos T CD4+ após o início do tratamento que estava maior nos indivíduos coinfectados (p=0.03). A análise multivariada longitudinal mostrou que a media de linfócitos T CD4+ ao longo do tratamento, foi estatisticamente maior nos pacientes coinfectados levando em consideração características demográficas, carga viral, fatores relacionados a terapia, entre outros. Nos pacientes coinfectados também não foram encontrados marcadores de HLA relacionados com os supressores de elite do HIV-1. Os dados deste trabalho sugerem que os pacientes coinfectados em terapia antirretroviral deveriam ter um acompanhamento clínico diferenciado dos indivíduos apenas infectados pelo HIV-1, pois a coinfecção poderia estar levando ao aumento do número dos linfócitos T CD4+ sem um possível ganho de resposta imune.

Palavras-chaves: HTLV-1; HIV-1; LTR; resistência aos antirretrovirais; coinfecção

REGO, Filipe Ferreira de Almeida. Genetic and clinical features in the HIV-1 and HTLV-1 infection. 109 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2014.

ABSTRACT

We performed three studies to analyze risk factors associated with retroviruses infections. In the first study we attempted to analyze mutations related to TSP/HAM development or proviral load. For that purpose we have sequenced the LTR 5' region of HTLV-1 by Ion Torrent. We found that mutations in 52 positions were present in more than one individual, but only 11 were present in the previously described TFBS. Three mutations that were not present in the previously described TFBS were statistically significant comparing groups. Despite the absence of previously described TFBS, these sites might be important for the viral transcription. In the second study we analyzed the prevalence of HIV-1 wild type genotype in adults failing first-line ART. A total of 220 adults were included. The wild type genotype was detected by population sequencing in 28 (12.7%). No major drug resistance mutations were detected by deep sequencing for 81.8% (9/11) of those sampled. Higher baseline CD4+ cell count was associated with a greater likelihood of wild type genotype as was a higher viral load prior to resistance testing but there was no evidence of an association between self-reported adherence and the presence of wild type genotype. Approximately one in eight adults failing first-line ART had wild type genotype and this result was confirmed trough deep sequencing in some samples. Access to genotypic resistance testing may be required in this region to achieve optimal individual-level and population-level outcomes. In the third study we proposed to verify the prevalence of HTLV-1 and to statistically assess differences in CD4+ counts between HTLV-1/HIV-1 co-infected and HIV-1 mono-infected patients living in rural KwaZulu-Natal. The HTLV-1 seroprevalence was 2.1% (8 out 381 patients). The patients were grouped by HTLV-1/HIV-1 co-infected and HIV-1 mono-infected status for the statistical analysis. There were no cross-sectional differences between the groups regarding CD4+ count before therapy, CD4+ count at genotype, age, gender, viral load, duration of ART, immunological failure, ART failure and first ART regimen. However, the first CD4+ count after treatment was higher in the co-infected group ($p=0.03$). A multivariate, longitudinal model showed that the mean CD4+ count over time for the HTLV-1/HIV-1 co-infected group was significantly higher than the HIV mono-infected group ($p<0.05$) when adjusting for demographic characteristics, viral load and ART treatment factors. This finding was independent of expression of HLA Class 1 genotypes previously associated with HIV-1 infected elite suppressors. This study suggests that the increase of CD4+ count after therapy suggests a differential clinical management for the HTLV-1/HIV-1 co-infected patients should be implemented.

Key-words: HTLV-1; HIV-1; LTR; Drug resistance; coinfection

LISTA DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

3TC	lamivudina
ABC	abacavir
AIDS	*Acquired immune deficiency syndrome* (Síndrome da imunodeficiência adquirida)
ART	*Antiretroviral therapy* (Terapia antiretroviral)
ATF	*Activating transcription factor* (Fator transcricional ativante)
ATL	*Adult T-cell leukemia/lymphoma* (Leucemia/Linfoma de células T do adulto)
ATV	atazanavir
AZT	zidovudina
bp	*base pairs* (pares de bases)
cells/μL	*cells per microliter* (células por microlitro)
CI	*confidence interval* (intervalo de confiança)
copies/ml	*copies per milliliter* (cópias por mililitro)
CREB/ATF	*cAMP response element-binding protein*
CTL	*Cytotoxic T lymphocytes* (linfócitos T citotóxicos)
D4T	estavudina
ddi EC	didanosina
DLV	delavirdina
DNA	ácido desoxirribonucléico
DRV	darunavir
EDTA	*Ethylenediamine tetraacetic acid* (Ácido etilenodiamino tetra-acético)
EFV	efavirenz
ELISA	*Enzyme linked immuno sorbent assay* (Ensaio imunoenzimático)
ESS	*Effective sample size* (Tamanho de amostra efetivo)
ETR	etravirina
FPV	fosamprenavir
FTC	emtricitabina
gp21	Glicoproteína 21
gp46	Glicoproteína 46
GSS	*Genomic Similarity Score* (Escore de similaridade genômica)
GTR	*general time-reversible* (Modelo evolutivo geral com tempo reversível)
HAART	*Highly active antiretroviral therapy* (Terapia antirretroviral de alta potência)

HAU *HTLV-1 associated uveitis* (Uveite associada ao HTLV-1)

HBZ HTLV-*1 basic leucine zipper* (ziper de leucina do HTLV-1)

HIV *Human Immunodeficiency Virus* (Vírus da imunodeficiência humana)

HLA *Human leukocyte antigen* (Antígeno leucocitário humano)

HPDs *Highest Probability Densities* (Densidade de maior probabilidade)

HTLV *Human T lymphotropic virus type* (Vírus linfotrópico de células T humanas)

IDH - *HTLV-1 –associated infective dermatitis* (Dermatite infectiva associada ao HTLV-1)

IDV indinavir

IN integrase

Kb kilobase

Km Quilômetro

km^2 Quilômetro quadrado

LPV lopinavir

LTR longas terminais repetitivas

MCMC Markov chain Monte Carlo (Cadeia de Markov/Monte Carlo)

ml/min mililitro por minuto

mRNA RNA mensageiro

NC nucleocapsídeo

NEF *Negative Regulatory Factor* (Fator regulatório negativo)

NFV nelfinavir

ng nanograma

Nm nanômetro

NNRTI RT não nucleosídico

NRTI RT nucleosídico

NVP nevirapina

OH hidroxila

OMS Organização Mundial da Saúde

OR *odds ratio*

ORF quadros abertos de leitura

p13 proteínas de 13 kilodaltons

p17 proteínas de 17 kilodaltons

p24 proteínas de 24 kilodaltons

p30 proteínas de 30 kilodaltons

p7 proteínas de 7 kilodaltons

p8	proteínas de 8 kilodaltons
PBMC	*Peripheral blood mononucleated cell* (Células mononucleares do sangue periférico)
PCR	reação em cadeia da polimerase
PDZ	Domínio que contém os sítios **P**SD95, **D**lgA e **z**o-1
PHC	*primary health care* (Centro de cuidados primários)
PI	inibidores de PR
PP	*posterior probability* (Probabilidade posterior)
PR	protease
RES249	Isolado do HTLV-1 proveniente do interior da Bahia
RNA	ácido ribonucléico
SNP	*single nucleotide polymorphism* (polimorfismo de nucleotídeo único)
SQV	saquinavir
TDF	tenofovir
TFBS	*transcription factor binding site*
TMRCA	*The time of most recent common ancestor* (data do ancestral comum mais recente)
TPV	tipranavir
TR	transcriptase reversa
TSP/HAM	paraparesia espática tropical/mielopatia associada ao HTLV
VIF	*Viral infectivity factor* (fator de infectividade viral)
VPU	*Viral Protein U* (proteína viral U)
VRP	*virus-like replicon particles* (partículas similares ao replicon viral)
µL	microlitro

SUMÁRIO

1. INTRODUÇÃO

1.1 DESCOBERTA DOS RETROVÍRUS HUMANOS

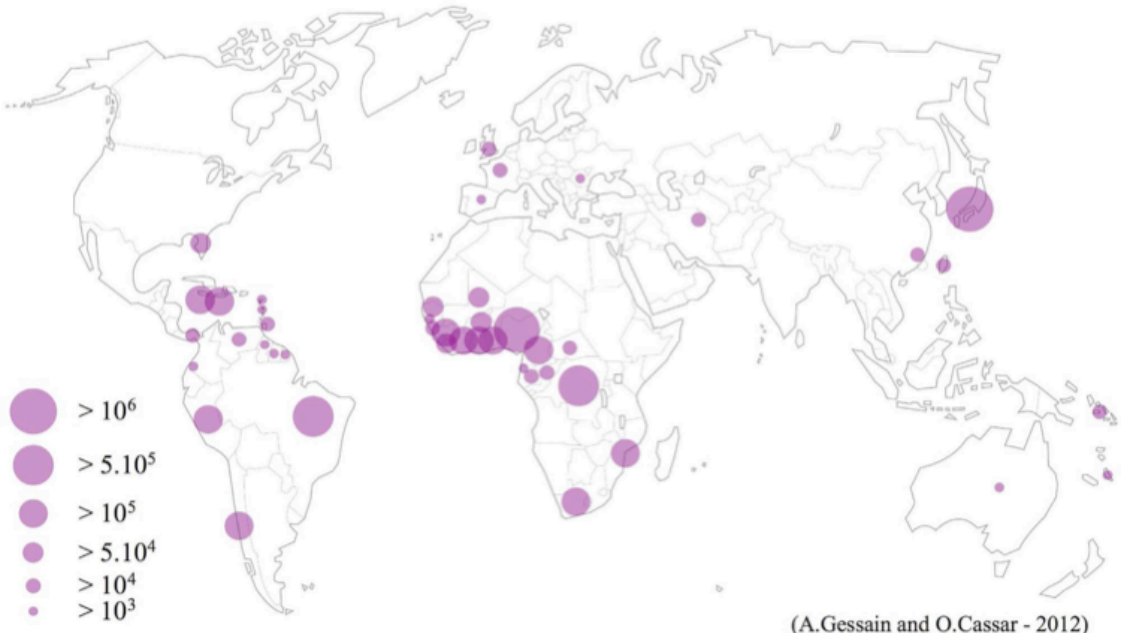
O vírus linfotrópico de células T humanas (HTLV) do tipo 1 foi o primeiro retrovírus humano descrito, isolado pela primeira vez em células T de um paciente com linfoma cutâneo (POIESZ et al, 1980). Em seguida um outro tipo deste vírus foi descoberto, o HTLV-2, identificado pela primeira vez numa linhagem de células T imortalizadas de um paciente com tricoleucemia (KALYANARAMAN et al, 1982). Em 1983 um terceiro retrovírus que infectava humanos foi identificado e isolado, o vírus da imunodeficiência humana (HIV) (BARRE-SINOUSI et al, 1983), responsável pela Síndrome da Imunodeficiência Adquirida (AIDS) que vinha assolando os Estados Unidos no início da década de 80.

Após a descoberta destes vírus, outros tipos do HIV e HTLV foram identificados. O HIV-2 foi identificado em 1986 em dois pacientes do Oeste Africano (CLAVEL et al, 1986), enquanto que os HTLV-3 e 4 foram descritos pela primeira vez em indivíduos de Camarões, na África Central, que foram expostos a contato com primatas não humanos mais recentemente (WOLFE et al, 2005). Devido as suas características moleculares, o HTLV e o HIV foram incluídos na família retroviridae, pertencente à subfamília oncovirinae, porém o HTLV foi classificado como sendo do gênero Deltaretroviruse o HIV do gênero Lentivírus.

1.2 EPIDEMIOLOGIA DOS RETROVÍRUS HUMANOS

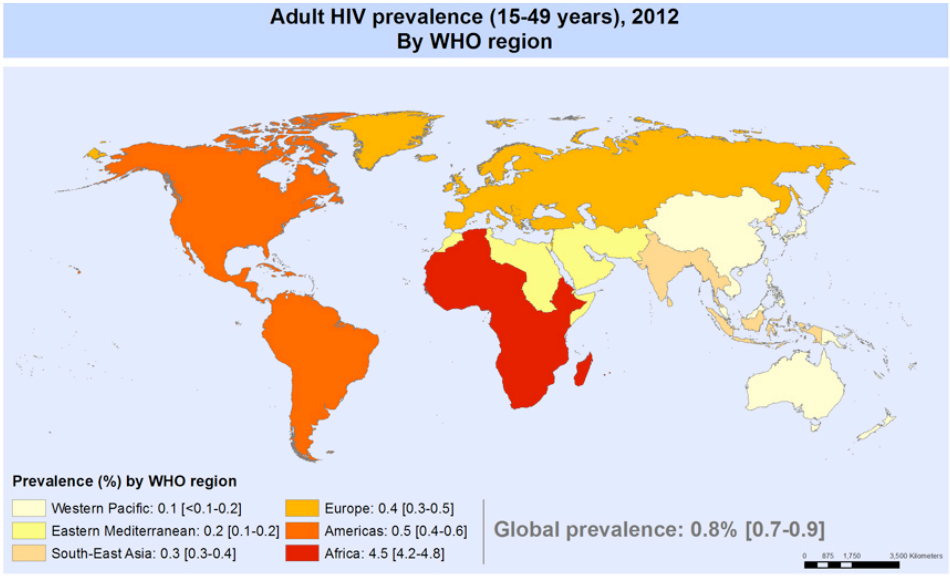
O HTLV e o HIV podem ser transmitidos por via sexual, parenteral e vertical, esta última principalmente através da amamentação. Dentre os tipos virais, o HIV-1 e o HTLV-1 são os principais responsáveis pelas infecções no mundo. O HIV-1 é responsável por uma pandemia enquanto que o HTLV-1 é endêmico em algumas regiões. As principais áreas endêmicas no mundo para infecção pelo HTLV-1 são Japão, Caribe, América Central e do Sul, África Equatorial, Oriente Médio e Melanésia (Figura 1). Estima-se que este vírus infecte aproximadamente 5 a 10 milhões de pessoas em todo o mundo (GESSAIN e CASSAR, 2012). No caso da infecção pelo HIV-1, estima-se que existam cerca de 34milhões de pessoas

vivendo com este vírus no mundo, sendo a prevalência da infecção maior na África subsaariana (Figura 2) (WHO, 2014).



Fonte: GESSAIN e CASSAR, 2012

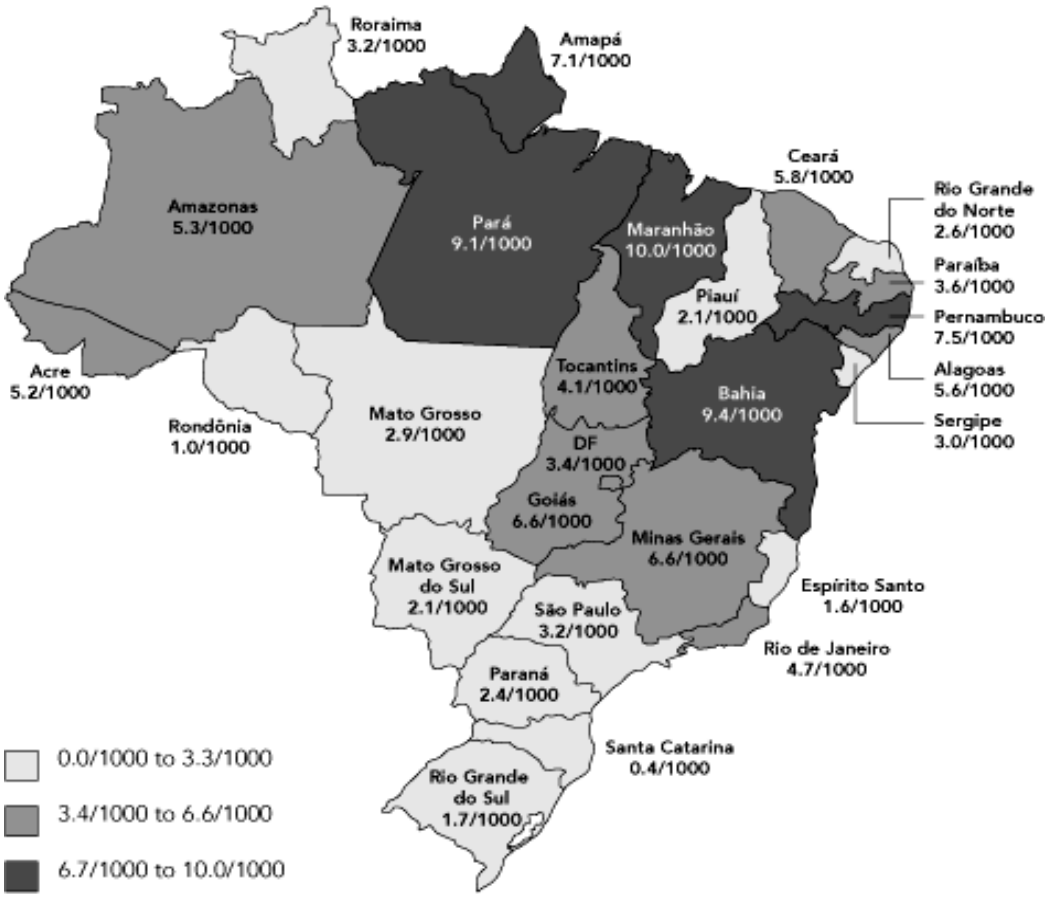
Figura 1. Distribuição geográfica por número de infecções pelo HTLV-1 no mundo



Fonte: WHO, 2013

Figura 2. Prevalência do HIV-1 em adultos no mundo em 2012.

O Brasil é uma área endêmica para o HTLV-1, porém com índice baixo de prevalência, variando nas capitais segundo Catalan-Soares e colaboradores (2004) (Figura 3). Porém, a maioria destes dados não demonstra a prevalência real da infecção, podendo estar subestimados, pois muitos foram feitos a partir de amostras provenientes de bancos de sangue, que conhecidamente trata-se de uma população mais jovem e com menos fatores de risco. O único estudo que demonstra dados com base populacional é o estudo de soroprevalência em Salvador, o qual estima uma prevalência de cerca de 1,8 % (DOURADO et al, 2003), provavelmente devido a grande entrada de escravos durante o tráfico negreiro.



Fonte: CATALAN-SOARES et al, 2004

Figura 3. Prevalência do HTLV-1 em doadores de sangue no Brasil por 1000 habitantes.

Estima-se que mais de 700 mil pessoas estejam infectadas pelo HIV-1 no Brasil (BRASIL, 2013), sendo esta infecção mais prevalente nas regiões sul e sudeste, porém, de acordo a Organização Mundial da Saúde (OMS), a epidemia no país esta controlada. A infecção pelo HIV-1 no Brasil iniciou-se entre indivíduos do sexo masculino, no início da década de 80, que possuíam comportamento homossexual, expandindo-se para usuários de drogas endovenosas, e então, disseminou-se para a população de uma forma geral, aumentando o número de mulheres infectadas no Brasil (DOURADO et al, 2007).

1.3 ESTRUTURA DOS RETROVÍRUS HUMANOS

1.3.1 HTLV-1

A morfologia do HTLV-1 é de esfera pleomórfica medindo de 80 a 100 nm de diâmetro, e são constituídos, basicamente, por um envelope, uma matriz protéica e um nucleocapsídeo. O vírus possui dentro do nucleocapsídeo duas fitas no seu capsídeo, sendo estas de ácido ribonucleico (RNA) fita simples positiva.

O HTLV-1 possui os genes *gag* (grupo antigênico), *pol* (polimerase), *env* (envelope) e uma região denominada *Px* (Figura 4). O envelope constitui o complexo lipoproteico da superfície da partícula viral, contendo a gp46 (proteína de superfície) e a gp21 (proteína transmembrana), codificadas pelo gene env. A matriz protéica viral é composta pela proteína p19, enquanto o capsídeo é composto pela proteína p24, ambas codificadas pelo gene gag. Estas proteínas codificadas pelos genes env e gag são as proteínas estruturais virais. As proteínas responsáveis pela maturação viral são codificadas pelo gene pol: protease, integrase e transcriptase reversa. Estas proteínas possuem as funções de clivagem proteica, integração do genoma viral no genoma do hospedeiro e transcrição reversa do RNA em DNA complementar (cDNA), respectivamente.

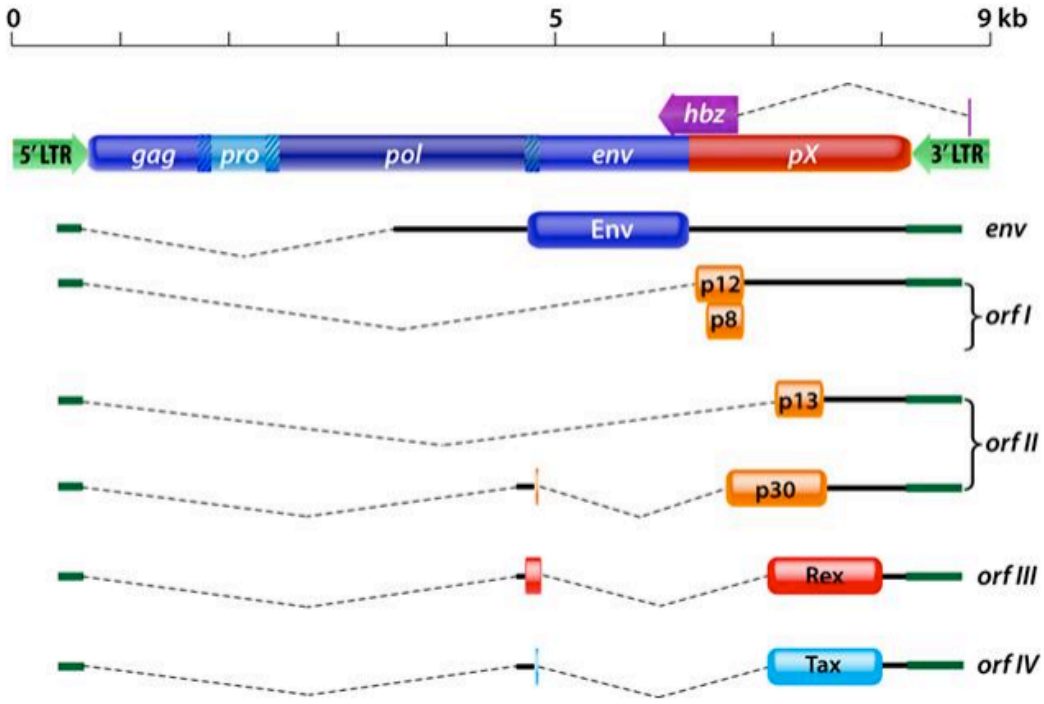
O genoma dos retrovírus são flanqueados por regiões longas terminais repetitivas (LTR) que estão divididas em três regiões: U3, R e U5. A região U3 contêm sinais importantes para o controle da expressão dos genes do HTLV-1, como as três repetições imperfeitas de 21 pares de bases, conhecidos como elementos responsivos de tax (TxRE), na LTR5', responsável pela transcrição do genoma proviral através da interação com fatores de transcrição principalmente do tipo CREB/ATF. Além das regiões TxREs, necessárias para a transativação viral, o TATA Box, onde se inicia a transcrição, o sinal de poliadenilação e outros sítios importantes como o E-BOX também estão presentes na região U3 da LTR5'.

Além disto, o HTLV possui uma região particular com cerca de 2 Kb, situada imediatamente antes da região LTR3', denominada inicialmente pX, em razão da sua natureza anteriormente desconhecida. Essa região contém pelo menos quatro quadros abertos de leitura (ORF) que codificam diferentes proteínas regulatórias e acessórias, além de codificar uma importante proteína na fita negativa chamada HBZ (Figura 4).

Tax, Rex e HBZ são as principais proteínas regulatórias do HTLV codificadas pelo genepX, e são responsáveis pela regulação da replicação viral e patogênese da infecção. A proteína Tax contém diversas regiões funcionais e domínios de interação, e já foi descrita por interagir com diversas proteínas: fatores transcricionais, proteínas do citoesqueleto, sinalização celular, proteínas do ciclo celular, proteínas PDZ e proteínas de transporte (BOXUS et al, 2008). A Tax é, portanto, uma das principais proteínas envolvida na patogênese do HTLV-1.

A proteína Rex regula a expressão das outras proteínas virais, modulando a transcrição nas fases do ciclo de replicação viral. A proteína HBZ, codificada pela fita complementar do HTLV-1 (3'→5') possui um domínio leucine zipper, interagindo diretamente com fatores transcricionais, aumentando sua degradação e consequentemente suprimindo a transcrição viral mediada pela proteína Tax (MATSUOKA, 2005).

Além de Tax, Rex e HBZ a região 3' do gene produz diversas outras proteínas, as proteínas p8 e p12, codificadas pela ORF-I do gene pX, (KORALNIK et al, 1992; CIMINALE et al, 1992; KORALNIK et al, 1993; FUKUMOTO et al, 2009) e as proteínas p13 e p30, codificadas pela ORF-II do gene pX (KORALNIK et al, 1992, CIMINALE et al, 1992). Estudos do papel funcional destas proteínas acessórias demonstraram que elas são essenciais para a infectividade e persistência da infecção in vivo, além de contribuírem para a manutenção da carga proviral (COLLINS et al, 1998; ALBRECHT e LAIRMORE 2002, MATSUOKA e JEANG, 2007).



Fonte: EDWARDS et al, 2011

Figura 4. Representação esquemática do genoma do HTLV-1.

1.3.2 HIV-1

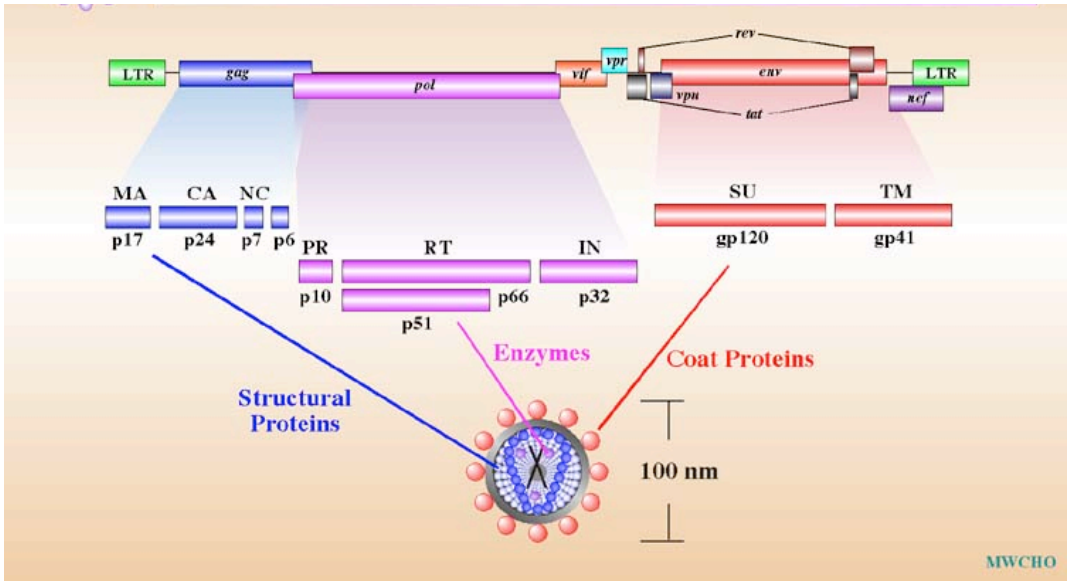
A partícula do HIV-1 possui um formato icosaédrico com tamanho em torno de 110 nm de diâmetro. O envelope viral, estrutura mais externa, é formado por uma bicamada lipídica que contém duas glicoproteínas: a de superfície com 120 kD (gp120) e a transmembrana de 41 kD (gp41) (CHAN, 1998). A matriz proteica viral localizada abaixo do envelope, é formada pela proteína p17 e mais interiormente encontra-se o capsídeo viral formado pela proteína p24. Já o nucleocapsideo éformado pelas proteínas p7 e p9 (NC) onde se localizam as duas fitas simples de RNA além das proteínas necessárias para a inserção viral no genoma do hospedeiro.

No interior do capsídeo viral estão localizadas as enzimas transcriptase reversa (TR), integrase (IN) e protease (PR), estas são envolvidas nos processos de replicação, integração do genoma viral e maturação respectivamente. As chamadas proteínas acessórias VIF, VPR e NEF também são encontradas no capsídeo, são essenciais para a persistência viral após integração no genoma humano, porém suas funções dentro do capsídio não foram completamente esclarecidas.

O genoma do HIV-1 possui um comprimento aproximado de 9,5 kD e é formado por duas fitas simples de RNA que contem 9 genes flanqueados por duas regiões LTR (Figura 5). Estas regiões, assim como no HTLV-1,contêm os sítios responsáveis pela transcrição gênica das proteínas virais (GREENE, 2002).

O HIV-1 apresenta 2 genes estruturais: *gag* (gene antígeno de grupo) e *env* (envelope). O gene *env* codifica a glicoproteínade 160 kD (gp160) que sofre o processo de clivagem gerando duas proteínas, a gp120 e a gp41. Essas proteínas estão presentes no envelope viral e são responsáveis pelo reconhecimento e fusão da célula alvo, portanto influenciam na infectividade e tropismo viral (CHAN, 1998). O gene gag codifica uma proteína precursora de 55 kD (p55) que sofrerá processo de clivagem dando origem às proteínas da matriz (p24), do capsídeo (p17) e do nucleocapsídeo viral (p9 e p7). Além dos genes estruturais, o gene pol codifica as enzimas TR, IN e PR. A TR é responsável pela transcrição reversa do RNA viral em cDNA, a IN integra o cDNA ao genoma da célula hospedeira e a PR irá participar do processo de maturação viral para geração de novos vírions.

Assim como o HTLV-1, o HIV-1 também possui proteínas reguladoras e acessórias. As proteínas reguladoras são codificadas pelos genes tat e rev. A proteína TAT é transativadora da transcrição viral tendo um papel importante na replicação viral enquanto que a proteína REV auxilia no transporte de mRNAs viral do núcleo para o citoplasma da célula (HOPE, 1997). As proteínas acessórias do HIV-1 são codificadas pelos genes vif, vrp, vpu e nef e possuem funções distintas. A proteína VIF auxilia no transporte de componentes virais, a VRP participa no transporte do cDNA para ser integrado ao genoma da célula alvo, já a VPU atua no brotamento da partícula viral e a proteína NEF está relacionada com processos de escape viral da resposta imune do hospedeiro, pois reduz a expressão de moléculas CD4 e das moléculas de antígeno leucocitário humano (HLA) de classe I e II.

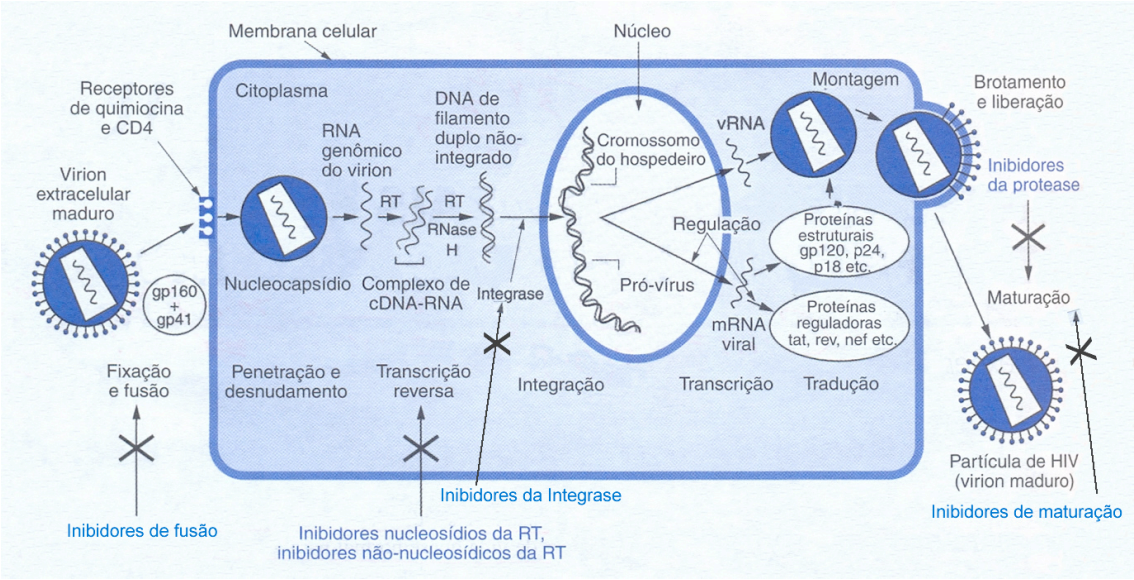


Fonte: https://web.stanford.edu/group/virus/retro/2005gongishmail/HIV.htmlhttps://web.stanford.edu/group/virus/retro/2005gongishmail/HIV.html

Figura 5. Representação esquemática do genoma do HIV-1.

1.4 CICLO DE REPLICAÇÃO DOS RETROVÍRUS HUMANOS

No ciclo de replicação típico dos retrovírus, primeiro o vírus se liga pelo domínio de ligação do amino terminal da proteína de superfície, ao receptor da membrana da célula hospedeira. Vale lembrar que cada retrovírus possui um receptor celular específico. Após isto, ocorre a transcrição do genoma viral de RNA para cDNA pela enzima transcriptase reversa ainda dentro do cerne. O DNA viral entra no núcleo e a proteína integrase insere o DNA linear no cromossomo do hospedeiro, formando o provírus. Então, a partir deste ponto, são gerados RNAs virais pelo maquinário intracelular do hospedeiro. Estes RNAs formarão os novos vírus e após sua formação, o vírion sai da célula por brotamento, de forma que, os precursores não são clivados até o brotamento (Figura 6). Esse mecanismo de replicação clássico dos retrovírus acontece principalmente no caso do HIV-1. Já no HTLV-1, sua proliferação no organismo decorre principalmente, pela expansão clonal das células do hospedeiro ou pelo contato célula-célula, principalmente através da sinapse virológica. Nesse fenômeno as células são polarizadas para facilitar a passagem viral quando entram em contato com uma célula não infectada, permitindo o acúmulo de proteínas de gag e de RNA e assim infectando uma nova célula (MATSUOKA & JEANG, 2007).



Fonte: LAURENCE et al 2010

Figura 6. Ciclo de replicação do HIV-1 e os principais alvos terapêuticos

1.5 PATOLOGIAS ASSOCIADAS AO HTLV

O HTLV-1 é o agente etiológico da paraparesia espática tropical/mielopatia associada ao HTLV (TSP/HAM), sendo esta uma doença neurológica crônico-degenerativa que atinge o sistema nervoso central, causando principalmente um aumento da espasticidade dos membros inferiores (GESSAIN et al, 1985, OSAME et al, 1986). Diversas hipóteses buscam explicar a patogênese da TSP/HAM (IJICHI et al, 1993; TAYLOR, 1998; JACOBSON, 2002; OSAME, 2002). A primeira hipótese sugere que linfócitos T citotóxicos (CTL) específicos circulantes atravessam a barreira hematoencefálica, destruindo células da glia infectadas pelo HTLV-1. A segunda hipótese sugere a existência de um antígeno da célula da glia similar ao do HTLV-1, mimetizando esta célula com uma infectada e a destruindo. A terceira hipótese sugere que a migração dos linfócitos infectados pelo HTLV-1 (T CD4+), juntamente com CTL específicos anti-HTLV-1 atravessam a barreira hematoencefálica.

O HTLV-1 também é o agente etiológico da Leucemia/Linfoma de Células T do Adulto (ATL), que é uma neoplasia de linfócitos T maduros, que ocorre devido a expansão monoclonal dos linfócitos T infectados (YOSHIDA et al, 1982). A ATL é caracterizada pela infiltração de células malignas em alguns tecidos e pela presença de células anormais com núcleo em forma de flor (MATSUOKA, 2005). A ATL é causada principalmente pela

inibição de proteínas relacionas ao reparo do dano ao DNA, além da aceleração de fases do ciclo celular por proteínas virais.

O HTLV-1 também já foi associado a algumas doenças inflamatórias, como dermatite (LA GRENADE, 1996), uveíte (MOCHIZUKI et al, 1996), além de poder estar associado a doenças reumáticas como síndrome de Sjögren e artrite reumatóide (MC COLLUM et al, 1997, NISHIOKA, 1996), manifestações psiquiátricas e aumento da susceptibilidade a infecção por helmintos, com aumento da patogênese destas parasitoses. Apesar de ser associado a diversas patologias, poucos indivíduos infectados apresentam alguma sintomatologia associada ao vírus. Apenas 2 a 5 % dos indivíduos infectados desenvolvem alguma doença que tem como agente etiológico determinado o HTLV-1. Um estudo de associação sugeriu que tanto fatores genéticos virais, quanto do hospedeiro influenciam no desfecho da infecção, no qual o sistema imune possui um papel crítico (BANGHAM et al, 2009).

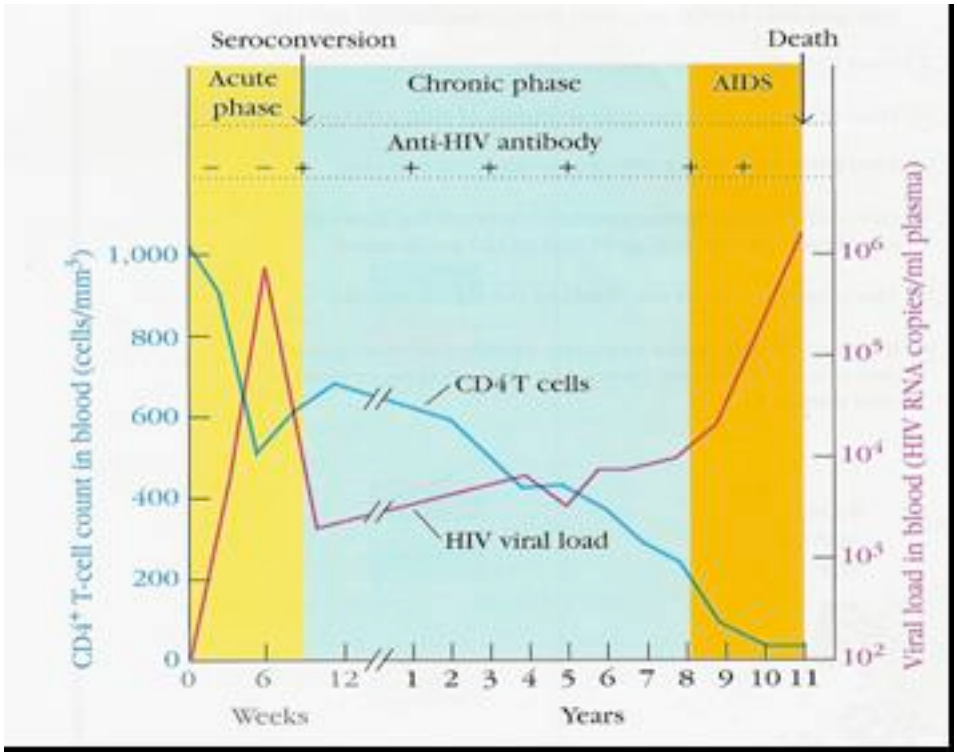
1.6 HIV-1 E AIDS

A infecção pelo HIV-1 leva ao desenvolvimento da AIDS, porém as fases de infecção são distintas. No início da infecção, entre duas a seis semanas, ocorre um pico da replicação viral, com aumento da carga viral, um aumento de linfócitos T CD8+ no sangue periférico (FAUCI, 1993) e uma queda de linfócitos T CD4+, como demonstrado na Figura 7 (PANTALEO & FAUCI, 1996). Durante esta fase da infecção, cerca de metade dos indivíduos infectados irão apresentar sintomas semelhantes aos de uma gripe que dura uma ou mais semanas (COHEN et al, 1997). Neste período não era \ possível diagnosticar a infecção com o uso de testes para detecção de anticorpos, pois os seus níveis são muito baixos e não eram detectados nos testes de triagem (FAUCI, 1993), porém com o avanço das técnicas de Biologia Molecular, já é possível detectar o vírus por PCR em tempo real.

Após esta fase, inicia-se o período de latência clínica, onde o número de linfócitos T CD4+ volta ao normal e diminui gradativamente ao longo do tempo (Figura 7). Esta fase também é caracterizada pelo alto título de anticorpos específicos contra o HIV, aumento de células T CD8+ (CTL) específicas, que controlam a replicação viral e, consequentemente, mantem a carga viral em níveis baixos. Esta fase dura normalmente entre 2 a 10 anos, e a

diferença de tempo irá depender, principalmente de fatores relacionados ao hospedeiro (COHEN et al,1997).

Quando os níveis de células T CD4+ chegam a valores inferiores a 200 células/μL, inicia-se a AIDS (COHEN et al, 1997). Durante este período, além da diminuição de células T CD4+, também ocorre a diminuição de células T CD8+ e anticorpos neutralizantes, e um consequentemente aumenta a carga viral do indivíduo (Figura 7). Também observa-se o aparecimento principalmente de infecções oportunistas, como tuberculose, além de neoplasias secundárias e manifestações neurológicas (COFFIN et al, 1995; KAHN et al, 1998).



Fonte: FAUCI et al, 1996.

Figura 7. Contagem de linfócitos T CD4 e carga viral nas diferentes fases de infecção pelo HIV-1

O tratamento com as drogas específicas contra o HIV surgiu em 1987 com a zidovudina, um análogo de nucleosídio que inibe a atividade da enzima RT impedindo a replicação viral (YARCHOAN et al, 1986). Subsequentemente, outras drogas que agem não apenas sobre a RT, mas também na PR e IN começaram a surgir no intuito de controlar a carga viral, aumentando a sobrevida do paciente. Em 1996, surgiu a chamada terapia antirretroviral de alta potência (HAART) ou "Coquetel Anti-AIDS", que se baseia na combinação de drogas de três classes diferentes (PERELSON et al, 1996; PALELLA et al, 1998; SEPKOWITZ, 2001). Com esta terapia foi possível reduzir a carga viral e reconstruir o sistema imune do paciente, retardando assim a progressão para AIDS e melhorando a qualidade de vida dos portadores do vírus (PERELSON et al, 1996; PALELLA et al, 1998; SEPKOWITZ, 2001).

Existem cinco tipos de antirretrovirais no combate ao HIV-1: os inibidores de PR (PI), os inibidores da RT nucleosídico (NRTI), os inibidores da RT não nucleosídico (NNRTI), os inibidores de fusão e os inibidores da IN. Dentre estes os mais utilizados no tratamento da infecção pelo HIV-1 são os PI, NRTI e NNRTI. Os PI podem agir de algumas formas: se ligando ao sítio ativo da enzima; mimetizando o estado de transição durante a clivagem do

peptídeo; ou, agindo como um complemento simétrico ao sítio ativo da enzima, impedindo assim a maturação da partícula viral. Os PI existentes no mercado atualmente são atazanavir (ATV), darunavir (DRV), fosamprenavir (FPV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), saquinavir (SQV) e tipranavir (TPV), sendo que o LPV e o ATV são os mais utilizados.

Os inibidores da enzima RT podem ser classificados em NRTI e NNRTI. Os NNRTI são drogas que também atuam inibição da RT, porém se ligam a um sítio próximo do sítio ativo da enzima, inibindo sua ação e diminuindo, consequentemente, a replicação viral. As drogas da classe dos NNTRIs disponíveis no mercado são: delavirdina (DLV), efavirenz (EFV), etravirina (ETR) e nevirapina (NVP), sendo que o EFV e o NVP são os mais indicados para uso. Os NRTI são substâncias análogas dos nucleosídios que não apresentam o grupo hidroxila (OH) no carbono 3', que impede que a RT continue a síntese do cDNA durante a transcrição reversa, pois se liga na nova fita que está sendo sintetizada. Dentre os principais NRTIs em uso terapêutico estão a lamivudina (3TC), zidovudina (AZT), didanosina (ddi EC), tenofovir (TDF), abacavir (ABC), estavudina (D4T) e emtricitabina (FTC).

Apesar destas terapias estarem contribuindo para a sobrevida dos pacientes por manter a carga viral baixa e número de linfócitos T CD4+ suficiente para combater a infecção, muitos pacientes apresentam falha terapêutica. Esta falha pode ocorrer devido a não adesão do paciente a terapia, a dificuldade de acesso ao medicamento e/ou ao acompanhamento médico, a resistência adquirida ou transmitida aos antirretrovirais ou a fatores do hospedeiro, como alelos específicos de HLA (CARRINGTON et al, 1999; MAGIEROWSKA et al, 1999; BAKER et al, 2009), que podem levar a uma maior susceptibilidade a progressão para a AIDS. No entanto, a falha terapêutica ocorre principalmente devido a resistência aos antirretrovirais, causada por mutações no gene *pol* (COFFIN, 1995), que normalmente é identificada por sequenciamento de DNA, podendo ajudar a definir qual o melhor e mais eficaz tratamento para o paciente.

A falha terapêutica é detectada principalmente pelo aumento da carga viral, pela queda da contagem de linfócitos T CD4+ ou pela progressão clínica. No Brasil, quado se observa a falha a múltiplos esquemas terapêuticos, é realizada a detecção da resistência pela genotipagem por sequenciamento utilizando principalmente a plataforma Sanger. Desta forma, é possível detectar as mutações que conferem resistência tanto na enzima transcriptase reversa quanto na protease, que são as enzimas alvo das drogas utilizadas no mundo.

1.7 COINFECÇÃO HIV-1/HTLV-1

A coinfecção entre HIV-1 e HTLV-1 tem sido detectada em muitos países da América do Sul e África, onde estes vírus são endêmicos (HISHIDA et al, 1994; BEILKE et al, 1994; CLEGHORN et al, 1995; CÉSAIRE et al, 2001; BHATT et al, 2009; de ALMEIDA REGO et al, 2010). Entretanto os efeitos que a coinfecção com estes importantes retrovírus provoca não são bem estudados. Sabe-se que ambos os vírus infectam preferencialmente linfócitos T CD4+, porém com efeitos controversos dentro destas células. O HTLV-1 estimula a proliferação desta célula por um mecanismo mediado pela proteína tax ou se dissemina através do contato célula-célula, enquanto que o HIV-1 utiliza principalmente a maquinaria celular para produção de novos virions que depletam os linfócitos T CD4+ do indivíduo.

Alguns estudos analisaram as diferenças na contagem de linfócitos T CD4+ em indivíduos coinfectados com HTLV-1/HIV-1 comparando com indivíduos infectados apenas por HIV-1 antes do início da terapia antirretroviral e demostraram que os indivíduos coinfectados possuíam uma contagem de linfócitos T CD4+ aumentada em relação aos demais indivíduos (SCHECHTER et al, 1997; HARRISON et al, 1997; BEILKE et al, 2004; BEILKE et al, 2007; PEDROSO et al, 2011). Em adição, alguns relatos de caso demostraram que indivíduos coinfectados apresentam imunodepressão mesmo com o número de CD4+ elevado (NADLER et al, 1996; REGIS et al, 2009). Um outro estudo sugeriu ainda que indivíduos coinfectados tem uma progressão mais rápida para a AIDS (GUDO et al, 2009). Entretanto, outro estudo não demonstra a diferença na contagem de linfócitos T CD4+ após o início do tratamento, porém, por este estudo ter analisado os pacientes em um corte transversal, os dados não são completamente claros (SILVA et al, 2012).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Identificar fatores genéticos e clínicos relacionados à desfechos clínicos na infecção pelo HIV-1 e HTLV-1.

2.2 OBJETIVOS ESPECÍFICOS

- Identificar mutações na região LTR que possam estar relacionadas ao desenvolvimento da TSP/HAM ou ao aumento da carga proviral do HTLV-1;
- Identificar a frequência do HIV-1 com genótipo selvagem em indivíduos com falha terapêutica em Kwazulu-Natal;
- Verificar os fatores de risco relacionados com o genótipo selvagem do HIV-1 em Kwazulu-Natal;
- Comparar a contagem de linfócitos T CD4+ ao longo do tempo entre indivíduos infectados pelo HIV-1 e indivíduos coinfectados HIV-1/HTLV-1;

3 RESULTADOS

3.1 AVALIAÇÃO ENTRE MUTAÇÕES NA REGIÃO LTR DO HTLV-1 COM A TSP/HAM E CARGA PROVIRAL

O HTLV-1 é o agente etiológico da TSP/HAM, uma doença neurológica, crônica e degenerativa. Poucos indivíduos infectados desenvolvem esta doença. O seu desenvolvimento, apesar de não estar completamente esclarecido, pode ser associado a fatores ambientais, do hospedeiro e do vírus, como mutações virais e carga proviral. A região flanqueadora LTR5', promotor do HTLV-1, é de extrema importância para a persistência viral e multiplicação do vírus. Devido a esta importância da região promotora, mutações poderiam estar levando a alterações na transcrição viral e consequentemente na carga proviral e o desenvolvimento da TSP/HAM. Neste trabalho nós objetivamos identificar mutações que poderiam estar relacionadas com o desenvolvimento da TSP/HAM e/ou carga proviral. Para isto sequenciamos a região LTR5' do HTLV-1 por Ion Torrent para verificar mutações com frequência de até 1% em portadores do vírus com e sem TSP/HAM e com carga proviral abaixo de 50.000 cópias/10^6 células ou com carga proviral acima de 100.000 cópias/10^6. Nós encontramos mutações em 136 posições dos 520 pares de base analisados em uma frequência de pelo menos 1%. Destas apenas 52 estavam presentes em mais de um indivíduo e apenas 11 em TFBS previamente descritos porém sem diferença estatística entre os grupos, provavelmente devido ao número amostral. Três mutações que não estavam presentes em TFBS previamente descritos foram estatisticamente significantes quando comparadas entre os grupos (G126C/T, G306C e C479T) e devido a carência de estudos na região promotora do HTLV-1, estes sítios podem ser importantes para a mediação da transcrição viral.

Title: Deep sequencing analysis of Human T cell Lymphotropic Virus type 1 (HTLV-1) long terminal repeat (LTR) 5'region from patients with Tropical Spatic Paraparesis (TSP)/HTLV-1 associated myelopathy (HAM) and asymptomatic carriers.

Authors: Filipe Ferreira de Almeida Rego[1,2,3]; Tulio de Oliveira[2]; Bernardo Galvão-Castro[4]; Marilda de Souza Gonçalves[1]; Luiz Carlos Junior Alcantara[1]

[1] Laboratory of Hematology, Genetic and Computational Biology, Gonçalo Moniz Research Center, Oswaldo Cruz Foundation (LHGB/CPqGM/FIOCRUZ), Salvador, Bahia, Brazil; [2]Africa Centre for Health and Population Study, University of KwaZulu-Natal, South Africa; [3] Universidade Católica do Salvador, Salvador, Bahia, Brazil; [4]HTLV Center/Bahia School of Medicine and Public Health, Salvador, Bahia, Brazil.

Corresponding authors:

Tulio de Oliveira, Ph.D.
PoBox 198
Mtubatuba, 3935
South Africa
Telephone: +27 (35) 550 7500
Fax: +27 (35) 550 7560
email: tdeoliveira@africacentre.ac.za

Luiz Carlos Junior Alcantara, Ph.D.
LHGB/CPqGM/FIOCRUZ.
Rua Waldemar Falcão 121, Candeal, Salvador, Bahia, Brazil 40295-001.
Telephone: +55 (71) 3176-2255
Fax: +55 (71) 3176-2300
email: lalcan@bahia.fiocruz.br

e-mail addresses:

Filipe Ferreira de Almeida Rego: filipefar@hotmail.com

Marilda de Souza Gonçalves: mari@bahia.fiocruz.br

Bernardo Galvão-Castro: bgalvao@bahiana.edu.br

Keywords: HTLV-1, deep sequencing, LTR, TSP/HAM

Background:

Human T-cell Lymphotropic Virus type 1 (HTLV-1) is known to be the etiological agent of Tropical Spatic Paraparesis/HTLV-1 Associated Myelopathy (TSP/HAM) [Gessain et al, 1985; Osame et al, 1986], Adult T-cell leukemia/lymphoma (ATL) [Yoshida et al, 1982], and other inflammatory diseases such as HTLV-1 associated uveitis (HAU) [Mochizuki et al, 1996] and infective dermatitis associated with HTLV-1 (IDH) [La Grenade, 1996].

The development of TSP/HAM in HTLV-1 infected individuals remains unknown, being that few infected individuals have this disease outcome, and it is probably related to environmental, host and viral factors, such as proviral load, the methylation profile of proviral DNA, the integration site of HTLV-1 into the host genome and viral mutations [Taniguchi et al, 2005; Gillet et al, 2011; Olavarria et al, 2012]. Recent studies have showed trough Sanger sequencing and real-time PCR that mutations in the LTR region may increase the HTLV-1 proviral load [Neto et al, 2011; Magri et al, 2012] and it could consecutively affect the TSP/HAM outcome.

The HTLV-1 genome encodes gag, pol, env and pX genes, regulated by LTR flanking region. The flanking regions are divided into three regions: U3, R and U5, which contain signals to HTLV-1 expression control, such as TxRE region (Tax responsive elements) in U3 LTR5' (Reviewed at Grant at al, 2002), responsible for proviral genome transcription through the interaction with transcription factors like CREB/ATF, the TATA box motif and the polyA signal.

The aim of this study was to analyze patients by deep sequencing the HTLV-1 LTR region in order to determine if minor and/or major mutations in this promoter region might be associated with TSP/HAM outcome or proviral load or HTLV-1 expression.

Methods:

Study Design

This study corresponded to a cross-sectional analyze of 29 HTLV-1 infected patients with TSP/HAM or asymptomatic carriers from the HTLV Center, Salvador, Brazil. Basic demographic and clinical information (Age, sex, TSP/HAM status and proviral load) was obtained from patient's clinical record. The patients were then classified according to their proviral load (high or low) and TSP/HAM status (present or absent). The Research Ethics Committee of the Oswaldo Cruz Foundation approved the project (Protocol number 368/2011).

HTLV-1 LTR 5' amplification:

Patient nuclear DNA was extracted from peripheral blood mononuclear cells (PBMC) samples using a QIAGEN QIAamp® DNA Blood Kit following the manufacture's protocol. DNA sample were submitted to a nested-PCR using the HTLV-1 long terminal repeat (LTR) 5' region primers as described previously [Mboudjeka et al, 1997]. The PCRs products were purified by PureLink® PCR Purification Kit. HTLV-1 sequencing was performed using the Taq FS Dye terminator cycle sequencing kit (Applied Biosystems) on an automated 3130xl Genetic Analyzer (Applied Biosystems Inc, Foster City, CA) using the identical nested-PCR inner primers in order to analyze the major strains and create the consensus sequence for further deep sequencing analysis.

HTLV-1 deep sequencing

The HTLV-1 LTR5' purified products were submitted to deep sequencing using the Ion Torrent™ sequencing technology (Life Technologies, Carlsbad, CA). At least 500 sequences with a minimum of 100 base pairs (bp) were produced for each of the specimens, which allowed characterization of the viral population at 1% level. For the Ion Torrent™ analysis the purified PCR products were submitted to enzymatic fragmentation following the manufacturer's conditions for the 100ng long amplicon input (Ion Xpress™ Plus Fragment Library Kit Part no. 4471269, Life Technologies, Carlsbad, CA), with 15 minutes of incubation time in a heat block followed by Agencourt AMPure XP® purification (Beckman Coulter, Brea, CA). The ligation of the Ion Xpress™ P1 and barcode adapters and nick repair was performed (Ion Xpress™ Barcode Adapters 1-16 Cat. No. 4471250 and Ion Xpress™ Barcode Adapters 17-32 Cat. No. 4474009) followed by the second Agencourt purification. The fragmented DNA was then size-selected (E-Gel® SizeSelect™ 2% Agarose, Life

Technologies, Carlsbad, CA) for the 200bp sequencing run. The samples were amplified and purified for the third time using Agencourt. The samples were then quantified using the Qubit™ dsDNA HS Assay Kit (Invitrogen).

The amplicons were pooled and then submitted to the One Touch system for clonal amplification trough emulsion PCR (Ion OneTouch™ 200 System Template Kit Cat. No. 4471263, Life Technologies, Carlsbad, CA) followed by the enrichment step. Finally, the pooled enriched amplicon bound to the Ion Sphere Particles were submitted to the sequencing for 200bp reads (Ion PGM™ 200 Sequencing Kit Cat. No. 4474004, Life Technologies, Carlsbad, CA) in a 314 chip (Ion 314™ Chip 8-pack Cat. No. 4462923, Life Technologies, Carlsbad, CA).

Sequencing assembling and analisis

The sequence data was trimmed then assembled to the consensus reference sequence using the highest sensitivity plus the fine tuning followed by manual alignment of the mapped reads and single nucleotide polymorphisms (SNP) calling using the Geneious R6 Software application [Kearse et al, 2012]. The SNP were manually filtered and statistically compared between groups using STATA.

The consensus sequence from each sample was exported and a maximum likelihood phylogenetic tree with bootstrap support constructed to confirm that there was no contamination (Supplementary material) using the PAUP v4.0b10 software. The HTLV-1 sequences were also submitted to the LASP HTLV-1 Automated Subtyping Tool Version 1.0 [Alcantara et al, 2008] for genotyping in order to exclude subtype specific mutations. To analyze the TFBS modifications related to specific mutations we used the TFScan plugin of Geneious software.

Results:

The selected patients were analyzed regarding age and proviral load. The median age of asymptomatic carriers with low proviral load was lower than the other groups but with no statistical significance (Table 1). The log proviral load was similar among the groups characterized as high proviral load (5.39 Asymptomatic X 5.27 TSP/HAM p= 0.1551) but slightly different in the low proviral load group (2.99 Asymptomatic X 4.57 TSP/HAM p=0.0172) possibly due to the age difference.

The Ion Torrent sequencing showed a variable coverage depth between the samples ranging from 108.6x to 3740.5X (Table 2), because of this variation we have decided to analyze the

mutations in frequency as low as 1% if the reads with the variant mutation has no other mutation. To avoid detect subtype specific mutations we submitted the sequences to the LASP HTLV-1 Automated Subtyping Tool Version 1.0 and all the sequences were classified as subtype a subgroup A. We described the mutations found on Ion Torrent as minor (if the mutation prevalence was lower than 50%) and major (if the mutation prevalence was higher or equal to 50%). We found that samples with low proviral load showed more detected minor than the samples with high proviral load.

We found mutations in 136 positions over the 520 bp analyzed fragment of HTLV-1 LTR 5' with at least 1% frequency. Just 52 mutations were present in more than one patient being eleven out of 52 present in the previously determined major TFBS (Table 3). Other three mutations were statistically significant using the Fisher non-parametric test between the groups but were not present in previously determined TFBS (G126C/T, G306C and C479T). The complete list of samples and the respective mutations are found in the Supplementary Table 1.

We performed an *in silico* analyze using TFScan of eleven mutations in the previously described TFBD plus those mutations with statistical difference between groups and one deletion of eleven base pairs to see possible modifications in the TFBS. We found the A153G, C167T, G199A, A207G, C215T, C219G/T, C268T and G306C abrogated possible TFBS while the T165C, C219G/T, T301A/C and G306C/A created new possible TFBS (Table 3).

Discussion:

The Ion Torrent DNA sequencing was able to provide further information on HTLV-1 quasispecies than the Sanger sequencing, which lacks the sensitivity to show mutations at lower levels (20%). For HTLV-1 studies is important to analyze quasispecies instead of viral population because the virus is highly conserved with low evolutionary rates [Gessain et al, 1992; Sherman et al, 1992; Switzer et al, 2009]. In addition, only around 1% of the infected cell will produce HTLV-1 proteins in a day [Asquit et al, 2007], becoming important to analyze the mutations at 1% prevalence or even lower in studies whereas the HTLV-1 mutations are being analyzed as disease outcome. The detection of mutations in approximately one in four analyzed site by the Ion Torrent sequencing with at least 1% frequency supports the use of this technique to search for minor mutations.

The found that samples with low proviral load showed more detected minor variants than the samples with high proviral load and it could be partly explained by the highest hate of clonal expansion of HTLV-1 infected cells (Gillet et al, 2011).

Eleven mutations were present in the previously determined major TFBS and in more than a patient, indicating that might be a differential HTLV-1 expression comparing individuals or in different cells from the same individual. Studies indicated that two mutations might be a marker for increase of viral load [Neto et al, 2011; Magri et al, 2012] and those mutations were found usually in the TFBS, We have searched one of those mutations to confirm the data previously described but we did not find associations with proviral load, unfortunately all studies lack on sample size.

Three mutations that were not present in previously determined TFBS were statistically significant in this study and most frequent in patients with low proviral load or in asymptomatic carriers. Despite those mutations being not present in previously determined TFBS, one of those mutations (G306C/A) was present in a Sp-1 binding site determined by *in silico* analyze, and its presence abrogated the site for Sp-1 binding and created a new possible ATF binding site. The HTLV-1 promoter binding sites are not completely understood mainly because the absence of chromatin immune precipitation analysis in the LTR promoter of HTLV-1 infecting T CD4+ cells, and those mutations could decrease or increase the viral expression abrogating or creating TFBS, but a further study must be conducted in order to determine if there is or not transcription factors bound to those positions as well as in the eleven positions where mutations were found.

List of abbreviations:

Human T-cell Lymphotropic Virus type 1 (HTLV-1)

Adult T-cell leukemia/lymphoma (ATL)

Tropical spatic paraparesis/HTLV-1 associated myelopathy (TSP/HAM)

Infective dermatitis associated with HTLV-1 (IDH)

HTLV-1 associated uveitis (HAU)

Base pair (bp)

Competing interests:

All authors have declared no conflict interests.

Authors' contributions:

FFAR and TO wrote the paper and did the analysis. FFAR did the experiments. TO, BG and LCJA helped with discussion. FFAR, BGC and LCJA designed the study. BG provided the samples.

Acknowledgments:

The authors are grateful to Ms. Prevashine Padayache for technical assistance and M.Sc. Justen Manasa for DNA Sanger sequencing support. This project was supported by Brazil Ciencia Sem Fronteiras project from CNPQ. FFAR was supported by CAPES.

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Table 1. Age and proviral load of analyzed groups

	Asymptomatic		TSP/HAM	
Characteristic	Low proviral load (n=9)	High proviral load (n=8)	Low proviral load (n=7)	High proviral load (n=6)
Age, years				
Median (IQR)	33.1 (23.5-45.6)	55.3 (39.3-68.6)	53.1 (27.8-55.8)	45.8 (40.9-64.4)
Proviral load, copies per 10^6 cells				
Median (IQR)	964 (386-10591)	248792 (196473-507858)	36776 (18929-44396)	188060 (164213-232227)
Proviral load, log10				
Median (IQR)	2.98 (2.85-3.71)	5.39 (5.29-5.69)	4.57 (4.28-4.65)	5.27 (5.22-5.37)

IQR, interquartile range; n, number of patients

Table 2. Mutations description in the LTR regulation sites

	Asymptomatic		TSP/HAM	
	Low proviral load (n=9)	High proviral load (n=8)	Low proviral load (n=7)	High proviral load (n=6)
Sanger sequencing mutations				
Median	3	2.5	3	2
Ion Torrent depth coverage				
Median (IQR)	445 (340-759)	389 (244-1139)	551 (196-886)	352 (165-1203)
Ion Torrent major mutations				
Median	5	2.5	3	2.5
Ion Torrent minor mutations				
Median	7	3.5	7	6

Table 3. Mutations frequency by groups and TFBS change according to mutation

Mutation position	Change	Previously described TFBS	TFBS by TFSCAN	TFBS change		Asymptomatic (n=17)	TSP/HAM (n=13)	Low pvl (n=16)	High pvl (n=14)	Asy & low pvl (n=9)	Asy & high pvl (n=8)	TSP/HAM & low pvl (n=7)	TSP/HAM & high pvl (n=6)
				Creation	Abrogation								
126	G -> C/T	none	none	-	-	0,35	-	0,25	0,14	0,44	0,25	-	-
151Delta11	Delta11	TxRE-2	Sp-1, CREB, ATF	-	Sp-1, CREB, ATF	0,06	-	-	0,07	-	0,13	-	-
153	G -> A	TxRE-2	Sp-1	-	Sp-1	0,35	0,46	0,44	0,36	0,33	0,38	0,57	0,33
165	T -> C	TxRE-2	none	c-ets-2, CACCC-binding factor	-	0,12	-	0,13	-	0,22	-	-	-
167	C -> T	TxRE-2	c-ets-2	-	c-ets-2	0,12	-	0,06	0,07	0,11	0,13	-	-
199	G -> A	c-ets-2	c-ets-2	-	c-ets-2	-	0,15	-	0,14	-	-	-	0,33
207	A -> G	Sp1	TGT-3	-	TGT-3	0,12	-	0,13	-	0,22	-	-	-
215	C -> T	Sp1	GammaCAC2, CACCC-binding factor GammaCAC2, CACCC-binding factor	-	GammaCAC2, CACCC-binding factor GammaCAC2, CACCC-binding factor	0,06	0,15	0,19	-	0,11	-	0,29	-
219	C -> G/T	c-ets-2		H4TF-1		0,29	0,38	0,31	0,36	0,22	0,38	0,43	0,33
268	C -> T	TxRE-3	c-ets-2	-	c-ets-2	0,12	0,08	0,13	0,07	0,22	-	-	0,17
298	A -> G	E-Box	none	-	-	-	0,15	0,13	-	-	-	0,29	-
301	T -> A/C	E-Box	none	Sp-1	-	-	0,15	0,06	0,07	-	-	0,14	0,17
306	G -> C/A	none	Sp1	ATF	Sp-1	0,24	0,08	0,31	-	0,44	-	0,14	-
345	T -> C	Inr	none	-	-	0,12	-	0,13	-	0,22	-	-	-
479	C -> T	none	none	-	-	0,35	0,08	0,31	0,14	0,56	0,13	0,00	0,17

Position related to J02029

3.2 ADULTOS COM FALHA TERAPÊUTICA NA TERAPIA ANTIRRETROVIRAL DE PRIMEIRA LINHA CONTRA O HIV-1 APRESENTAM O GENÓTIPO SELVAGEMEM KWAZULU-NATAL, ÁFRICA DO SUL.

A África do Sul é o país com o maior número de infecções pelo HIV-1 no mundo. A presença do genótipo selvagem em adultos na terapia antirretroviral de primeira linha que falham a terapia (aumento da carga viral) é associada com desfechos clínicos desfavoráveis quando a terapia de segunda linha é introduzida. Além disto a terapia de segunda possui um custo muito mais alto do que a terapia de primeira linha na África do Sul, de tal forma que, se 12% ou mais dos indivíduos apresentarem falha terapêutica torna-se mais barato realizar a genotipagem de todos os indivíduos do que alterar a terapia. Devido a estes fatos, foi realizado um estudo com o objeto de determinar a prevalência do genótipo selvagem em Hlabisa além de identificar fatores associados a presença deste genótipo. Para realizar estas análises foram estudados 220 adultos com falha virológica que estavam sobre regime da terapia antirretroviral de primeira linha e foi realizado o sequenciamento do gene pol do HIV-1 pelo método de Sanger em todas as amostras. Das amostras com genótipo selvagem pelo sequenciamento de Sanger (28), 11 foram submetidas ao sequenciamento pelo Ion Torrent para confirmar a ausência de genótipos com resistência aos antirretrovirais. Os dados clínicos dos pacientes foram adquiridos através do SATuRN. O genótipo selvagem foi detectado em 28 amostras (12,7%). Dos 11 pacientes selecionados para realizar o sequenciamento pelo Ion Torrent, nove confirmaram não ter mutações de resistência aos antirretrovirais em alta frequência. Foi encontrada uma alta contagem de CD4+ no início da terapia associado a falha terapêutica assim como uma alta carga viral antes da genotipagem e não foi encontrado associação entre aderência a terapia auto-reportada e a presença do genótipo selvagem.

Wild type human immunodeficiency virus type 1 in adults with virological failure of first-line antiretroviral therapy in KwaZulu-Natal, South Africa

Baljinder SINGH[1,2], Tulio DE OLIVEIRA[2,3], Nuala McGRATH[2,4], Justen MANASA[2], Filipe Ferreira de Almeida REGO[2,5], Richard J LESSELLS*[1,2]

[1] Department of Clinical Research, London School of Hygiene and Tropical Medicine, London, UK
[2] Africa Centre for Health and Population Studies, University of KwaZulu-Natal, Mtubatuba, South Africa
[3] Research Department of Infection, University College London, UK
[4] Academic Unit of Primary Care and Population Sciences, and Department of Social Statistics and Demography, University of Southampton, Southampton, UK
[5] Gonçalo Moniz Research Center/Oswaldo Cruz Foundation, Salvador, Brazil

* Corresponding author:

Dr Richard J Lessells
London School of Hygiene and Tropical Medicine
Keppel Street
London
WC1E 7HT
United Kingdom
Phone: +44-207-958-8201
Fax: +44-207-612-7860
Email: richard.lessells@lshtm.ac.uk

Running title: Wild type HIV-1 in adults with virological failure
Article type: Original article
Word count: 4000 words (main text); 278 (abstract)
Tables/Figures: 4 Tables, 1 Figure, 1 Supplementary Figure
References: 48

Source of funding: This work was supported by the European Union (SANTE 2007 147–790), the US Centre for Diseases Control via CAPRISA (Health Systems Strengthening and HIV Treatment Failure) and the Swiss South African Joint Research Programme (SSJRP) research grant (Swiss Prot / South Africa: Protein Bioinformatics Resource Development for Important Health-related Pathogens). The Hlabisa HIV Treatment and Care Programme has received support through the United States Agency for International Development (USAID) and the President's Emergency Plan (PEPFAR)

under the terms of Award No. 674-A-00-08-00001-00. RJL and NMcG were supported by the Wellcome Trust (grant numbers 090999/Z/09/Z and WT083495MA respectively). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ABSTRACT

Background

The presence of wild type genotype (i.e. absence of drug resistance-associated mutations) in adults with virological failure on first-line antiretroviral therapy (ART) is associated with poor clinical outcomes after switch to second-line therapy. Identification of factors associated with wild type genotype could help to prevent unnecessary switches to second-line therapy.

Methods

Cross-sectional analysis of adults (≥18 years) with virological failure (at least one viral load >1000 copies/ml) on first-line ART in a large primary health care treatment programme. Genotypic drug resistance testing (population sequencing) was performed using the SATuRN/Life Technologies affordable and open access method. Deep sequencing was performed on a random sample (N=11) using Ion Torrent™ technology to determine the frequency of minority drug-resistant variants. Factors associated with wild type genotype were explored by logistic regression.

Results

A total of 220 adults were included (73.2% female, mean age 38.1 years). Wild type genotype was detected by population sequencing in 28 (12.7%). No major drug resistance mutations were detected by deep sequencing for 81.8% (9/11) of those sampled. Higher baseline CD4+ cell count was associated with a greater likelihood of wild type genotype (per 50 cells/ μL increase, adjusted odds ratio (aOR) 1.45, 95% confidence interval (CI) 1.15-1.82, $p<0.001$) as was a higher viral load prior to resistance testing (per 1 $\log_{10}$ increase, aOR 3.03, 95% CI 1.44-6.36, $p=0.002$) but there was no evidence of an association between self-reported adherence and the presence of wild type genotype.

Conclusions

Approximately one in eight adults failing first-line ART had wild type genotype which, for the majority of those sampled, was confirmed by deep sequencing. Access to genotypic resistance testing may be required in this region to achieve optimal individual-level and population-level outcomes.

Keywords: HIV-1; anti-retroviral agents; drug resistance; microbial sensitivity tests; treatment failure; medication adherence

INTRODUCTION

The scale-up of HIV treatment services in the last decade has enabled the delivery of antiretroviral therapy (ART) to almost seven million individuals in sub-Saharan Africa by 2012[1]. The public health approach involves standardised ART regimens, simplified laboratory monitoring strategies and clear criteria for switching from first-line to second-line regimens[2,3]. Studies from the region have demonstrated that the majority of adults with virological failure on first-line ART regimens harbour drug-resistant HIV strains; yet in these same studies up to one-third of adults had no drug resistance-associated mutations detected using standard genotyping techniques, suggesting that wild type virus was the predominant strain in the viral population (Table 1)[4-15].

In the presence of wild type genotype, there is the potential with appropriate adherence interventions for viral re-suppression on first-line therapy, thus preserving future treatment options for the individual as well as offsetting costs associated with second-line ART[5,13]. Clinical outcomes after switch to second-line ART have been shown to be poorer in adults with wild type genotype, which may be related to on-going and unaddressed poor adherence[7]. This is strengthened by data from studies in South Africa, reporting very high frequency of wild type genotype (39-67%) in adults with virological failure on second-line regimens[11,16,17]. The failure to identify these patients who are more likely to benefit from intensified adherence support or specific adherence interventions, rather than a switch in ART regimen, will therefore lead to worse clinical outcomes and will increase patient and health system costs[18].

Standard genotypic techniques using population (Sanger) sequencing have the limitation that mutations present at a low frequency (below 20%) in the viral population are not reliably detected and therefore the presence of minority drug-resistant quasispecies cannot be excluded[19]. This problem can be at least partly overcome by next-generation sequencing techniques, which have higher sensitivity and can therefore detect minority drug-resistant quasispecies[20]. It is important to know whether or not, at time of virological failure, the absence of major drug resistance-associated mutations is confirmed by deep sequencing techniques.

Here we use data from a cross-sectional drug resistance study embedded in a large primary health care treatment programme in rural South Africa to determine the frequency of wild type genotype at time of failure of first-line ART. We also use next-generation deep sequencing methodology to determine the presence of minority drug-resistant quasispecies at time of first-line ART failure in those with wild type genotype on Sanger sequencing. Finally, we explore factors associated with the presence of wild type genotype to understand whether the presence of wild type genotype might be predictable in the absence of genotypic resistance testing.

MATERIALS AND METHODS

Setting

The Hlabisa HIV Treatment and Care Programme provides comprehensive HIV services at 17 primary health care (PHC) clinics and one district hospital in the predominantly rural Hlabisa sub-district (1430 km^2) in northern KwaZulu-Natal[21,22]. The programme is co-ordinated by the Department of Health (DoH) with support from the Africa Centre for Health and Population Studies (www.africacentre.ac.za). HIV treatment and care is delivered largely by nurses and counsellors. The programme adheres to national antiretroviral treatment guidelines. Between 2004 and April 2010, eligibility for adults was based on CD4+ cell count <200 cells/μL[23]; this was expanded initially in April 2010 to 350 cells/μL for pregnant women and individuals with active TB disease[24]; in August 2011, this was extended to 350 cells/μL for all[25]. ART was recommended throughout for individuals with World Health Organization (WHO) clinical stage 4 disease and, since April 2010 for all individuals with drug-resistant TB. First-line ART regimens were until April 2010 based on a combination of stavudine (d4T), lamivudine (3TC) and either efavirenz (EFV) or nevirapine (NVP). During this time, substitution of zidovudine (ZDV) for d4T was permitted in the event of serious toxicity. After April 2010, tenofovir (TDF) replaced d4T in the recommended first-line regimen and ZDV was an alternative option in the presence of baseline renal impairment (creatinine clearance <50 ml/min).

Patients are required to attend monthly appointments to collect ART and for review of any problems. Routine monitoring includes CD4+ cell count and viral load testing after six months, 12 months and then annually (if viral load <400 copies/ml). Switch to second-line ART is recommended in the event of two viral load measurements >1000 copies/ml, provided that adherence issues have been addressed[24].

Study design

Since late 2010, genotypic resistance testing has been accessible through a drug resistance study nested within the treatment programme[26,27]. Adult patients (≥18 years) on a standard first-line regimen for greater than 12 months with evidence of virological failure (defined for the study as a single viral load >1000 copies/ml) were reviewed at their local clinic by a medical officer. Following discussion of study objectives and procedures (in isiZulu, the local language), informed written consent was obtained. During the initial consultation, demographic and clinical information was recorded on a standardized data collection form. This incorporated details of all ART regimens, CD4+ cell count and viral load measurements, and factors plausibly associated with adherence and treatment failure. Patients were asked to rate their overall adherence at the time of genotype using a modified Likert-type scale, as recommended in the national guidelines[24]. A rating of ‘good’ implied that 90% or greater doses were taken and ‘suboptimal’ adherence implied fewer than 90% of doses taken. Any

treatment interruptions as disclosed by the patient were documented but not cross-referenced with pharmacy details. Patients were asked whether they currently or had ever used traditional medicine and other non-prescribed medications (e.g. immune boosters). A 5ml ethylenediaminetetraacetic acid (EDTA) blood sample was taken and transported the same day to the Africa Centre virology laboratory in Durban.

Population HIV-1 sequencing

Genotypic resistance testing was performed using the SATuRN/Life Technologies affordable open access method, which has been described in detail previously[26,28]. The nucleotide sequences were interpreted using the Stanford HIVSeq algorithm version 6.0.5, generating a genotypic susceptibility score (GSS) for each antiretroviral drug. A GSS of 1 suggested full drug activity for the specific drug, 0.5 partial drug activity, and 0 no drug activity. Interpretation of the results and treatment recommendations from an HIV specialist physician were then incorporated into the final report, which was returned to the medical officer and clinic within 30 days in order to guide clinical management[27].

All clinical and genotypic data were entered into the SATuRN RegaDB clinical resistance database[29,30]. Data were anonymised at entry and individuals were identified only by a unique study identification number.

Deep HIV-1 sequencing

A subset of specimens with wild-type HIV-1 genotype was selected for deep sequencing by simple random sampling. The sequences were produced using the Ion Torrent™ sequencing technology (Life Technologies, Carlsbad, CA). At least 15,000 sequences were produced for each of the specimens, which allowed characterization of the viral population at 1% level. For the Ion Torrent analysis the purified PCR products were submitted to enzymatic fragmentation following the manufacturer's conditions for the 100ng-long amplicon input (Ion Xpress™ Plus Fragment Library Kit Part no. 4471269, Life Technologies, Carlsbad, CA), with 15 minutes of incubation time in a heat block followed by Agencourt® AMPure® XP purification (Beckman Coulter, Brea, CA). The ligation of the Ion Xpress P1 and barcode adapters and nick repair was performed (Ion Xpress Barcode Adapters 1-16 Cat. No. 4471250 and Ion Xpress Barcode Adapters 17-32 Cat. No. 4474009) followed by the second Agencourt purification. The fragmented DNA was then size-selected (E-Gel® SizeSelect™ 2% Agarose, Life Technologies, Carlsbad, CA) for the 200 base pair (bp) sequencing run. The samples were amplified and purified for the third time using Agencourt. The samples were then quantified and analysed using the Experion™ DNA 1K system (Bio-Rad Laboratories, Inc., Hercules, CA). In the next step, the amplicons were pooled and then submitted to the One Touch system (Ion OneTouch™ 200 System Template Kit Cat. No. 4471263, Life Technologies, Carlsbad, CA) followed

by the enrichment step. Finally, the pooled enriched Ion Sphere Particles (ISPs) were submitted to the sequencing for 200bp reads (Ion PGM™ 200 Sequencing Kit Cat. No. 4474004, Life Technologies, Carlsbad, CA) in a 314 chip (Ion 314™ Chip 8-pack Cat. No. 4462923, Life Technologies, Carlsbad, CA). The sequence data were assembled using the Geneious Pro Software application[31]. Single nucleotide polymorphisms (SNPs) were estimated by Geneious, followed by *de novo* assembly and manual alignment of the sequences for each participant. The consensus sequence from each sample was exported and a maximum likelihood phylogenetic tree with bootstrap support constructed using phyML in order to confirm that there was no contamination (Figure 2)[32].

Data analysis

Wild type genotype was defined based on population (Sanger) and Ion Torrent sequencing as the absence of major nucleoside/nucleotide reverse-transcriptase inhibitor (NRTI) resistance mutations (at reverse transcriptase (*RT*) positions 41, 65, 67, 69, 70, 74, 115, 151, 184, 210, 215 and 219) and non-nucleoside reverse-transcriptase inhibitor (NNRTI) resistance mutations (at *RT* positions 100, 101, 103, 106, 138, 181, 188, 190 and 230), as listed by the Stanford HIV Drug Resistance Database[33]. No major protease mutations were observed in this study. The overall proportion of wild type genotype was determined and the characteristics of individuals with wild type genotype were compared with those harbouring drug resistance-associated mutations. Descriptive analysis used Pearson's chi-squared test for categorical data. The distribution of all continuous variables was assessed by histogram. If data were normally distributed, Student's *t*-test was used and if not normally distributed the Wilcoxon rank-sum test was used.

The date of genotype was defined as the date on which the specimen was collected for genotypic resistance testing. Baseline CD4+ cell count was the measurement closest to the date of ART initiation (no more than seven months prior to, or two weeks immediately after date of ART initiation). The pre-genotype CD4+ cell count and viral load were the closest measurements prior to (no more than thirteen months) or up to four weeks after the date of genotype. Duration of ART was calculated from the date of ART initiation to the date of genotype. Duration of virological failure was calculated from the date of the earliest viral load >1000 copies/ml, unless that was followed by a measurement <50 copies/ml. If all viral load measurements were >1000 copies/ml then duration of virological failure was calculated from the date of ART initiation[26]. Immunological failure was based on the pre-genotype CD4+ cell count and was defined according to WHO definitions: pre-genotype CD4+ cell count lower than baseline CD4+ cell count; pre-genotype CD4+ cell count less than 50% of the peak CD4+ cell count on treatment; or persistent CD4+ cell count <100 cells/μL[2].

Logistic regression models were used as part of an exploratory analysis of factors associated with the presence of wild type genotype (as defined by Sanger population sequencing). All variables considered significant at 10% in the univariate model were considered in the multivariable model. Self-reported adherence at the time of genotype (categorised as suboptimal (<90%) or good (≥90%)) was included in the multivariable model based on previous studies that highlighted a strong association between adherence and the absence of drug resistance following treatment failure. A separate category was created for variables with missing data. All analyses were performed in STATA 10 (StataCorp, College Station, TX).

Ethics statement

The project was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BF052/010), the Provincial Health Research Committee of the KwaZulu-Natal Department of Health (HRKM176/10) and the Ethics Committee of the London School of Hygiene and Tropical Medicine (011/476).

RESULTS

Subjects

A total of 305 adults had a specimen submitted for genotypic resistance testing between December 2010 and June 2012. Thirty-four (11.5%) individuals were excluded from this analysis for protocol violations and five (1.6%) patients were excluded due to unsuccessful genotyping (Figure 1). A further 45 (14.0%) with missing baseline CD4+ cell count, pre-genotype CD4+ cell count or pre-genotype viral load data were excluded from the analysis, as these data were central to the multivariable analysis. There were no significant differences between the excluded and included individuals (12% of those excluded but with a genotype result had wild type virus). Therefore, 220 patients were included in the final analysis; 161 (73.2%) female, mean age at genotype 38.1 years (95% confidence interval (CI) 36.8-39.3); median baseline CD4+ cell count 97.5 cells/μL (interquartile range (IQR) 45-166.5). The median duration of treatment was 38.5 months (IQR 29-50). One-third of the participants (n = 74, 33.6%) had immunological failure at the time of resistance testing. Over half of the participants (n = 128, 58.2%) rated their adherence as good (>90% doses taken) but around a quarter (n = 56, 25.5%) reported at least one instance of ART interruption. A minority of patients disclosed current or past traditional medicine (n = 69, 31.4%) or alcohol (n = 25, 11.4%) use (Table 2). Although all patients in the analysis met the study definition for virological failure, the majority of individuals (83.8%) also satisfied the national definition for virological failure (two consecutive viral loads >1000 copies/ml)[24].

HIV-1 population sequencing and deep sequencing

Using standard population (Sanger) sequencing, 28 patients (12.7%) had wild type genotype; the majority (n=192, 87.3%) had mutations conferring resistance to both nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs) and non-nucleoside reverse-transcriptase inhibitors (NNRTIs), as reported previously[26]. In total, 11 of the 28 specimens with wild type HIV-1 on population sequencing underwent deep sequencing using the Ion Torrent platform. The mean number of sequences per specimen was 5304, the mean deep coverage was 568.7 and the mean read size was 121.1bp (Table 3). Nine of the eleven samples did not have any minority drug-resistant population (>5%). However, one sample had the K103N mutant in 56.8% of reads covering this position. A second sample had the E138A mutation, which is polymorphic at a prevalence of around 5% in subtype C viruses; although this mutation is associated with decreased susceptibility to second-generation NNRTIs (rilpivirine and etravirine), it is not known to be selected by NNRTIs and so was considered in this case to be a polymorphism.

Factors associated with wild type genotype

The characteristics of those with and without drug resistance-associated mutations are summarized in Table 2. The duration of ART was similar between the two groups (median 35.5 months for wild type group vs. 39.5 months for drug resistance group, $p = 0.18$), as was the duration of virological failure (median 27.5 months vs. 22 months, $p = 0.37$). The wild type group had higher baseline CD4+ cell count than the resistance group (median 170.5 cells/μL vs. 88 cells/μL, $p = 0.001$) and were more likely to have immunological failure at the time of genotype (53.6% vs. 30.7%, $p = 0.02$). Patients in the wild type group had significantly fewer blood monitoring tests (CD4+ cell count and viral load) and a longer median time between tests.

The results of the logistic regression analysis exploring factors associated with wild type genotype are shown in Table 4. The likelihood of having wild type virus increased with every 50 cells/μl increase in the baseline CD4+ cell count (odds ratio (OR) 1.31, 95% CI 1.09-1.57, p=0.002). A greater duration between monitoring blood tests (per 30 day increase: CD4+ cell count OR 1.19, 95% CI 1.04-1.36; viral load OR 1.17, 95% CI 1.06-1.28, p=0.002) and fewer recorded blood test measurements (CD4+ cell count OR 0.74, 95% CI 0.59-0.92, p=0.005; viral load OR 0.63, 95% CI 0.49-0.81, p<0.001) were significantly associated with a higher likelihood of harbouring wild type virus. The presence of immunological failure at time of genotype was associated with higher odds of having wild type virus (OR 2.6, 95% CI 1.16-5.81, p=0.02). There was no strong evidence to support the impact of duration of ART, duration of virological failure and treatment interruption, as was the case with self-reported adherence, use of traditional medicine or alcohol. ART substitution was strongly associated with a reduced likelihood of having wild type virus (OR 0.29, 95% CI 0.10-0.87, p<0.001).

In the multivariable model, baseline CD4+ cell count remained strongly associated with wild type genotype (adjusted odds ratio (aOR) 1.45, 95% CI 1.15-1.82 p<0.001) (Table 4). A higher pre-genotype viral load was also associated with higher odds of wild type genotype (for every 1 $\log_{10}$ increase, aOR 3.03, 1.44-6.36, p=0.002). Every additional month delay between the last viral load and subsequent resistance test increased the likelihood of having wild type virus (aOR 1.27, 95% CI 1.07-1.51, p=0.005). Individuals that experienced a NRTI or NNRTI substitution were significantly less likely to have wild type genotype compared to those who had remained on the same antiretroviral regimen throughout (aOR 0.29, 95% CI 0.10-0.87, p=0.03). Despite strong associations at the univariate level, duration between viral load/CD4+ cell count measurements, the total number of these blood tests and immunological failure did not contribute to the final model. Self-reported adherence was also not statistically significant in the univariate or multivariable analysis.

DISCUSSION

The presence of wild type genotype in adults failing first-line ART has been consistently observed in studies from South Africa[4-15]; and has been associated with adverse clinical outcomes after switch to second-line ART[7]. Here, in adults with virological failure on first-line ART in a large primary health care programme in rural KwaZulu-Natal, we found that around one in eight had wild type genotype, despite long durations of ART and long durations of virological failure. In the majority of cases for whom deep sequencing was performed, the absence of drug resistance-associated mutations was confirmed. The presence of wild type genotype was strongly associated with baseline CD4+ cell count; in that individuals who had started ART at higher CD4+ cell counts had a greater risk of wild type genotype, suggesting a possible association between higher CD4+ cell count and poorer adherence.

Studies from South Africa exploring drug resistance in adults failing first-line antiretroviral regimens have demonstrated that between 5% and 33% have wild type genotype using standard genotypic resistance testing (Table 1)[4-15]. Our finding of 12.7% with wild type genotype is comparable to the pooled proportion of 14.0% from these twelve other studies. Minority drug-resistant variants can be present at low levels in the viral population, especially where there has been undocumented interruption of ART prior to sampling. We used deep sequencing techniques to confirm that in almost all cases drug resistance-associated mutations were not present, providing more confidence that first-line ART regimens would remain effective in this group and that switch to second-line therapy might be unnecessary.

The relationship between adherence and the likelihood of emergence of drug resistance is complicated and differs among the antiretroviral drug classes[34-36]. Resistance to NNRTIs develops rapidly at moderate to low levels of adherence due to the limited effect of mutations on viral fitness. As all

adults in the study sample were on NNRTI-based first-line regimens, the absence of drug resistance in the face of prolonged virological failure (median 27.5 months on failing regimen for wild type group) suggests that adherence in this group might have been so low that there was no selective pressure on the virus, at least at the time of the genotype, supported by our finding of significantly higher levels of viraemia in patients with wild type virus. The lack of association between self-reported adherence and isolation of wild type virus corroborated findings of a similar study in South Africa, which used pill-count as an objective adherence measure[12]. Many of the instruments used for measuring adherence (including the Likert-type scale used in this study) have been shown to lack sensitivity or specificity to virological outcomes in this setting and may not measure adherence very well[37]. A particular limitation in the context of this study was that adherence was only assessed at a single time point (at the time of genotyping) and no robust measures of adherence over the whole treatment course were available. Poor adherence to HIV care and monitoring, in terms of having fewer viral load and CD4+ cell count measurements, greater delays between blood tests and greater delays to resistance testing, were associated in the univariate analysis with having wild type virus in this cohort, suggesting that these may be potential markers of intermittent adherence or undocumented treatment interruptions. Single drug substitutions in this programme have been most commonly due to adverse effects, particularly the long-term metabolic complications related to stavudine. The reduced risk of wild type genotype with previous drug substitution therefore could be explained by the fact that patients with the poorest adherence to their ART would be at lower risk of adverse effects due to lower overall exposure to antiretroviral drugs.

The observation that wild type genotype was associated with higher baseline CD4+ cell counts is of particular concern at a time of expanding ART eligibility criteria and increasing interest in the use of antiretroviral treatment for prevention[38,39]. This finding correlates with results from one North American study where higher baseline CD4+ cell counts (>350 cells/μL) were associated with a lower risk of drug resistance at first-line ART failure[40]. Higher CD4+ cell counts at ART initiation have been associated in sub-Saharan Africa with higher risk of treatment interruption and higher likelihood of disengagement from care, which may be broader measures of poor adherence compared to patients self-estimation of adherence[41-44]. Individuals with a higher baseline CD4+ cell count are less likely to have symptomatic disease at presentation and could plausibly have less incentive to optimise ART adherence in order to improve their health[45]. Healthier people with less symptomatic disease may also face additional barriers to adherence, for example challenges of regular clinic attendance if in employment or with care responsibilities. However, with evidence from Uganda showing high levels of programme retention in patients with CD4+ >350 cells/μL[46], the association between higher baseline CD4 count and adherence is complex and likely to be multi-factorial in the Sub-Saharan Africa setting.

These findings have broader relevance for the public health approach to ART delivery in low-income and middle-income countries. The cost differential between first-line and second-line ART regimens remains high and there is therefore a need to ensure that patients are switched to second-line regimens appropriately. At present, genotypic resistance testing is not included in South African Department of Health ART guidelines to guide treatment switch from first-line to second-line ART in adults, although other guidelines in the region have recommended its use[47]. Mathematical models based on South African HIV treatment programs have suggested that genotypic resistance testing at first-line ART failure would be cost-effective in this setting, especially when the prevalence of wild type genotype was 12% or more, as in our study[18]. If the occurrence of wild type genotype increases with further expansion of ART and particularly with treatment at higher CD4+ cell counts then the rationale for genotypic resistance testing to guide treatment switch may become stronger.

The results of this analysis need to be interpreted in light of certain limitations. The adults included in the study were enrolled during routine clinical care and were a subset of adults with virological failure in the programme; we cannot be sure that they were representative of all adults with virological failure. Adherence was measured at a single time-point (at time of virological failure) and would not have captured the dynamics of adherence over time[48]. No other objective measures of adherence, such as pharmacy refill data or medication possession ratios, were available routinely in the programme. Self-declared treatment interruptions were recorded in clinic records? but this was neither systematic nor corroborated with pharmacy records. Methodologically, the multivariable analysis reflected an exploratory analysis, and further work on larger or combined cohorts would provide further supporting evidence for the associations seen.

In conclusion, about one in eight HIV-infected adults with virological failure on first-line ART has wild type virus. Although certain factors were associated with wild type genotype, based on these data it would be difficult to predict the presence of wild type genotype using only clinical and demographic details. The use of genotypic resistance testing at time of first-line ART failure may therefore be a cost-effective intervention to avoid unnecessary treatment switches and preserve antiretroviral regimens within the public health model. There is now a need for empirical evidence of the impact of genotypic resistance testing in this region.

Sequence data

All sequences were submitted to Genbank (accession numbers KC951632 - KC951853 for Sanger sequences and accession numbers KF561837-KF561847 for Ion Torrent sequences)

Acknowledgements

The authors would like to thank study participants; Zakhona Gumede, Xolile Kineri and Lungani Ndwandwe for their assistance with data entry and curation; Siva Danaviah and Sureshnee Pillay for their help with genotypic resistance testing; and the medical officers and nurses involved with the enrolment of subjects and patient care.

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Figure 1 Flow diagram showing study participants

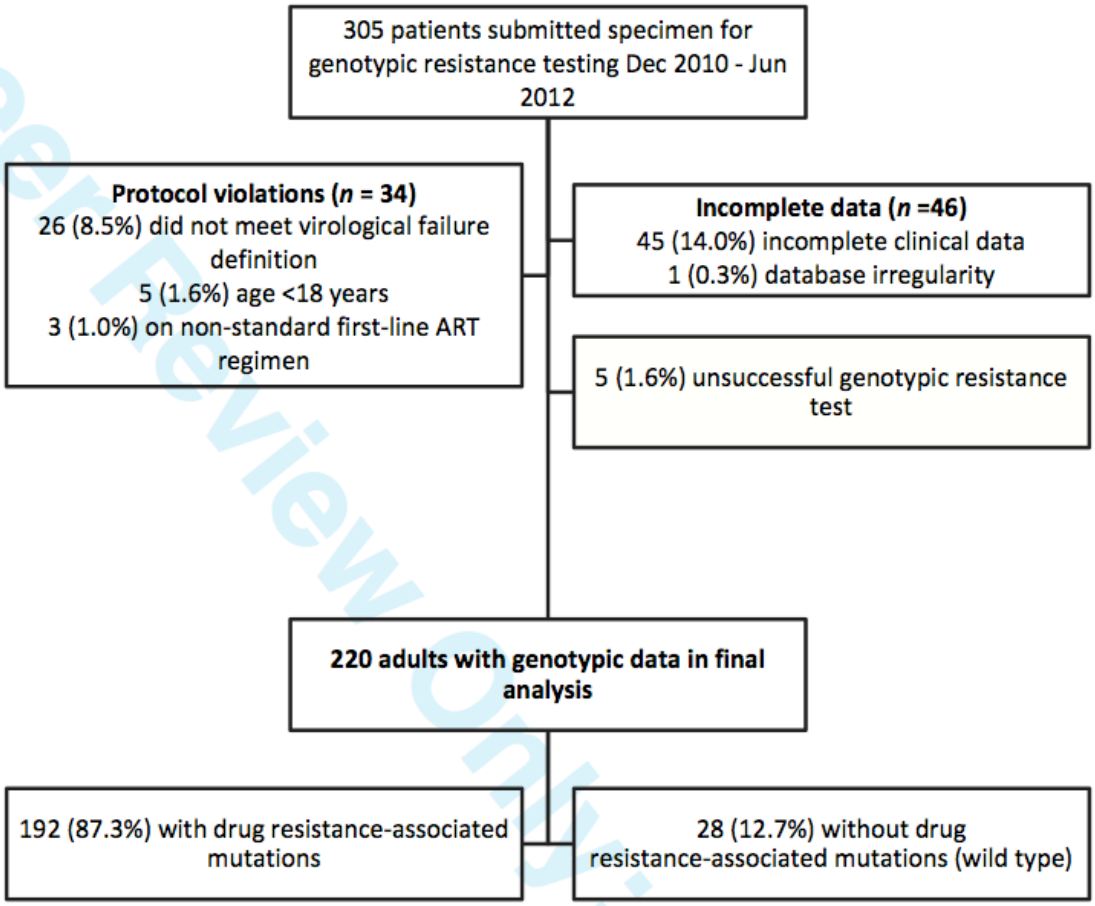

Figure 2 Maximum likelihood phylogenetic tree showing relationship between paired sequences from population sequencing and consensus sequences from Ion Torrent sequencing. The sequences in this study are marked in red. All of the pairings are support by bootstrap of 100%. Scale bar units are nucleotide substitutions per site.

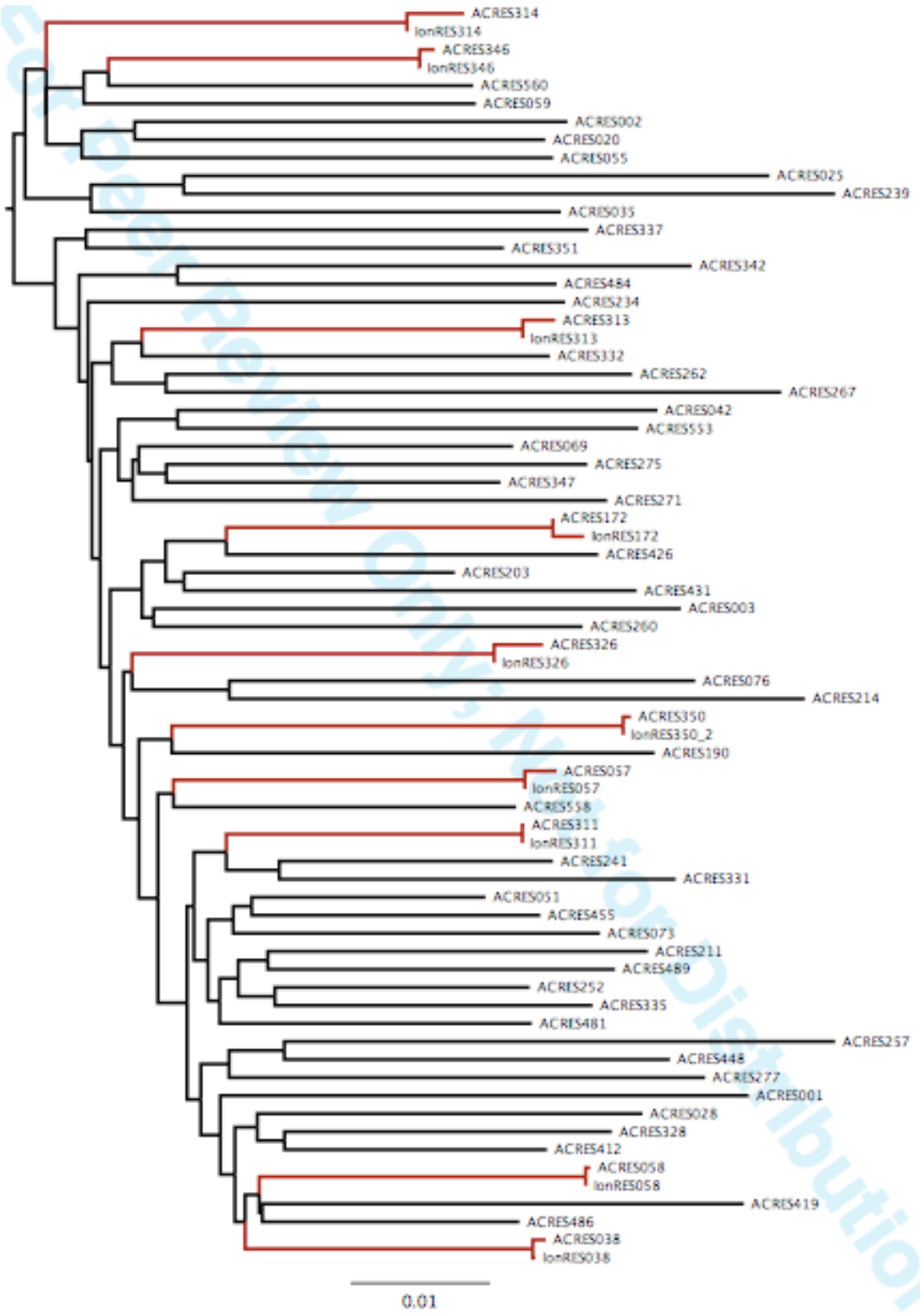

Table 1 Summary of previous South African studies of drug resistance in adults with virological failure on first-line antiretroviral therapy showing proportion with wild type genotype

Table 1 Summary of previous South African studies of drug resistance in adults with virological failure on first-line antiretroviral therapy showing proportion with wild type genotype

Study author	Period of enrolment	Setting	Indication for genotype	N	Duration of ART (median)	Wild type (n, %)
Barth[4]	NS	Limpopo (one rural clinic)	1 x VL >1000	21	8 months	2 (9.5%)
Hoffmann[5]	2002-2006	Multi-site workplace programme	1 x VL >1000	68	NS	23 (33.8%)
Orrell[6]	2002-2007	Cape Town (eight urban clinics)	2 x VL >1000	110	9 months	7 (6.0%)
Murphy[7]	2004-2006	Durban (two urban hospitals)	1 x VL >1000	141	12 months	19 (13.5%)
Marconi[8]	2005-2006	Durban (two urban hospitals)	1 x VL >1000	115	11 months	19 (16.5%)
Sigaloff[9]	2006-2009	Johannesburg (one hospital)	2 x VL >5000	43	22 months	5 (11.6%)
Sigaloff[10]	2007-2009	Multicentre study*	1 x VL >1000	183	26 months	22 (12.0%)
Sigaloff[11]	2007-2009	Multicentre study*	1 x VL >1000	232	27 months	22 (9.5%)
Van Zyl[12]	2007-2010	Western Cape (one hospital and one CHC)	1 x VL >400	167	14 months	28 (16.8%)
El Khatib[13]	2008	Soweto (one hospital)	1 x VL >400	38	NS	7 (18.4%)
Wallis[14]	NS	Johannesburg (two urban hospitals)	2 x VL >1000 or 2 x VL >5000†	226	NS	38 (16.8%)
Singh[15]	NS	Durban (one urban hospital)	1 x VL >1000	43	29 months	2 (4.7%)

ART, antiretroviral therapy; CHC, community health clinic NS, not stated; VL, viral load

* Multicentre studies involving urban and rural sites in South Africa, Zambia, Zimbabwe, Uganda, Kenya and Nigeria

† Different entry criteria for the two hospitals but aggregated results presented

Table 2 Characteristics of participants with and without evidence of drug resistance-associated mutations

Patient characteristic	Drug resistance [n=192]	No drug resistance (wild type) [n=28]	Overall [n=220]	p value
Sex, female, n (%)	137 (71.4)	24 (85.7)	161 (73.2)	0.11
Age, years, mean (95% CI)	38.2 (36.7-39.6)	36 (34.2-40.6)	38.1 (36.8-39.3)	0.70
Baseline CD4+ cell count, cells/μL, median (IQR)	88 (42-156.5)	170.5 (126.5-189.5)	97.5 (45-166.5)	0.001
Pre-genotype CD4+ cell count, cells/μL, median (IQR)	221 (119-314.5)	175 (91.5-291)	217 (113-312)	0.29
Duration between CD4+ tests, median days (IQR)	255 (218-298)	288 (267-355)	263 (221-300)	0.005
CD4+ measurements prior to genotype, median (IQR)	5 (3-7)	4 (2-6)	5 (3-7)	0.007
Immunological failure*, n (%)	59 (30.7)	15 (53.6)	74 (33.6)	0.02
Pre-genotype VL, log_{10} copies/ml, median (IQR)	4.21 (3.69-4.84)	4.67 (4.41-5.33)	4.30 (3.74-4.89)	<0.001
Duration between VL test, median days (IQR)	240 (195-317)	288 (228-387)	250 (200-323)	0.005
VL measurements prior to genotype, median (IQR)	5 (4-7)	4 (3-4.5)	5 (4-6)	<0.001
Time between last VL and genotype, days, median (IQR)	77 (41-138)	153 (105-224)	86 (43-153)	<0.001
Duration of ART, months, median (IQR)	39.5 (29-51.5)	35.5 (29-44)	38.5 (29-50)	0.18
Duration of virological failure†, months, median (IQR)	22 (15-32.5)	27.5 (15.5-35)	22.5 (15-33)	0.37
ART substitution‡, n (%)	70 (36.5)	4 (14.3)	74 (33.6)	0.02
ART interruption, n (%)	51 (26.6)	5 (17.9)	56 (25.5)	0.32
Self-reported adherence§, n (%)				
Suboptimal	53 (32.5)	6 (25.0)	59 (26.8)	0.46
Good	110 (67.5)	18 (75.9)	128 (58.2)	
Use of traditional medicines or immune boosters, n (%)‖	58 (31.7)	11 (39.3)	69 (32.7)	0.43
Denies current alcohol use‖	149 (86.6)	25 (92.6)	174 (87.4)	0.39

ART, antiretroviral therapy; IQR, interquartile range; VL, viral load

* Immunological failure was based on the pre-genotype CD4+ cell count and was defined according to WHO definitions[2]: pre-genotype CD4+ cell count lower than baseline CD4+ cell count; pre-genotype CD4+ cell count less than 50% of the peak CD4+ cell count on treatment; or persistent CD4+ cell count <100 cells/μL

† Duration of virological failure measured from first viral load >1000 copies/ml (unless subsequent viral load <50 copies/ml); if all viral load measurements >1000 copies/ml then duration measured from date of ART initiation

‡ Any substitution of single NRTI or NNRTI, or both

§ Self-reported adherence based on visual analogue scale corresponding to overall adherence (suboptimal <90%, good ≥90%). 33 patients had missing data for self-reported adherence, one was in the wild type group

‖ Nine patients had missing data for traditional medicine use; 21 patients had missing data for alcohol use

Table 3 Results of deep sequencing analysis using Ion Torrent sequencing technology

Sequence Name	# Sequences	Deep coverage	Mean size (bp) read	Treatment experience	SNP position, codons and mutant amino acid at resistance sites
RES038	2308	231	124	d4T/3TC/NVP	Y181 (TAC -> TAA (3.2%) = stop codon), T215 (ACC ->ATC (6.8%) = I)
RES057	5347	605	119	d4T/3TC/EFV/NVP	None
RES058	27736	3017	122	TDF/3TC/EFV	**E138 (GAA -> GCA (99.1%) = A)***, L210 (TTG -> CTG(2.6%) = L), P255 (CCC -> GCC(2.6%) = A)
RES172	3519	403	129	d4T/3TC/NVP	T215 (AAC ->ACA(5.8%) -> T)
RES255	1519	174	122	d4T/3TC/EFV	None
RES311	1559	179	123	d4T/3TC/EFV	None
RES313	3417	368	120	d4T/3TC/EFV	None
RES314	1473	175	125	d4T/3TC/EFV	L100 (TTA ->CTA(3.8%) =L)
RES326	5454	467	96	d4T/3TC/NVP	None
RES346	1586	170	127	d4T/3TC/EFV	**K103 (AGA -> AGT(56.8%) = N)**, M230 (ATG ->AAG(2.5%) =K)
RES350	646	72	126	d4T/3TC/EFV/NVP	None
Average (mean)	**4960**	**533**	**121**		

3TC, lamivudine; d4T, stavudine; EFV, efavirenz; NVP, nevirapine; TDF, tenofovir; #, number; bp, base pairs; SNP, single nucleotide polymorphism

* E138A is a resistance mutation associated with reduced susceptibility to rilpivirine and etravirine, but not selected by efavirenz

Table 4 Univariate and multivariable logistic regression analysis of factors associated with wild type genotype

Variable	*n*	Univariate analysis			Multivariable analysis		
		OR	95%CI	p value	aOR	95%CI	p value
Sex							
Male	83	1			1		
Female	137	2.41	0.80-7.26	0.09	3.48	0.85-14.22	0.06
Age, per 10 years	220	0.92	0.61-1.40	0.70			
Baseline CD4+ cell count*	220	1.31	1.09-1.57	0.002	1.45	1.15-1.82	<0.001
Pre-genotype CD4+ cell count*	220	0.94	0.82-1.07	0.32			
Duration between CD4+ tests, days†	220	1.19	1.04-1.36	0.01	1.18	0.88-1.59	0.28
CD4+ measurements prior to genotype, per test	220	0.74	0.59-0.92	0.005	1.35	0.63-2.87	0.45
Immunological failure							
No	146	1			1		
Yes	74	2.60	1.16-5.81	0.02	0.74	0.23-2.34	0.60
Pre-genotype VL, log_{10} copies/ml	220	2.42	1.40-4.17	0.001	3.03	1.44-6.36	0.002
Duration between VL test, days†	220	1.17	1.06-1.28	0.002	1.01	0.78-1.30	0.97
VL measurements, prior to genotype	220	0.63	0.49-0.81	<0.001	0.57	0.24-1.35	0.20
Time between last VL & genotype, days†	220	1.25	1.10-1.41	<0.001	1.27	1.07-1.51	0.005
Duration of ART, months‡	220	0.77	0.55-1.07	0.11			
Duration of virological failure, months‡	220	1	0.99-1.01	0.53			
ART substitution							
No	146	1			1		
Yes	74	0.29	0.10-0.87	<0.001	0.22	0.05-0.96	0.03
ART interruption							
No	164	1					
Yes	56	0.60	0.22-1.66	0.31			
Self-reported adherence							
Suboptimal (<90%)	59	1			1		
Good (≥90%)	128	1.45	0.54-3.85		1.31	0.35-4.94	
Missing	33	1.22	0.32-4.67	0.75	1.17	0.09-3.39	0.55
Use of traditional medicines or immune boosters							
No	141	1					
Yes	69	1.39	0.61-3.17	0.43			
Denies current alcohol use							
No	25	1					
Yes	174	0.52	0.11-2.34				
Missing	21	0.30	0.04-2.32	0.27			

CI, confidence interval; OR, odds ratio; aOR, adjusted odds ratio

* per 50 cells/μL increments

† per 30 day increments

‡ per 12 month increments

3.3 EFEITO DA COINFECÇÃO HIV-1/HTLV-1 NA CONTAGEM DE LINFÓCITOS T CD4+ EM PACIENTES COM FALHA TERAPÊUTICA NA ZONA RURAL DE KWAZULU-NATAL, ÁFRICA DO SUL

Poucos estudos descrevem esta coinfecção no mundo, e muito menos investigam avaliam a relação entre estes retrovírus. É conhecido que o HTLV-1 gera uma expansão clonal dos linfócitos T CD4+, enquanto que o HIV-1 faz o oposto, depletando estas células. Além disto a contagem de linfócitos T CD4+ é utilizada como marcador de progressão para AIDS em alguns países e nos indivíduos coinfectados, a infecção pelo HTLV-1 poderia mascarar a contagem de linfócitos T CD4+. Neste estudo nós analisamos as diferenças na contagem de linfócitos T CD4+ entre indivíduos infectados apenas com o HIV-1 e indivíduos coinfectados HIV-1/HTLV-1 com falha terapêutica, além de analisarmos a soroprevalência do HTLV-1 em indivíduos infectados pelo HIV-1. Para estes objetivos triamos 381 pacientes infectados pelo HIV-1 com falha terapêutica para a infecção pelo HTLV-1 e comparamos as contagens de linfócitos T CD4+ adquiridas ao longo do tempo dos pacientes coinfectados com os infectados apenas com o HIV-1. Neste trabalho também foi realizada a análise filogenética e haplotipagem de HLA para evitar viés na comparação entre os grupos. Foram encontramos 8 pacientes coinfectados (2,1%). Nós não observamos nenhuma diferença estatística quando analisamos transversalmente os dados clínicos dos pacientes, exceto na primeira contagem de linfócitos T CD4+ após o início do tratamento que estava maior nos indivíduos coinfectados ($p=0.03$). A análise multivariada longitudinal mostrou que a média de linfócitos T CD4+ ao longo do tratamento foi estatisticamente maior nos pacientes coinfectados levando em consideração características demográficas, carga viral, fatores relacionados a terapia, entre outros. Nos pacientes coinfectados também não foram encontrados marcadores de HLA relacionados com os supressores de elite do HIV-1. Os dados deste trabalho sugerem que os pacientes coinfectados em terapia antirretroviral deveriam ter um acompanhamento clínico diferenciado dos indivíduos apenas infectados pelo HIV-1, pois a coinfecção poderia estar levando ao aumento do número dos linfócitos T CD4+ sem um possível ganho de resposta imune.

Title: HIV-1 and HTLV-1 co-infection effect on CD4+ level of patients failing anti-retroviral therapy in rural KwaZulu-Natal, South Africa.

Authors: Filipe Ferreira de Almeida Rego[1,2]; Alain Vandormael[2,3]; Siva Danaviah[2]; Denis Chopera[4,5]; Bernardo Galvão-Castro[1,6]; Luiz Carlos Junior Alcantara[1]; Tulio de Oliveira[2]

[1]Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil; [2]Africa Centre for Health and Population Study, University of KwaZulu-Natal, South Africa; [3]Department of Sociology & Statistics, University of Minnesota, USA; [4]KwaZulu-Natal Research Institute for Tuberculosis and HIV, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [5]HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [6]HTLV Center/ Bahia School of Medicine and Public Health/Bahia Foundation for Science Development, Salvador, Bahia, Brazil.

Corresponding author

Tulio de Oliveira, PhD
PO Box 198
Mtubatuba, 3935
South Africa
Telephone: +27 (35) 550 7500
Fax: +27 (35) 550 7560
email: tdeoliveira@africacentre.ac.za

Filipe Ferreira de Almeida Rego, M.Sc.
LHGB/CPqGM/FIOCRUZ.
Waldemar Falcão Street 121, Brotas, Salvador, Bahia, Brazil 40295-001
Telephone: +55 (71) 3176-2246
Fax: +55 (71) 3176-2300
email: filipefar@hotmail.com

e-mail addresses:

Alain Vandormael: vando026@umn.edu

Siva Danaviah: sdanaviah@africacentre.ac.za

Denis Chopera: denis.chopera@gmail.com

Bernardo Galvão-Castro: bgalvao@bahiana.edu.br

Luiz Carlos Junior Alcantara: lalcan@bahia.fiocruz.br

Abstract:

Background: HTLV-1/HIV-1 co-infection may result in the increase of patient CD4+ count. In KwaZulu-Natal, a province of South Africa, the presence of HTLV-1 and HIV-1, as well as HTLV-1/HIV-1 co-infection, has been described previously in cohorts of untreated HIV-1 patients. In this paper we propose to verify the prevalence of HTLV-1, to determine the molecular epidemiology of patients failing ART and to statistically assess differences in CD4+ counts between HTLV-1/HIV-1 co-infected and HIV-1 mono-infected patients living in rural KwaZulu-Natal. For this purpose, 381 HIV-1 patients failing ART were screened for HTLV-1 by ELISA, and from some of these patients PCR and genotypic sequences were produced.

Results: The HTLV-1 seroprevalence was 2.1% (8 out of 381 patients). The phylogenetic analysis showed no epidemiological relationship between the co-infected patient samples, and that the sequences grouped into 3 different monophyletic clusters in the subtype *a* subgroup *A* cluster. The TMRCA analysis for 3 clusters with South African sequences showed an introduction in the past 300 years. The patients were grouped by HTLV-1/HIV-1 co-infected and HIV-1 mono-infected status for the statistical analysis. There were no cross-sectional differences between the groups regarding CD4+ count before therapy, CD4+ count at genotype, age, gender, viral load, duration of ART, immunological failure, ART failure and first ART regimen. However, the first CD4+ count after treatment was higher in the co-infected group ($p=0.03$). A multivariate, longitudinal model shows that the mean CD4+ count over time for the HTLV-1/HIV-1 co-infected group was significantly higher than the HIV mono-infected group ($p<0.05$) when adjusting for demographic characteristics, viral load and ART treatment factors. This finding was independent of expression of HLA Class 1 genotypes previously associated with HIV-1 infected elite suppressors.

Conclusions: The HTLV-1 prevalence among HIV-1 infected patients in KwaZulu-Natal is higher than previously described. This study suggests that the HTLV-1 subtype *a* subgroup *A* is the circulating strain in KwaZulu-Natal, and that this was introduced at least 3 times from West-Central Africa. In addition, the increase of CD4+ count after therapy suggests a differential clinical management for the HTLV-1/HIV-1 co-infected patients.

Keywords: HIV-1, HTLV-1, CD4+, co-infection, South Africa

Background:

Human T-cell Lymphotropic Virus type 1 (HTLV-1) is known to be the etiological agent of Adult T-cell leukemia/lymphoma (ATL) [1], tropical spatic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) [2,3] and other inflammatory diseases such as HTLV-1 –associated infective dermatitis (IDH) [4] and HTLV-1 associated uveitis (HAU) [5]. HTLV-1 is transmitted via sexual contact, breastfeeding, blood transfusion and intravenous drug use, in a similar way to the transmission of human immunodeficiency virus type 1 (HIV-1). HTLV-1 is present in many areas where HIV-1 infection is found [6-11].

Around 5.6 million people are living with HIV in South Africa [12]. KwaZulu-Natal, a province of South Africa, has the highest HIV-1 prevalence in the country; 24.7% of 15-49 year-olds are infected [13]. HTLV-1/HIV-1 co-infection has been detected in many African countries, such as Ghana, where prevalence among AIDS hospitalized patients has reached 11.3%. [14,15]. In South Africa, two surveys showed HTLV-1 prevalence to range from 1.7% to 6.1% in HIV-1 seropositive patients [14] and another survey showed an HTLV-1 prevalence of 0.49% in non HIV-1 tested patients [17].

HIV-1 subtype *C* is the most prevalent HIV subtype circulating in KwaZulu-Natal, as well as worldwide, and is responsible for nearly half (48%) of global infections [18]. HTLV-1 is classified into 7 subtypes (a to g), 6 of which (subtypes *a, b, d, e, f, g)* are present in Africa. Subtype *a* is the most common subtype around the world [19] and in South Africa [16, 20].

HIV-1 and HTLV-1 viruses both infect CD4+ T-cells. HTLV-1 stimulates the proliferation of CD4+ infected lymphocytes through a mechanism mediated by one of its proteins (trans activator x – *tax protein)* [21]. This is unlike HIV infection, which depletes CD4+ cells in patients over time, causing acquired immunodeficiency syndrome (AIDS). This difference is attributed to the virus replication process: HTLV is a virus with clonal replication and cell-to-cell transmission while HIV proliferation is determined by active replication. In addition, several studies have shown the importance of HLA types controlling the CD4+ count on HIV-1 infection. In this infection, some HLA types such as HLA-I B*27 have been linked with HIV's slow progression to AIDS and the increase of CD4+ count [22-24].

Previous studies have attempted to show differences in CD4+ counts between HTLV-1/HIV-1 co-infected patients and HIV mono-infected patients before treatment [25-29]. While there is

no consensus, a number of these studies suggest that HTLV-1 infection has the important effect of increasing patient CD4+ count. Some case series and case reports show the development of AIDS despite high CD4+ counts in co-infected patients [30,31], and one study suggests that AIDS might progress faster in co-infected patients with higher CD4+ counts and higher levels of activation markers [32]. On the other hand, one study [33] did not show any statistically supported difference in the CD4+ count after HAART in co-infected patients.

In this study, we verified HTLV-1 prevalence, determined the molecular epidemiology of patients with HIV-1 and HTLV-1 failing ART, and statistically assessed differences in CD4+ T-cell counts between HTLV-1/HIV-1 co-infected and HIV-1 mono-infected patients living in rural KwaZulu-Natal, South Africa.

Results

We screened 381 HIV-infected plasma samples for HTLV-1/2 antibodies from patients failing first-line ART. Eight samples were reactive by ELISA (2.1%), 7 of which were confirmed by nested-PCR as HTLV-1 (1.8%) and were successfully genotyped. The phylogenetic analysis suggested no epidemiological relationship between the sequences from co-infected patients (Figure 1), given that all the HIV sequences from the co-infected patients clustered separately in the phylogenetic tree (Figure 1A). The LASP HTLV-1 Automated Subtyping Tool (Version 1.0) and the Bayesian analysis classified all the sequences as HTLV-1 subtype *a* subgroup *A* with a bootstrap support of >90% and a posterior probability (PP) value of 1.00 respectively. The HTLV-1 tree showed that 7 South African LTR sequences grouped into 3 different clusters: 2 sequences grouped with southern African sequences (PP=0.91), another 2 sequences grouped exclusively with South African sequences (PP=1.00) and the remaining 3 sequences with South American and southern African sequences (PP=1.00) (Figure 1B). The multiple clustering and non-epidemiological linkages suggest at least 3 introductions of HTLV-1 subtype *a* subgroup A to KwaZulu-Natal, South Africa. The time of most recent common ancestor (TMRCA) of the clusters of South African sequences from this work and previously published sequences from South Africa and Mozambique were estimated using molecular clock techniques. The TMRCA of the first cluster was 221 (HPDs ranging from 81 to 395 years ago, ESS=1411). The second cluster, which also contained 2 South African sequences, grouped with sequences from Mozambique and South Africa, showed a common ancestor (TMRCA) of 225 (HPDs: 139 to 321 years ago, ESS=2084). The third group, which

consisted of 3 new sequences, clustered with Brazilian and Mozambican sequences and showed the TMRCA of 180 (HPDs: 100 to 264 years ago ESS=1905). The phylogeny of the South African sequences suggests multiple introductions of HTLV-1 into the KwaZulu-Natal area, and has a close relationship with sequences from Mozambique. The TMRCA suggests that these multiple introductions occurred relatively recently and that this was most probably related to Bantu migration from West-Central Africa to southern Africa.

In order to analyze the clinical and demographic characteristics of HTLV-1/ HIV-1 co-infected and HIV-1 mono-infected patients, a detailed statistical analysis was conducted on the dataset. Table 1 shows the bivariate statistics for the demographic, CD4+, viral load and ART treatment characteristics for the total sample of 381 patients; the results are stratified by HTLV-1/HIV-1 co-infection (n=8) and HIV-positive only (n=373) group status. Females comprise 75% of the sample, with the gender distribution not significantly different by group status (p=1.00). There was no significant difference in median age by group status (p=0.452), though we do report that 7 of the 8 HTLV-1/HIV-1 co-infected patients were more than 34 years of age. Cross-sectional, bivariate analyses show no significant differences between the 2 groups for CD4+ count at baseline (p=0.410) and at genotype (p=0.151); there was, however, a significant difference between groups for first CD4+ counts after treatment initiation (p=0.03). There was no statistical difference between groups for the bivariate analyses with regard to immunological failure at the time of the genotype, log10 viral load, the duration on ART treatment (in months), the duration of ART failure (in months) and the initial ART regimen (see Table 1). 6 of the 8 (75%) and 5 of the 8 (62.5%) HTLV-1/HIV-1 co-infected patients have achieved virological suppression below 1000 copies/ml and 50 copies/ml respectively after therapy introduction. The chronological RegaDB charts for co-infected patients with CD4+ counts, therapy regimens and viral load are shown in the Supplementary Figure 1.

Table 2 shows the results of the random-effects analysis, which we used to model trends in CD4+ counts by HTLV-1/HIV-1 co-infected and HIV-1 mono-infected group status over time. The time variable was scaled in months from the start of ART treatment until the date of genotypic resistance testing. All the reported variables from Table 1 were included in the final analysis. We report a mean baseline CD4+ count of 353.7 units. The interquartile range of the patients' predicted mean CD4+ counts was 113.43 units. (This result gives an estimation of the deviation of the random intercepts from the baseline mean). Time was significantly

associated with variation in CD4+ trajectories (slopes): a 1 month increase in time since the start of ART treatment was associated with a 0.63 unit increase in CD4+ count (SE= 0.26, $p<0.05$), holding all else constant. The patients' predicted CD4+ count trajectories (slopes) ranged from -14.26 to 6.24 units with an interquartile range of 1.63 units. (The interquartile range estimates for the predicted intercept and slope values are not shown in Table 2.)

Results show that the mean baseline CD4+ count for the HTLV-1/HIV-1 co-infection group (445.04 units) was significantly higher (93.32 units, SE=43.71, $p<0.05$) than the HIV-1 mono-infected group, holding all else constant. Log10 viral load count and immunological failure at time of genotypic resistance testing were significant at the 0.001 level. For example, a 1log unit increase in viral load was associated with a 25.04 unit decrease in CD4+ count (SE=2.78), holding all else constant. We further report that a 1 month increase in ART treatment failure was associated with a 1.91 unit decrease in CD4+ count (SE=0.52, $p<0.001$), adjusting for the remaining covariates. Age and viral load suppression below 50 and 1000 units were not significant at the 0.05 level (holding all else constant).

In order to determine if host HLA typing associated with HIV-infected elite controllers were responsible for the CD4+ proliferation, we decided to type the 8 co-infected individuals and a random sample of 30 mono-infected individuals from our cohort of 381 patients. Known protective HLA types were noted in only 1 of the 8 co-infected patients namely, RES249 (HLA B58:01 with HLA A02:05). The remaining 7 patients displayed genotypes commonly noted in the South African population, which are not related to protection or have been associated with HIV-infected elite controllers (table 3, supplementary table 1). An additional 30 patients infected with HIV but not with HTLV were also HLA typed. Of these, 2 of 30 displayed the B*81 HLA type while 1 patient displayed the HLA B58:01 genotype. Both of these are associated with elite controllers. These findings therefore indicate no association between HLA type and HTLV/HIV co-infection.

Discussion:

Our results show an HTLV-1 prevalence of 2.1% among HIV-1 infected patients in KwaZulu-Natal failing ART. This result is slightly higher than the HTLV-1 prevalence reported in our previous study that analyzed 1435 samples from both HIV-1 non-infected and mono-infected treatment-naive patients in the KwaZulu-Natal region [16]. The highest prevalence information from South Africa pointed to a prevalence of 6.1% in 293 HIV-1 positive patients

from Free State province [14], which suggests the HTLV-1 prevalence could be heterogeneous in South Africa.

The HTLV-1 LTR phylogenetic analysis showed that the new South African sequences grouped into 3 different monophyletic clusters in the subtype *a* subgroup *A* cluster, as previously discussed [16]. The larger number of sequences from South Africa allowed us to estimate the ancestry of HTLV-1 subtypes *a* subgroup A cluster. The genetic diversity among the new HTLV-1 strains and molecular clock analysis of the different clustering suggests an introduction into the region prior to HIV-1. According to historical reports, HTLV-1 emerged from Central Africa and spread throughout the world [34]. The recent introduction of HTLV-1 into South Africa is confirmed by molecular clock analysis. The introduction of HTLV-1 into South Africa is the result of the past 300 years of migration of the Bantu speaking population into southern Africa. The relationship of South African and Mozambican sequences is probably due to the close distance between the geographic area of the study and the capital city of Mozambique, Maputo (about 380 km) and this warrants further investigation.

The CD4+ count is an important marker for immunological efficiency in HIV-1 mono-infected patients. Previous studies have reported increases in the CD4+ counts of HTLV-1/HIV-1 co-infected patients before therapy [25-29], but to the best of our knowledge, only one of these studies attempted to assess patient CD4+ count after the introduction of therapy in a cross-sectional analysis [33]. Our study finds that the median CD4+ count of the HTLV-1/HIV-1 co-infected group was higher than the HIV-1 mono-infected group after the beginning of therapy (acknowledging that there was no cross-sectional difference in median baseline CD4+ counts between the two groups). In addition, results from our longitudinal analysis show that, over time, the mean CD4+ count for the HTLV-1/HIV-1 co-infected group was significantly higher than the HIV mono-infected group, taking demographic, viral load and ART treatment factors into account. A study in Brazil [35] found the median CD4 cell count in the HIV-1 monoinfected group was lower than in the co-infected group. This data in agreement with others studies [10,25] suggesting that CD4+ cells are functionally impaired in co-infection and it should be taken into account for clinical management.

We propose that CD4+ count differences between HTLV-1/HIV-1 co-infected and HIV-1 mono-infected be considered in the clinical management of HIV-1 progression, HIV-1 and

HTLV-1 associated diseases, and the treatment management of HTLV-1/HIV-1 co-infected patients.

Methods:

Study Design

This study is a retrospective, longitudinal data analysis of patients failing ART at the Hlabisa HIV Treatment and Care Programme at Unkanyakude district in rural KwaZulu-Natal. The Hlabisa HIV Treatment and Care Programme provides comprehensive HIV services at 17 primary health care (PHC) clinics and one district hospital in the predominantly rural Hlabisa sub-district (1430 km2) in northern KwaZulu-Natal. In late 2010, HIV genotypic resistance testing was incorporated in the treatment programme through a drug resistance study nested within the treatment programme [36]. Briefly, adult patients (≥16 years) on a first-line ART regimen for longer than 12 months and who showed evidence of virological failure (defined by the study as a single viral load >1000 copies/ml) were informed of the study protocol and consent was obtained. Basic demographic and clinical information was recorded on a standardized data collection form. This information included details of ART regimens, CD4+ cell counts and viral load measurements. A 5ml ethylenediamine tetraacetic acid (EDTA) blood sample was taken from all patients analysed in this study. The Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BF052/010) approved the project.

HTLV-1 detection:

All plasma samples obtained from HIV-1 infected patients were submitted to HTLV Ab ELISA (Diagnostic Automation, Inc) to determine HTLV-1 positive status. To determine poatient nuclear DNA was extracted was extracted from positive blood samples using a QIAGEN QIAamp® DNA Blood Kit. Positive samples were then submitted to a nested-PCR using the HTLV-1 long terminal repeat (LTR) 5' region as described previously [37]. The positive PCRs were used for HTLV-1 genotyping.

HIV-1 and HTLV-1 genotyping

HIV-1 genotyping was performed using the in-house method of SATuRN Life Technologies [38]. From the HTLV-1 LTR PCR positive samples, the amplicons were purified using the PureLink® PCR Purification Kit. HTLV-1 sequencing was performed using the Taq FS Dye terminator cycle sequencing kit (Applied Biosystems) on an automated 3130xl Genetic

Analyzer (Applied Biosystems Inc, Foster City, CA) using the identical nested-PCR inner primers. The sequences were then analyzed using the Geneious 5.6.5 software [39]. The HTLV-1 sequences were submitted to the LASP HTLV-1 Automated Subtyping Tool Version 1.0 [40] for genotyping. HIV-1 sequences were analysed with the REGA HIV-1 subtyping Tool version 3.0 [41].

HLA Typing:

DNA was extracted from DBS and whole blood (where available) from 8 HIV/HTLV co-infected patients and 30 HIV mono-infected patients randomly selected from our cohort of 381 patients. The HLA Class 1A and 1B alleles were amplified in a nested PCR strategy modified from Cotton et al [2012] using the Kapa2G™ Robust HotStart kit (Kapabiosystems Inc.) and 7µl extracted DNA. Each of the 1kb fragments generated were sequenced using primers described in Cotton et al [2012] producing 6 sequencing fragments for each of the Class 1 alleles. Sequences were produced using an ABI 3100xl genomic analyzer. The sequences were analysed using the Assign SBT software (Connexio Genomics INC).

Phylogenetic analysis

For the phylogenetic analysis, all available South African HTLV-1 LTR sequences plus sequences related to all subtypes and subgroups were downloaded from the GenBank and the dataset was aligned using the ClustalX [43] software and edited manually using the Se-Al programme. We constructed Bayesian trees in duplicate (to enhance reliability) using the MrBayes programme [44]. The MrBayes parameters used were the following: the selected model was GTR with gamma distribution; 1 tree was sampled every 1000 trees during 10 million generations; the initial 10% of trees were excluded from analysis. The analysis convergence was verified using Tracer. Trees posterior probabilities (PP) were summarized using TreeAnnotator (http://beast.bio.ed.ac.uk). The PP value was considered significant if >0.9. The genetic variability analysis was performed using 1000 bootstrap and Tamura-Nei plus Gamma distribution model by the MEGA software [45].

Molecular clock analysis:

The BEAST package [46] was used to determine the time of the most recent common ancestor (TMRCA) for the clusters with the new sequenced strains from South Africa with PP support >0.9. For the Beast analysis, we have assumed the previously described evolutionary rate (2X10-5 substitutions/site/year for one fixed mutation) [16]. The analysis was done in

three independent MCMC runs in order to enhance the reliability of the results. The analysis used the strict molecular clock with the constant growth tree priors and the effective sample size (ESS) was considered good if greater than 200. The statistical parameters were analyzed using Tracer. We report the point estimate of TMRCA and the 95% Highest Probability Densities (HPDs).

Statistical analysis of clinical parameters:

The data was derived from anonymized patient clinical records stored in the RegaDB database of the SATuRN Treatment Network [47,48]. Our analysis included variables indicating patient HTLV-1/HIV-1 co-infection status; demographic characteristics (age and sex); CD4+ counts (at baseline, after start of treatment therapy, at time of genotypic resistance testing); immunological failure (as defined according to WHO guidelines: fall of CD4+ cell count to baseline or below; 50% fall from on-treatment peak value; or persistent CD4+ cell count <100 cells/μl at time of genotypic resistance testing); viral load (at time of genotypic resistance testing); virological suppression (<1000 copies/ml and <50 copies/ml); and treatment characteristics (duration of ART therapy, duration of ART therapy failure and initial ART regimen). We performed bivariate analyses of the demographic, CD4+, viral load and treatment variables by HTLV-1/HIV-1 co-infection status. To determine cross-sectional statistical differences by group, we used Mood's non-parametric equality of median tests for the interval data and Pearson chi-squared tests for the categorical data (a Fisher's exact test was used for cells in which the expected frequencies were less than 5). We selected a longitudinal mixed-effects model to undertake the multivariate analysis. Our selection of this model was motivated by the multiple CD4+ count and viral load measures for each patient over time. We therefore specified a two-level model, and allowed each patient's CD4+ count intercept and slope to vary randomly in order to model the effect of time. All analyses were performed with Stata 12.1.

Sequence data:

The GenBank accession numbers of the new HTLV-1 LTR5' fragments included in phylogenetic study were as follows: KF042345 to KF042351.

List of abbreviations:

Human T-cell Lymphotropic Virus type 1 (HTLV-1)

Adult T-cell leukemia/lymphoma (ATL)
Tropical spatic paraparesis/HTLV-1 associated myelopathy (TSP/HAM)
Infective dermatitis associated with HTLV-1 (IDH)
HTLV-1 associated uveitis (HAU)
Human immunodeficiency virus type 1 (HIV-1)
Acquired immunodeficiency syndrome (AIDS)
Antiretroviral therapy (ART)
World Health Organization (WHO)

Competing interests:
All authors have declared no conflict interests.

Authors' contributions:
FFAR, AV and TO wrote the paper. FFAR did the genotyping and HTLV-1 detection. AV did the statistical analysis. TO, BG and LCJA helped with discussion. FFAR, TO, BG and LCJA designed the study. SD and DC performed the HLA typing sequencing and analysis.

Acknowledgments:
The authors are grateful to Ms. Prevashine Padayache for technical assistance and M.Sc. Justen Manasa for DNA sequencing support. This project was supported by Brazil Ciencia Sem Fronteiras project from CNPQ. Tulio de Oliveira & Siva Danaviah are funded by the Wellcome Trust (082384/Z/07/Z).

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Table 1. Demographic and clinical characteristics of HIV-1 infected and HIV-1/HTLV-1 co-infected patients.

Characteristic	HIV-1/HTLV-1 positive	HIV-1 positive	p value
Sex, *n* (%)			
Male	2 (25%)	100 (27%)	
Female	6 (75%)	273 (73%)	0.909
Age, years			
Median (IQR)	39 (35.5-43.5)	36 (31-43)	0.452
16-24	-	28 (7.5%)	
25-34	1 (12.5%)	128 (34.3%)	
35-44	5 (62.5%)	135 (36.2%)	
≥45	2 (25.0%)	82 (22.0%)	0.361
Baseline CD4+ cell count, cells/µl [a]			
Median (IQR)	109.5 (68-166)	105.5 (48-171)	0.410
<50	1 (12.5%)	81 (21.7%)	
50-100	1 (12.5%)	75 (20.1%)	
101-200	3 (37.5%)	133 (35.7%)	
>200	1 (12.5%)	33 (8.8%)	
Missing values	2 (25.0%)	51 (13.7%)	0.891
First CD4+ cell count after treatment, cells/µl			
Median (IQR)	282 (229-424)	206 (119-323)	**0.031**
<50	-	23 (6%)	
50-100	-	51 (14%)	
101-200	1 (12.5%)	109 (29%)	
>200	7 (87.5%)	190 (51%)	0.224
CD4+ cell count at time of genotype, cells/µl			
Median (IQR)	248.5 (219-461)	200 (107-307)	0.151
<50	-	43 (11.5%)	
50-100	-	48 (12.9%)	
101-200	1 (12.5%)	97 (26.0%)	
>200	7 (87.5%)	185 (49.6%)	0.192
Immunological failure at time of genotype, *n* (%) [a]	6 (75%)	288 (77%)	0.883
HIV viral load at time of genotype, log_{10} copies/ml			
Median (IQR)	4.36 (3.67-4.83)	4.35 (3.80-4.88)	0.994
Ever achieved virological suppression, *n* (%)			
Viral load <1000 copies/ml	6 (75.0%)	199 (53.4%)	0.224
Viral load <50 copies/ml	5 (62.5%)	137 (36.7%)	0.136
Duration of antiretroviral therapy, months			
Median (IQR)	58 (50-76)	55 (43-68)	0.116
Duration of antiretroviral failure, months [b]			
Median (IQR)	31 (16-41)	25 (15-38)	0.646
Initial antiretroviral regimen, *n* (%)			
d4T/3TC/EFV	5 (62.5%)	231 (61.9%)	
d4T/3TC/NVP	3 (37.5%)	95 (25.5%)	
AZT/3TC/EFV	-	4 (1.1%)	
Other	-	43 (11.4%)	0.694

IQR, interquartile range; d4T, stavudine; 3TC, lamivudine; EFV, efavirenz; NVP, nevirapine; AZT, zidovudine.

[a] Immunological failure was defined according to WHO guidelines: fall of CD4+ cell count to baseline or below; 50% fall from on-treatment peak value; or persistent CD4+ cell count <100 cells/μl

[b] Duration of antiretroviral failure was estimated from the date of the first viral load >1000 copies/ml to date of genotype, unless there was a viral load <50 copies/ml in-between, in which case the time was estimated from the next viral load >1,000 copies/ml. If there was no viral load ≤1,000 copies/ml then time was calculated from date of ART initiation.

Table 2: Results of the multivariate random-coefficients model for the CD4 counts

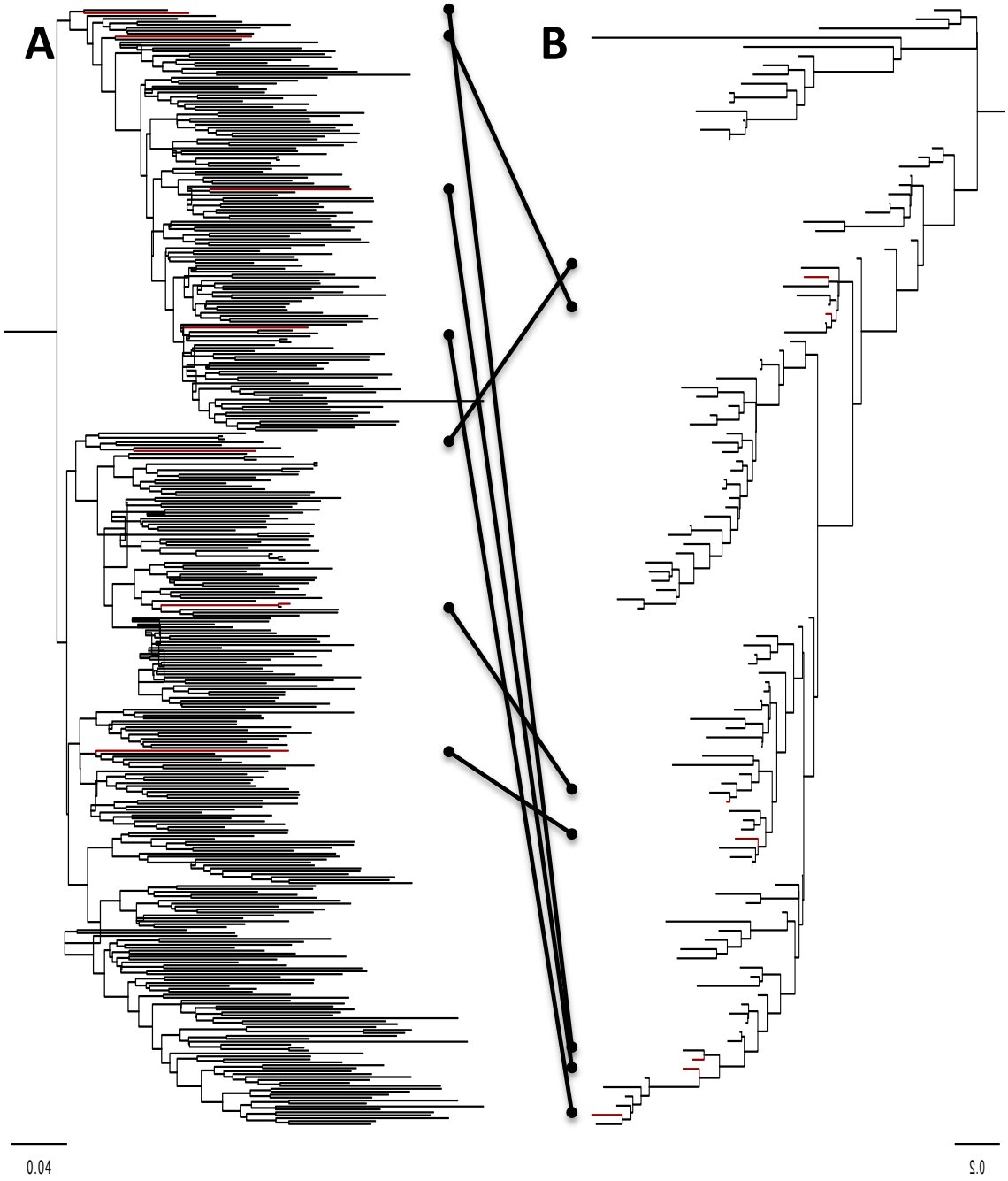
Variables	**CD4 Count**	**(SE)**
HTLV-1/HIV-1 co-infected	91.32**	(43.71)
Female	41.67***	(15.59)
Age	-0.923	(0.649)
Treatment Time (months)	0.628**	(0.265)
Viral Load (log10)	-25.04***	(2.781)
Immunological Failure	-90.92***	(16.36)
Virological Suppression (<1000)	19.15	(22.72)
Virological Suppression (<50)	-0.293	(20.00)
ART Treatment Failure (months)	-1.906***	(0.519)
Duration of ART Treatment (months)	2.190***	(0.484)
Regimen: d4T/3TC/NVP	-17.85	(16.23)
Regimen: AZT/3TC/NVP	-60.95	(69.45)
Regimen: Other	-59.91**	(26.23)
Constant	353.7***	(39.69)
Observations	2,130	
Number of groups	380	

Standard errors in parentheses
*** p<0.01, ** p<0.05, * p<0.1

Table 3: HLA Class I allele genotypes of the eight patients co-infected with HIV and HTLV-1.

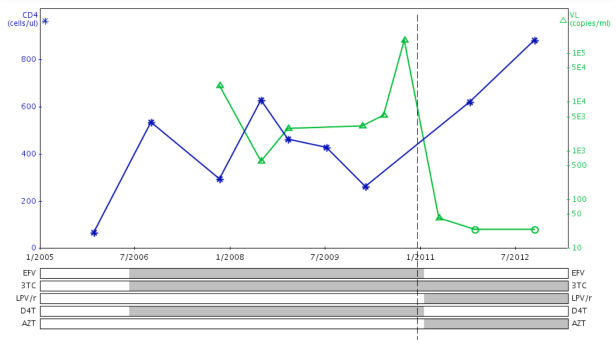
Patient Id	HLA A1	HLA A2	HLA B1	HLA B2
RES001	29:02	34:02	18:01	44:03
RES064	30:02	68:02	15:10	53:01
RES068	68:01	74:01	15:03	15:03
RES123	02:05	43:01	NA	NA
RES177	02:05	23:01	45:01	58:02
RES249	02:05*	74:01	47:01	58:01*
RES268	03:01	30:02	08:01	42:01
RES405	68:02	74:01	15:16	57:03

Figure 1. Bayesian tree of 380 HIV-1 pol subtype C sequences from the study (A) and 132 HTLV-1 LTR sequences (B) based on a 1050 and 566 base pair alignment, respectively. The red lines in the tree indicate the sample position while the oval arrows indicate the relationship between the samples in trees.

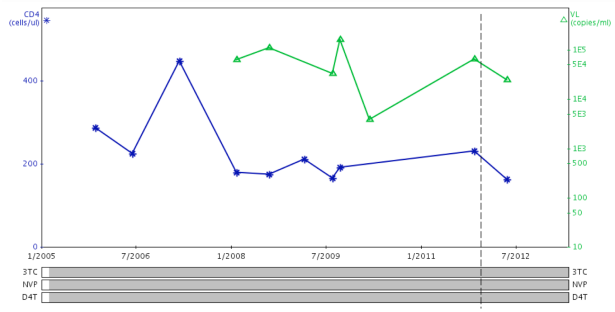


Supplementary figure 1. CD4+ count, viral load, treatment time and therapy RegaDB charts for HIV-1/HTLV-1 co-infected patients.

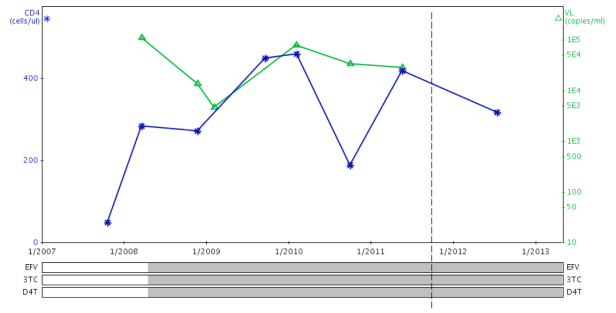
RES001



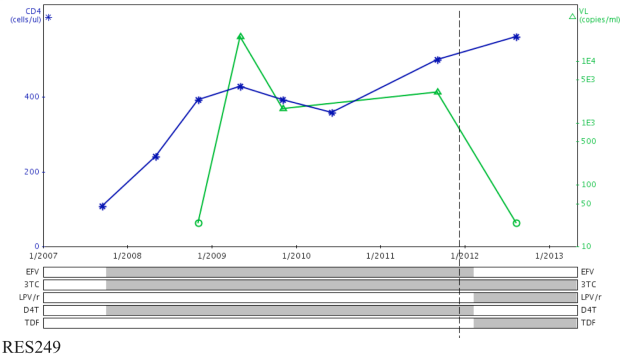
RES064



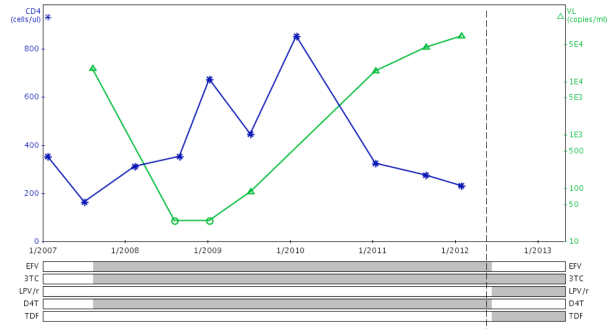
RES068



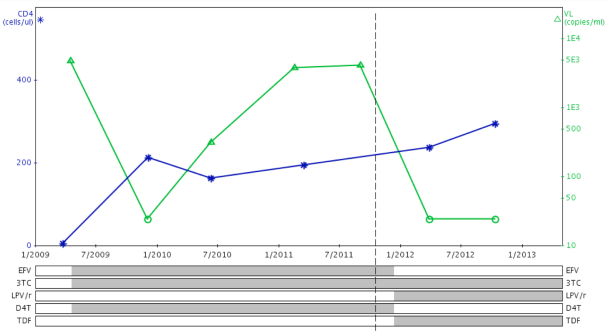
RES123



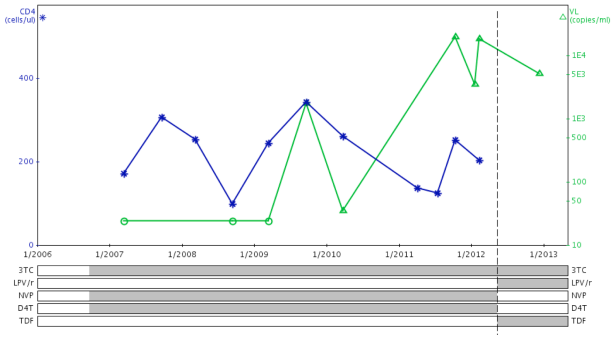
RES177



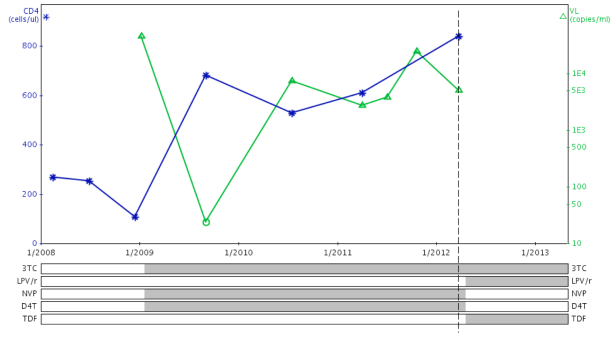
RES249



RES268



RES405



4 DISCUSSÃO

Foram encontradas mutações em uma a cada quatro posições na região LTR do HTLV-1 utilizando o Ion Torrent e parte destas mutações estavam presente em TFBS, sugerindo que o HTLV-1 pode ter uma expressão diferencial em pacientes com TSP/HAM. Estudos indicaram que algumas mutações nesta região podem ser marcadores para o aumento da carga proviral e consequentemente para o desenvolvimento da TSP/HAM (NETO et al, 2011; MAGRI et al, 2012). Neste estudo não foi identificado uma diferença estatística para uma destas mutações, porém, todos os estudos, incluindo este, possuem um número de amostras limitado. Uma outra falha em todos os estudos que buscam a associação de mutações na região LTR com algum desfecho clínico é a análise apenas de mutações na região LTR como marcador de expressão para o HTLV-1, sendo que a metilação do DNA assim como o sitio de integração do HTLV-1 no genoma humano poderiam também estar interferindo na expressão viral.

Mutações não presentes em TFBS previamente descritos foram encontradas com uma diferença estatística entre os grupos analisados, sendo sempre mais frequentes em indivíduos com baixa carga proviral ou indivíduos assintomáticos. Os TFBS do promotor do HTLV-1 não são completamente entendidos devido à falta de estudos utilizando tecnologias modernas, como a imunoprecipitação de cromatina da região promotora do HTLV-1 em linfócitos T CD4, podendo estas mutações estarem presente em TFBS ainda não identificados, porém um estudo mais profundo deveria ser realizado para esclarecer a ligação de fatores de transcrição na região LTR do HTLV-1.

Foi encontrado que amostras com carga proviral baixa possuíam uma quantidade de mutações maior que as amostras com alta carga proviral, o que poderia ser parcialmente explicado pela a alta taxa de expansão clonal do HTLV-1 em indivíduos com alta carga proviral (GILLET et al, 2011).

Com a chegada das novas metodologias de sequenciamento, novas abordagens vem sendo feitas para esclarecer mecanismos de patogênese viral e para identificar mutações virais com frequência muito baixa. No caso de infecções pelo HTLV-1 é de grande importância que estudos que busquem associação entre mutações virais e desenvolvimento de doenças sejam realizados através destas metodologias, pois além deste vírus ter uma taxa evolutiva muito baixa, apenas cerca de 1% das células infectadas irão expressar a proteína tax por dia (GESSAIN et al, 1992b; SHERMAN et al, 1992; SWITZER et al, 2009; ASQUIT et al,

2007). Nas infecções pelo HIV-1 a utilização destas novas tecnologias de sequenciamento de DNA possui uma importância ainda maior, pois é conhecido que após o início da terapia antirretroviral, o vírus irá desenvolver mutações que levam a resistência aos antirretrovirais e consequentemente irá desenvolver uma falha terapêutica. A identificação precoce destas mutações mesmo em baixa prevalência, poderia ajudar na escolha da terapia após falha terapêutica. Foi utilizada a metodologia do Ion Torrent para analisar mutações tanto no HTLV-1 quanto no HIV-1 e observamos que esta metodologia é eficaz na busca de mutações com baixa frequência, sendo mais eficaz em detectar mutações que o sequenciamento através da técnica de Sanger.

A presença do genótipo selvagem do HIV-1 em adultos falhando ART tem sido consistentemente observado em estudos da África do Sul com uma prevalência geral de 14%, podendo variar de 5% a 33% e tem sido associada a resultados clínicos adversos quando há a mudança para a segunda linha da ART (MARCONI et al, 2008; BARTH et al, 2008; HOFFMANN et al, 2009; ORRELL et al, 2009; MURPHY et al, 2010; WALLIS et al, 2010; EL-KHATIB et al, 2011; SIGALOFF et al, 2011; SINGH et al, 2011; VAN ZYL et al, 2011; SIGALOFF et al, 2012a; SIGALOFF et al, 2012b). Foi encontrado que um em cada oito adultos com falha terapêutica para a primeira linha de ART tinham o genótipo tipo selvagem (12,7%). Na maioria dos casos para os quais foi realizado o sequenciamento pelo Ion Torrent, a ausência de mutações associadas à resistência aos ART foi confirmada. Proporcionando mais confiança de que os regimes de primeira linha de ART continuaria sendo eficaz para estes indivíduos e que a mudança para a terapia de segunda linha seria desnecessária.

A relação entre a adesão e a probabilidade de surgimento de resistência aos ARV é uma medida difícil de ser aferida além de diferir entre as diferentes classes de ARV (BANGSBERG et al, 2006; GARDNER et al, 2009; ROSENBLOOM et al, 2012). A resistência aos NNRTI acontece rapidamente em níveis moderados a baixos de aderência. Como todos os adultos do estudo estavam em regimes a base de NNRTI, a ausência de resistência aos medicamentos em virtude da falha terapêutica prolongada sugere que a adesão destes pacientes pode ter sido tão baixa que sequer havia pressão seletiva sobre o vírus. A falta de associação entre a adesão auto-relatada e a identificação do genótipo selvagem corroboram resultados de outro estudo na África do Sul, que utilizou o método de contagem de pílulas como uma medida para verificar a adesão aos ARV (VAN ZYL et al, 2011). Muitos dos instrumentos utilizados para medir a adesão terapêutica (incluindo a escala do tipo Likert utilizada neste estudo) demonstram uma falta de sensibilidade ou especificidade e normalmente não mensuram a adesão de forma correta (CHAIYACHATI et al, 2012). Uma

limitação particular do presente estudo foi que a adesão foi avaliada apenas num único ponto de tempo e não há dados consistentes de aderência ao longo de todo o tratamento.

A presença do genótipo selvagem foi fortemente associado com uma maior contagem de células CD4+, sugerindo uma possível associação entre contagem de células CD4+ com baixa adesão ao tratamento. Este achado se correlaciona com os resultados de um estudo norte-americano, onde a contagem de células CD4+ acima de 350 células/ml foi associadas a um menor risco de resistência aos ARV de primeira linha (UY et al, 2009). Os indivíduos com uma maior contagem de células CD4+ são menos propensos a ter sintomas e poderiam ter interrompido o tratamento devido a isto, assim como foi mostrado em outro estudo, porém é muito difícil relacionar um único fator à interrupção do tratamento, sendo esta provavelmente um desfecho multifatorial (WARE et al, 2009).

O custo diferencial entre o regime de primeira linha e o regime de segunda linha na ART é bastante elevado e há, portanto, uma necessidade de garantir que os pacientes que entram na ART de segunda linha realmente tenham mutações que levam a resistência aos ARV. No momento, os testes de genotipagem não estão incluídos nas diretrizes do Departamento de Saúde Sul Africano, embora outras orientações na região têm recomendado a sua utilização (CONRADIE et al, 2012). Modelos matemáticos baseados em programas de tratamento ao HIV-1 da África do Sul sugerem que realizar a genotipagem em todos os indivíduos em falha terapêutica seria mais barato do que apenas alterar para a terapia de segunda linha caso a prevalência do genótipo selvagem fosse igual ou superior a 12% (LEVISON et al, 2013). A partir dos dados encontrados, é sugerido que a implementação do teste de genotipagem nesta região da África do Sul.

A contagem de CD4+ é um importante marcador para a eficiência imunológica em pacientes infectados pelo HIV-1. Estudos anteriores relataram aumentos nas contagens de CD4 + em indivíduos coinfectados HIV-1/HTLV-1 antes do início da ART (SCHECHTER et al, 1997; HARRISON et al, 1997; BEILKE et al, 2004; BEILKE et al, 2007; PEDROSO et al, 2011), porém, apenas um estudo avaliou a contagem de células T CD4+ após a introdução da ART em uma análise transversal e não encontrou diferenças significativas entre os grupos (SILVA et al, 2012). Foi observado que a média de linfócitos T CD4+ em indivíduos coinfectados era superior aos indivíduos infectados apenas pelo HIV-1 logo após o início da ART. Além disso, a contagem de linfócitos T CD4+ também foi maior ao longo do tempo no grupo de indivíduos coinfectados, quando os dados foram ajustados para carga viral, fatores demográficos e tratamento. De acordo com outros estudos, os linfócitos T CD4+ infectados pelo HTLV-1 são funcionalmente deficientes e portanto esta contagem deve ser levada em

consideração para o início do tratamento, assim como para o manejo clínico (SCHECHTER et al, 1997; BHATT et al, 2009). Propomos que o aumento da contagem de linfócitos T CD4+ em indivíduos coinfectados deve ser cautelosamente considerados no manejo clínico após o início da ART.

Foi encontrada uma prevalência de 2,1% da infecção pelo HTLV-1 entre indivíduos infectados pelo HIV-1 em KwaZulu-Natal. Esta prevalência foi um pouco maior quando comparada com o último estudo realizado na região em indivíduos infectados e não infectados pelo HIV-1(ALCANTARA et al, 2003). Interessantemente a prevalência para o HIV-1 aumentou nesta região e era esperado que o mesmo acontecesse em realção a prevalência para o HTLV-1, porém isto pode ser explicado pela maior eficiência da transmissão pelo HIV-1 devido à uma grande produção de partículas virais quando comparada à infecção pelo HTLV-1 Estudos de prevalência desta coinfecção na África do Sul mostraram resultados bastante diferentes, chegando em alguns caso a uma prevalência de até 6%, o que sugere que a prevalência desta coinfecção deve ser heterogênea no país.

A análise filogenética das sequências LTR classificou todos os isolados do HTLV-1 identificados no estudo em Kwazulu-Natal como subtipo Cosmopolita do subgrupo transcontinental. Este estudo reforça que o HTLV-1a, subgrupo transcontinental é a cepa circulante mais prevalente em Kwazulu-Natal (ALCANTARA et al, 2003). Além disto, a presença de sequências LTR do HTLV-1 em diferentes grupos na árvore filogenética sugere múltiplas introduções do HTLV-1 nesta população. A diversidade genética entre as novas sequências do HTLV-1 e a análise de relógio sugere uma introdução anterior ao HIV-1 nesta região. De acordo com relatos históricos, HTLV-1 surgiu da África Central e se espalhou por todo o mundo (VAN DOOREN et al, 2001). A introdução de HTLV-1 na África do Sul é provavelmente resultado dos últimos 300 anos de migração da população de lingua Bantu para esta região, o que pode ser confirmada através das análises do tMRCA.

5 CONCLUSÃO

- Foram identificadas mutações na região LTR do HTLV-1 relacionadas com a carga proviral e o desenvolvimento da TSP/HAM, porém estudos funcionais adicionais precisam ser realizados para confirmar se estas mutações estão relacionadas com a ligação de fatores de transcrição;
- Cerca de um em cada oito adultos infectados pelo HIV-1 em Kwazulu-Natal com falha terapêutica em ART de primeira linha possui o genótipo selvagem. O uso de testes de resistência genotípica no momento da falha ART de primeira linha podem, portanto, ser uma intervenção de baixo custo para evitar a interrupção desnecessária do tratamento e preservar ART;
- Embora a contagem de linfócitos T CD4+ seja associada com o genótipo selvagem é difícil prever a presença deste genótipo utilizando apenas este marcador;
- A contagem de linfócitos T CD4+ após o início da terapia em indivíduos coinfectados com HIV-1 é maior do que em indivíduos apenas infectados pelo HIV-1 e portanto esta contagem deve ser levada em consideração no manejo clínico dos pacientes coinfectados;

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APÊNDICE

Journal of Medical Virology 84:1428–1436 (2012)

Oral Health Profile in Patients Infected With HTLV-1: Clinical Findings, Proviral Load, and Molecular Analysis From HTLV-1 in Saliva

Liliane Lins,[1,2] Victor José Uchoa de Carvalho,[1,3] Filipe Ferreira de Almeida Rego,[1,3] Rochele Azevedo,[4] Simone Kashima,[4] Viviana Nilla Olavarria Gallazi,[1,3] Marcia Tosta Xavier,[1] Bernardo Galvão-Castro,[1,3] and Luiz Carlos Junior Alcantara[1,3]*

[1]*Bahiana School of Medicine and Public Health, Salvador, Bahia, Brazil*
[2]*Federal University of Bahia-UFBA School of Medicine, Salvador, Bahia, Brazil*
[3]*Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil*
[4]*National Institute of Science and Technology in Stem Cells and Cell Theraphy, University of São Paulo—USP School of Medicine, Ribeirao Preto, São Paulo, Brazil*

Human T-lymphotropic virus type 1 (HTLV-1) is associated with adult T-cell leukemia (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) and has also been implicated in several disorders, including periodontal disease. The proviral load is an important biological marker for understanding HTLV-1 pathogenesis and elucidating whether or not the virus is related to the clinical manifestation of the disease. This study describes the oral health profile of HTLV-1 carriers and HAM/TSP patients in order to investigate the association between the proviral load in saliva and the severity of the periodontal disease and to examine virus intra-host variations from peripheral blood mononuclear cells and saliva cells. It is a cross-sectional analytical study of 90 individuals carried out from November 2006 to May 2008. Of the patients, 60 were HTLV-1 positive and 30 were negative. Individuals from the HTLV-1 positive and negative groups had similar mean age and social-economic status. Data were analyzed using two available statistical software packages, STATA 8.0 and SPSS 11.0 to conduct frequency analysis. Differences of $P < 0.05$ were considered statistically significant. HTLV-1 patients had poorer oral health status when compared to seronegative individuals. A weak positive correlation between blood and saliva proviral loads was observed. The mean values of proviral load in blood and saliva in patients with HAM/TSP was greater than those in HTLV-1 carriers. The HTLV-1 molecular analysis from PBMC and saliva specimens suggests that HTLV-1 in saliva is due to lymphocyte infiltration from peripheral blood. A direct relationship between the proviral load in saliva and oral manifestations was observed. ***J. Med. Virol. 84:1428–1436, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: oral health; saliva; HTLV-1

INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) was the first human retrovirus described [Poiez et al., 1980]. HTLV-1 is endemic in Southwestern Japan, Melanesia, Caribbean Islands, Central and South America, as well as in parts of Africa. It has been estimated that 20 million people are infected worldwide [Catalan-Soares et al., 2001; Proietti et al., 2005]. Salvador, the capital city of the State of Bahia located in Northeastern Brazil, has the highest described prevalence of HTLV-1 in Brazil. A cross-sectional population-based study carried out in Salvador found a prevalence of HTLV-1 infection of 1.7%, estimating that approximately 50,000 individuals were infected

Grant sponsor: FAPESB; Grant number: 303/03; Grant sponsor: Brazilian Ministry of Health; Grant numbers: 306/04. 307/04.

This paper is a part of the Master Dissertations of V.J.U.C. and F.F.A.R.

Liliane Lins and Victor José Uchoa de Carvalho contributed equally to this study.

*Correspondence to: Luiz Carlos Junior Alcantara, Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Rua Waldemar Falcão 121, Candeal, Salvador, Bahia 40296-610, Brazil. E-mail: lalcan@bahia.fiocruz.br

Accepted 24 April 2012

DOI 10.1002/jmv.23327
Published online in Wiley Online Library (wileyonlinelibrary.com).

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 29, Number 00, 2013

DOI: 10.1089/aid.2013.0181

Molecular Characterization of the Human T Cell Lymphotropic Virus Type 2 Long Terminal Repeat Region: A Discussion about Possible Influences at Viral Gene Expression

Fernanda K. Barreto,[1] Felipe F.A. Rego,[1] Loianna M. Fonseca,[1] Bernardo Galvão-Castro-Filho,[2] Thessika H.A. Araújo,[1] Aline Cristina A. Mota-Miranda,[2,3] Joana P. Monteiro-Cunha,[2,3] and Luiz Carlos J. Alcantara[1]

Abstract

This study aimed to identify nucleotide signatures in the promoter region of human T cell lymphotropic virus type 2 (HTLV-2) isolated from infected individuals from Salvador, Brazil and in sequences from the GenBank database. DNA samples from HTLV-2-infected individuals were submitted to nested polymerase chain reaction (PCR) and sequencing, and molecular analyses were performed using bioinformatics tools. The phylogeny of HTLV-2 strains isolated from patients from Salvador reveals that all sequences were subtype c. One hundred and fifty-one sequences from GenBank were selected, among which 30 belong to subtype a, 88 to subtype b, 32 to subtype c, and one to subtype d. Subtype-specific signatures were identified as well as mutations resulting in loss or gain of motifs important to transcription regulation. The subtypes a and b have two E box motifs, while subtypes c and d have only one. These polymorphisms may impact viral fitness and infection outcome and should be more closely investigated.

Human T cell lymphotropic virus type 2 (HTLV-2) was described in 1982.[1] Currently this virus is found mainly among Brazilian Amerindians, African populations, and intravenous drug users (IVDU) from the United States, Europe, and Asia.[2] HTLV-2 is not clearly identified as the etiologic agent of human pathologies, although it seems to be linked to neurological disorders and appears to be associated with an increased incidence of autoimmune diseases and respiratory tract infections.[3,4]

Viral and host factors are probably involved in determining the infection outcome. It is believed that small variations in the HTLV-2 long terminal repeat (LTR) region may modify the binding ability of transcription factors and this could influence gene expression.

Although most of the HTLV-2 genome remains stable, considerable variations are noted in the promoter region of this retrovirus. Phylogenetic analysis, using this region and the *env* gene, demonstrated three main subtypes of HTLV-2: HTLV-2a, HTLV-2b,[5] and HTLV-2d.[6] In addition, one cluster within subtype a, formed exclusively by viral isolates from Brazil, was described in 1996, and it is called HTLV-2c.[7] Despite this genetic variability, to date there are no reports describing nucleotide signatures specific to the viral subtypes or evaluating the consequences of nucleotide variations at the gene expression.

Therefore, this study was performed with the objective of identifying nucleotide variations (signatures) in the HTLV-2 promoter region of isolates from different geographic regions and from different viral subtypes.

Blood samples were collected from 15 infected individuals followed at the HTLV reference center of Bahia School of Medicine and Public Health, located in Salvador city, Northeastern Brazil. All patients were monoinfected with HTLV-2. The Ethnic Committee of Centro de Pesquisa Goncalo Moniz/FIOCRUZ approved this study. Informed consent was obtained from all patients.

DNA was extracted using a spin column QIAamp DNA Blood Minikit (Qiagen) following the manufacturer's

[1]Laboratório Hematologia, Genética e Biologia Computacional, Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil.
[2]Escola Bahiana de Medicina e Saúde Pública, Salvador, Bahia, Brazil.
[3]Universidade Federal da Bahia, Instituto de Ciências da Saúde, Salvador, Bahia, Brazil.