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 worldwide 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-I 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, North America, 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-I AKT (cosmopolitan strain)

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
  - 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

**Sequence Alignment**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACGCTTTGCCTGACCCTGCTTGCTCAACTCTGCGTCTTTGTTTCGTTTT</td>
<td>68</td>
</tr>
<tr>
<td>QIACube</td>
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</tr>
<tr>
<td>GREEN</td>
<td>55</td>
</tr>
<tr>
<td>gag</td>
<td>55</td>
</tr>
<tr>
<td>MagNA Pure DNA</td>
<td>55</td>
</tr>
<tr>
<td>597</td>
<td>55</td>
</tr>
<tr>
<td>CA</td>
<td>55</td>
</tr>
<tr>
<td>region was selected for sequencing isolates from Central</td>
<td>55</td>
</tr>
<tr>
<td>gene was identified as very highly conserved</td>
<td>55</td>
</tr>
<tr>
<td>EZ1</td>
<td>55</td>
</tr>
<tr>
<td>EZ1 Region</td>
<td>55</td>
</tr>
<tr>
<td>Extraction</td>
<td>55</td>
</tr>
<tr>
<td>from Local HTLV</td>
<td>55</td>
</tr>
<tr>
<td>Sequencing HTLV</td>
<td>55</td>
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<tr>
<td>DNA recovered using different extraction platforms</td>
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</tr>
<tr>
<td>gi</td>
<td>1488238</td>
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<tr>
<td>gi</td>
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<tr>
<td>Z370093</td>
<td>55</td>
</tr>
<tr>
<td>Z365967</td>
<td>55</td>
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<tr>
<td>Z379025</td>
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<td>55</td>
</tr>
<tr>
<td>Z379025</td>
<td>55</td>
</tr>
<tr>
<td>Average Albumin Ct of platform vs Cell count</td>
<td>55</td>
</tr>
<tr>
<td>Cells/mL</td>
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</tr>
<tr>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>1 X 10^15 X 10^1</td>
<td>55</td>
</tr>
<tr>
<td>1 X 10^22.5 X 10^2</td>
<td>55</td>
</tr>
<tr>
<td>5 X 10^21 X 10^3</td>
<td>55</td>
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<tr>
<td>1 X 10^41 X 10^5</td>
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<td>1 X 10^6</td>
<td>55</td>
</tr>
<tr>
<td>1 X 10^7</td>
<td>55</td>
</tr>
</tbody>
</table>

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

**Aims**

- Develop an HTLV PVL assay
- Optimize 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

**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-la 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-I 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-la 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
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

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

<table>
<thead>
<tr>
<th>HTLV copies/mL</th>
<th>Runs</th>
<th>Detected</th>
<th>Mean</th>
<th>SD</th>
<th>% CV</th>
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</thead>
<tbody>
<tr>
<td>2x10⁶</td>
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<td>1</td>
<td>42.8</td>
<td>2.3</td>
<td>5.4</td>
</tr>
<tr>
<td>2x10⁷</td>
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<td>6</td>
<td>40.4</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>2x10⁸</td>
<td>20</td>
<td>20</td>
<td>37.2</td>
<td>1.8</td>
<td>5.0</td>
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<tr>
<td>2x10⁹</td>
<td>20</td>
<td>20</td>
<td>33.5</td>
<td>1.7</td>
<td>5.1</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Ali copies/mL</th>
<th>Runs</th>
<th>Detected</th>
<th>Mean</th>
<th>SD</th>
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<td>41.0</td>
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<td>4.6</td>
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<td>2x10⁷</td>
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<td>6</td>
<td>40.1</td>
<td>1.4</td>
<td>3.5</td>
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<tr>
<td>2x10⁸</td>
<td>20</td>
<td>16</td>
<td>39.2</td>
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<tr>
<td>2x10⁹</td>
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<tr>
<td>2x10¹⁰</td>
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<td>20</td>
<td>33.3</td>
<td>1.2</td>
<td>3.7</td>
</tr>
</tbody>
</table>

NRL HTLV-1 Testing Strategy

Clinical Sensitivity and Specificity

- A total of 1147 samples have been run through the assay

HTLV-I Quantification

- Standard curve set up using a ten fold serial dilution of SP cells from 1x10⁶ – 1x10³ cells/mL
- SP cells are cloned from P815 cells line a murine with adult T cell leukemia. They express surface recombinase activating gene (RAG) levels of TCRαβ or CD3. It represents a mature T cell since it lacks surface CD8 expression, intracellular deoxynucleotidyl transferase, and membrane expression for CD45 or CD18.

- HTLV-I Ct indicates the number of HTLV proviral copies in the sample
- Albumin Ct indicates the number of leukocytes in the sample

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 – 8.59) copies/reaction
- 95% detection limit for IC (albumin)
  - 15.56 (12.91 – 19.96) copies/reaction
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
  - Cutoff detection

- Specificity
  - False reactivity
  - Reproducibility
  - Inter-day
  - Intra-day

- 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
- 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

Thank you for your attention!