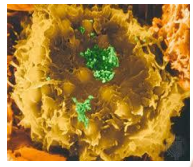
Development and Validation of a Human T-Cell Lymphotropic Virus Type I (HTLV-I) Proviral Load Assay



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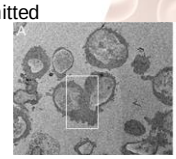
HTLV-I Epidemiology

- 15 to 20 million people world wide infected with HTLV-1
- Life long infection
  - 95% carriers remain asymptomatic
  - 5% associated with severe diseases
    - Neoplastic diseases (lymphoma, Adult T-cell leukaemia)
    - Inflammatory syndromes (HTLV-1 associated myelopathy/tropical spastic paraparesis etc)
    - Opportunistic infections (*Strongyloides stercoralis* hyperinfection etc.)
- HTLV remains cell associated and is transmitted by cell to cell contact during early infection
- In the later stages it is replicated by clonal expansion of the host cells by mitosis

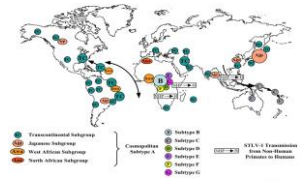


HTLV-I Transmission and Testing

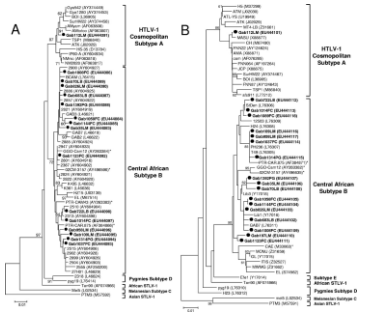
- **Modes of transmission include**
  - Breast feeding from an infected mother
  - Exposure to contaminated blood products
  - Sexual contact
- **Diagnosis and monitoring infection**
  - Anti-HTLV antibody assays
  - Proviral DNA

Global HTLV Subtype Distribution

- Positive sense, single-stranded RNA virus
- Deltaretrovirus genus of the Retroviridae family
  - HTLV-I subtypes A, B, C,D, E, F and G
    - Subtype A (Caribbean, NorthAmerica, Japan – ATK & MT-2)
    - Subtype B (African)
    - Subtype C (Australo-Melanesian found in PNG, Solomon Islands and Australia
  - HTLV-II subtypes A, B and D
  - HTLV-III
  - HTLV-IV



Phylogenetic Trees for HTLV-1



- Low degree of genetic variation (0.5 to 3%) in the cosmopolitan strains of the virus
- Subtype C shows a divergence of 8.5% at the nucleotide level
  - Amino acid sequence varying between 3 to 11% for the structural genes
  - Amino acid sequence varying between 8.5 to 25% for the regulatory and pX regions
  - More closely related to HTLV-II than HTLV-I
- Diagnostic assays are based on the prototype virus HTLV-IAKT (cosmopolitan strain)

Gessain, A. *et. Al.* 1993. J. Virol. 67(2):1015

Published NAT Assay for the Detection of HTLV-I and HTLV-II

- Multiplex PCR using real time DNA amplification for the rapid detection and quantitation of HTLV I or II[1]
- Amplifies a region of the tax gene
- Performed by NRL as a qualitative multiplex assay
- HTLV in samples from Central Australia were not consistently detected

[1] Michael C. Estes, J. Sanders Sevall. Molecular and Cellular Probes 17 (2003) 59–68

Sequence Alignment

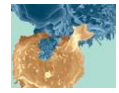
```
Z365967                    CACGCTTTGCCTGACCCTGCTTGCTCAACTCTGCGTCTTTGTTTCGTTTT   57
Z370093                    CACGCTTTGCCTGACCCTGCTTGCTCAACTCTGCGTCTTTGTTTCGTTTT   66
gi|50404473|gb|AY563954.1| CACGCTTTGCCTGACCCTGCTTGCTCAACTCTGCGTCTTTGTTTCGTTTT  481
gi|254273153|dbj|AB513134.1| CACGCTTTGCCTGACCCTGCTTGCTCAACTCTACGTCTTTGTTTCGTTTT  599
gi|1488238|gb|L02534.1|HTVMEL5 CACGCTTTGCCTGACCCTGCTTGCTCAACTCCACGTCTTTGTTTCGTTTT  597
Z379025                    CACGCTTTGCCTGACCCTGCTTGCTCAACTCCACGTCTTTGTTTCGTTTT   69

Z365967                    CTGTTCTGCGCCGCTACAGATCGAAAGTTCCACCCCTTTCCCTTTCATTC  107
Z370093                    CTGTTCTGCGCCGCTACAGATCGAAAGTTCCACCCCTTTCCCTTTCATTC  116
gi|50404473|gb|AY563954.1| CTGTTCTGCGCCGCTACAGATCGAAAGTTCCACCCCTTTCCCTTTCATTC  531
gi|254273153|dbj|AB513134.1| CTGTTCTGCGCCGTTACAGATCGAAAGTTCCACCCCTTTCCCTTTCATTC  649
gi|1488238|gb|L02534.1|HTVMEL5 CTGTTCTGCGCCGCTACCAATCGAAAGTTCCGCCCCCTTCCCTTTCATTC  647
Z379025                    CTGTTCTGCGCCGCTACCGATCGAAAGTTCCGCCCCTTTCCCTTTCATTC  119

Z365967                    ACGACTGACTGCCGGCTTGGCCCACGGCCAAGTACCGGCGACTCCGTTGG  157
Z370093                    ACGACTGACTGCCGGCTTGGCCCACGGCCAAGTACCGGCGACTCCGTTGG  166
gi|50404473|gb|AY563954.1| ACGACTAACTGCCGGCTTGGCCCACGGCCAAGTGCCGGCGACTCCGTTGG  581
gi|254273153|dbj|AB513134.1| ACGACTGACTGCCGGCTTGGCCCACGGCCAAGTACCGGCGACTCCGTTGG  699
gi|1488238|gb|L02534.1|HTVMEL5 ACGTCTGATTGCCGGCTTGGCCCACGGCCAAGCACCGGCACCTCTACTGG  697
Z379025                    ACGTCTGACTGCCGGCTTGGCCCACGGCCAAGCACCGGCACCCTTACTGG  169

Z365967                    CTCGGAGCCCGGCGACAGCCCATTCTATAGCA-CTCTCCAGG-AGAGAAA  205
Z370093                    CTCGGAGCC-AGCGACAGCCCATTCTATAGCAACTCTCCAGG-AGAGAAA  214
gi|50404473|gb|AY563954.1| CTCGGAGCC-AGCGACAGCCCATTCTATAGCA-CTCTCCAGG-AGAGAAA  628
gi|254273153|dbj|AB513134.1| CTCGGAGCC-AGCGACAGCCCATCCTATAGCA-CTCTCCAGG-AGAGAAA  746
gi|1488238|gb|L02534.1|HTVMEL5 CCCGGAGCC-AGTGACAGCCCATTCTATACCT-CTCTCCAGG-AGAGAAA  744
Z379025                    CTCGGAGCC-AGCGACAGCCCATTCTATACCT-CTCTCCAGGGAGAGAGA  217
```

Blue = HTLV-I subtype A sequence Black = HTLV-I subtype C sequence Red = Nucleotide mismatches

Numerous base pair substitutions in the regions where both the primers and probes bound

Aims

- Develop an HTLV PVL assay
  - Optimise DNA extraction efficiency
  - Identify an appropriate means of quantifying results
  - Improve assay sensitivity by examining HTLV-I sequences
  - Determine conserved primer and probe targets suitable for the population we are testing
  - Investigate the use of alternative samples (whole blood, buffy coats, dried blood spots)

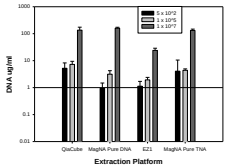
- Fully validated the HTLV PVL assay
- Determine if HTLV PVL correlates with clinical manifestations of HTLV infection

Optimisation of Nucleic Acid Extraction

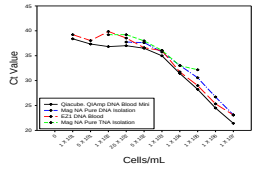
- Examination of several methods
  - Roche MagNA Pure LC -TNA Isolation Kit
  - Roche MagNA Pure LC -DNA Isolation Kit
  - Qiagen EZ1 -DNA Blood Kit
  - Qiagen QIACube -DNA Blood Mini Kit


- PBMC dilution series on each platform

DNA recovered using different extraction platforms



Average Albumin Ct of platform vs Cell count

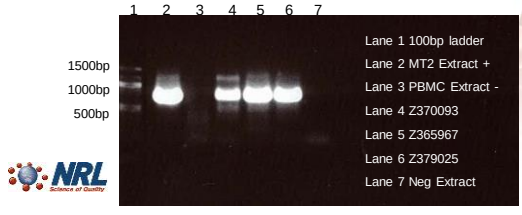


Identifying Appropriate HTLV-I Sequences

- Genebank search conducted comparing as many full length HTLV sequences as possible
- Data base contains approximately 20 full length HTLV-Ia sequences & many partial sequences, only 1 full length HTLV-Ic sequence
- Compared HTLV-II sequence for future multiplexing assay


Sequencing HTLV-I *Gag* Region from Local HTLV-1 Isolates

- A section of the *gag* gene was identified as very highly conserved across all isolates
- This *gag* region was selected for sequencing isolates from Central Australia presumed to be HTLV-Ic and archived HTLV-Ia extracted DNA
- Design primers to amplify a 760 bp region of interest

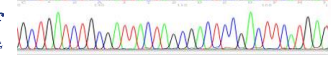


Sequencing HTLV-I *Gag* Region

- Design sequencing primers
- Sequence region of interest
- Produce electropherograms
- Align sequenced region to determine highly conserved areas

```
5'GCGCGCCCTACCTGAGGCCGCCATCCAGGCCGGTTGAGTCGCTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCCGTCTAGG
TAAGTTCGGAGCTCAGGTCGAGACCGGGCCTTTGTCCGGCGCTCCCTTAGAGCCCACCTAGATTCGGTCGGCTCTCCACGCTTTGCCTGACC
CTGCTTGCTCAACTCCACGTCTTTGTTTCGTTTTTCTGTTCTGCGCCGCTACCAATCGAAAGTTCCGCCCCCTTCCCTTTCATTCACGTCTGATTG
CCGGCTTGGCCCACGGCCAAGCACCGGCACCTCTACTGGCCCGGAGCCAGTGACAGCCCATTCTATACCTCTCTCCAGGAGAGAAACATAGAA
CACAGTTGGGGGCTCGTCCGGGATTCTAACGACGCCCCCTTTATTCCCTAGGCAATGGGCCAAATCTTCCCTCGTACGGCTAACCCTATCCCG
CGGCCGCCCCGGGGGCTGGCCACTCATCACTGGCTTAATTTCCTCCAGGCGGCATACCGCCTGGAGCCCGGTCCTTCCAGTTACGATTTTCAT
CAGTTAAAACAGTTCTTAAAATGCCCTAGAAACCCCAGTCTCGATCTGTCCCATTAACTACTCCTCCTCCTAGCTAGTCTACTCCTCCAAAGGATA
CCCTGGCCAGGTAAATGAAATTTTACAGGTACTCATCCAAACCCAAACCCAGATCCGTCCCACCCCGCGCCGCCGCCGCCGCTCATCCCCCAC
CCACGACCCCCAGATTCTGATCCACAGATCCCCCCTCCCTATGTTGAGCCTACAGCCCCCCAAGTCCTTCCAGTCATGCACCCACACGGTGT
CCCTCCCACCGCACCGCCCGTGGCAAATGAAAGACCTACAGGCCTATTAAGCAGGAAGTCTCC 3'
```

- Forward and reverse Gag region primers **RED** **GREEN**
- Forward sequence primers 1 & 2 **PINK**



Assay Target Selection

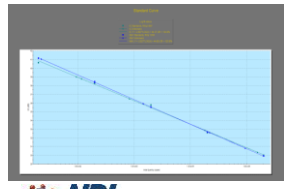
- Determined a suitable region of the *Gag* region as the assay target based on sequence
- Designed 15-30 bp primer and probe set over a highly conserved region of 50–150bp for the assay

5'GCCGCCCTACCTGAGGCCGCCATCCACGCCGGTTGAGTCGCTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCCGTCTAGG
TAAGTTCGGAGCTCAGXXXXXXXXXXXXCCTTGTCXXXXXXXXXXTAGAGCCCACTTAGATTCGGTCGGCTCTCCACGCTTXXXXXXXXXXXXX
XAGCTCAACTCCAGTCTTGTTTCGTTTTCTCTTCTGCCCCGCCTACCAATCGAAAGTTCGGCCCCCTTCCCTTTCATTCACGCTCTGATTGCCCC
CTTGGCCCACAGGCAAGCACCGGCACCTCTACTGGCCCGGAGCCAGTGACAGCCCATTCTATACCTCTCTCCAGGAGAGAAACATAGAACACA
GTTGGGGGCTCGTCCGGGATTCTAACGAGCGCCCTTTATTCCCTAGGCAATGGGCCAAATCTTCCCTCGTAGCGCTAACCCTATCCGCGGC
CGCCCCGGGGGCTGGCCACTCATCACTGGCTTAATTTCCTCAGGCGGCATACCGCCTGGAGCCCGGTCCTTCCAGTTACGATTTCATCAGTT
AAAAACAGTTCTTAAAATGGCCCTAGAAACGCCAGTCTGGATGTGTCCCATTAACTACTCCCTCCTAGCTAGTCTACTCCCAAAGGATACCCTG
GCCAGGTAAATCGAAATTTTACAGGTACTCATCCAAACCCAAACCCCAGATCCCGTCCCACCCCGCGCCGCCGCCGTCATCCCCCACCCACCG
ACCCCCCAGATTCTGATCCACAGATCCCCCCTCCCTATGTTGAGCCTACAGCCCCCCAAGTCCTTCCAGTCATGCACCCACACGGTGTCCCTCC
CACGCACCGCCCGTGGCAAATGAAAGACCTACAGGCCTATTAAGCAGGAAGTCTCC 3'

- **HTLV-I FWD Primer** CAGXXXXXXXXXXXXCCT
- **HTLV-I REV Primer** CTTXXXXXXXXXXXXXXXGCT
- **HTLV-I Probe** [6FAM] *GTCXXXXXXXXXXXXXXXGCC*[BHQ1]

HTLV-I Quantification

- Standard curve set up using a ten fold serial dilution of SP cells from $1x10^6$ – $1x10^1$ cells/ml
  - SP cells are cloned from PBMCs from a female with adult T-cell lymphoma. They **contain a single integrated, full-length copy of HTLV-1**. SP expresses mature T-cell specific antigens with a co-expression of CD4 and CD8 on its surface. It also expresses CD2 and CD3, but lacks detectable levels of TCRαβ or TCRγδ. It represents a mature T cell since it lacks surface CD1 expression, intracellular thymic terminal deoxynucleotidyl transferase, and message expression for V(D)J recombinase activating gene[1].
  - HTLV-I Ct indicates the number of HTLV proviral copies in the sample
  - Albumin Ct indicates the number of leukocytes in the sample



- Normalise proviral load by expressing as copies/number of leukocytes

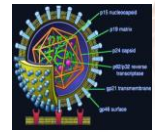
[1] Thomas Rowe, et al (1995) CHARACTERIZATION OF A HTLV-I-INFECTED CELL LINE DERIVED FROM A PATIENT WITH ADULT T-CELL LEUKEMIA WITH STABLE CO-EXPRESSION OF CD4 AND CD8. Leukemia Research Vol. 19, No. 9. pp. 621-628, 1995.

Reproducibility of HTLV-I PVL Results – Inter-run variability

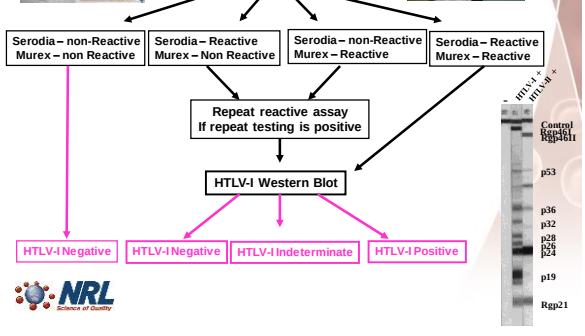
HTLV-I copies/mL	Runs	Detected	Mean	SD	% CV
$2x10^0$	20	1	42.8		
$2x10^1$	20	6	42.4	2.3	5.4
$2x10^2$	20	20	40.4	2.0	5.0
$2x10^3$	20	20	37.2	1.8	5.0
$2x10^4$	20	20	33.5	1.7	5.1
$2x10^5$	20	20	31.0	1.7	5.6
Alb copies/mL	**Runs**	**Detected**	**Mean**	**SD**	**% CV**
$2x10^0$	20	3	41.0	1.9	4.6
$2x10^1$	20	6	40.1	1.4	3.5
$2x10^2$	20	16	39.2	1.7	4.3
$2x10^3$	20	20	36.8	1.1	3.0
$2x10^4$	20	20	33.3	1.2	3.7
$2x10^5$	20	20	30.6	1.0	3.4

Limit of Detection

- **To determine the limit of detection more precisely**
  - A two fold serial dilutions of SP cells were made
  - Range from 1000 to 1 copy/ml
  - Creating a 10 member panel
  - A total of 27 replicates were analysed on 4 separate runs
- **A probit analysis was performed**
  - 95% detection limit for **HTLV-1**
  - **6.54** (5.44 – 8.39) copies/reaction
  - 95% detection limit for **IC** (albumin)
  - **15.56** (12.91 – 19.98) copies/reaction



NRL HTLV-1 Testing Strategy



Clinical Sensitivity and Specificity

- A total of 1147 samples have been run through the assay
  - 629 samples screened negative for HTLV-1
    - 4 were inhibited or contained insufficient cells
    - 4 produced discordant or indeterminate serology results
    - These samples were excluded from the calculations
    - 621 samples were used to determine the specificity of the assay
  - 518 samples screened positive for HTLV-1
    - 4 were inhibited or contained insufficient cells
    - 17 produced discordant or indeterminate serology results
    - These samples were excluded from the calculations
    - 497 samples were used to determine the sensitivity of the assay

Clinical Specificity

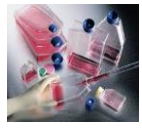
- A total of 621 samples that generated a negative serology status were tested for HTLV-I PVL
- These samples were all non-reactive on the :-
  - Murex EIA
- Of these 621 samples
- HTLV DNA was not detected in any samples
- HTLV DNA was below the limit of detection in all 621 samples
- **Resulting in a final specificity of 100%**

Clinical Sensitivity

- A total of 497 samples that generated a positive HTLV-1 serology status were tested for HTLV PVL
- These samples were reactive on the :-
  - Murex EIA
  - Serodia PA
  - MP Diagnostics Western blot
- Of the 497 samples
- HTLV DNA was detected in 463 samples
- HTLV DNA was below the limit of detection in 34 samples
- **Resulting in a final sensitivity of 93.2%**

Conclusion

- We have developed an HTLV-I PVL assay which reliably detects all subtypes of HTLV commonly encountered in our laboratory
- Paired whole blood, buffy coats and dried blood spots have been run in parallel and we obtained good concordance
- Validation Requirements
- Sensitivity
  - Dynamic range
  - Subtype detection
- Specificity
  - False reactivity
- Reproducibility
  - Inter-assay
  - Intra-assay
- Controls
  - Standards (SP cells)
  - Negative (PBMCs)
  - QC (MT-2 or MT-4 cells)



- This assay has the potential to be a valuable tool for predicting disease progression and clinical outcome

Acknowledgements

- This project was funded by NHMRC Project Grant # 1012945
- Dr. Lloyd Einsiedel and his staff at Flinders Medical Centre recruited participants and provided the samples
- Stirling Dick and the staff at NRL (Tam, Penny, Terri, Nilukshi, Jing, Alison and Frank) have performed diagnostic testing, assay development and data entry
- Alice Springs Hospital and the hospital laboratory staff (Ron Halliwell) collected samples, prepared the buffy coats and organised shipping of the samples
- IMVR who have helped with the shipping of samples
- Kevin Freeman and the staff at the Royal Darwin hospital provided screening test results and shipped samples
- **The participants who enrolled in the study** we are incredibly grateful for your support and we hope this work will improve the health of your communities in the future

Australian Government
National Health and Medical Research Council
N H M R C

Melbourne, Australia

Thank you for your attention!

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