Development of Diagnostics for Cysticercosis and Taeniasis—CDC Research

3rd European Cysticercosis Workshop
Antwerpen, Belgium
April 16, 2012

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Division of Parasitic Diseases & Malaria
Laboratory Objectives

Review progress to develop improved methods for

- Diagnosis of neurocysticercosis
- Detection of cysticercosis and taeniasis cases
LLGP Immunoblot for Cysticercosis

An Enzyme-Linked Immunoelectrotransfer Blot Assay and Glycoprotein Antigens for Diagnosing Human Cysticercosis (*T. solium*)

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From the Parasitic Diseases Branch, Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia
## Assay performance of the LLGP Immunoblot

<table>
<thead>
<tr>
<th>Patient type</th>
<th>Specificity</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 or more cysts</td>
<td>100%</td>
<td>98%</td>
</tr>
<tr>
<td>Single cyst (USA)</td>
<td>100%</td>
<td>~60%</td>
</tr>
<tr>
<td>Single cyst (Peru)</td>
<td>100%</td>
<td>~80%</td>
</tr>
<tr>
<td>Single cyst (India)</td>
<td>100%</td>
<td>~79%</td>
</tr>
</tbody>
</table>
LLGP immunoblot is available......

- Commercially from Immunetics, (Specialty Labs, Focus Labs) but expensive

- Technology transfer of CDC test requires high complexity laboratory capacity
What is needed?

- Simple
- Sustainable (recombinant antigens)
- Available
Proteomics Approach

- Purify individual native proteins to homogeneity
- Obtain aa sequence from tryptic peptides
- Design degenerate primers
- PCR amplify and clone genes
- Express proteins in baculovirus systems
- Evaluate diagnostic potential of proteins
Isolation of cyst LLGP proteins by preparative gel electrophoresis

SDS PAGE separation of fractions collected from preparative gel; Immunoblot probed with cysticercosis + serum pool
7 antigens in LLGP represent 3 diagnostic protein families
Immunoblot using recombinant proteins

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rGP50

---
rT24H

---
sTSRS1
Evaluation of recombinant proteins in immunoblot

<table>
<thead>
<tr>
<th>Proteins (s)</th>
<th>Sens(^1)</th>
<th>Sens(^2)</th>
<th>Spec</th>
<th>J-Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gp50 + rT24H + TSRS1</td>
<td>99</td>
<td>83</td>
<td>98</td>
<td>.99</td>
</tr>
<tr>
<td>Gp50 + rT24H</td>
<td>99</td>
<td>83</td>
<td>99</td>
<td>.98</td>
</tr>
<tr>
<td>Gp50 + TSRS1</td>
<td>97</td>
<td>80</td>
<td>98</td>
<td>.96</td>
</tr>
<tr>
<td>T24H + TSRS1</td>
<td>99</td>
<td>81</td>
<td>99</td>
<td>.99</td>
</tr>
<tr>
<td>Gp50</td>
<td>96</td>
<td>79</td>
<td>99</td>
<td>.95</td>
</tr>
<tr>
<td>rT24H</td>
<td>99</td>
<td>80</td>
<td>100</td>
<td>.99</td>
</tr>
<tr>
<td>TSRS1</td>
<td>75</td>
<td>57</td>
<td>99</td>
<td>.75</td>
</tr>
</tbody>
</table>

\(^1\) Sensitivity for 2+ viable cysts
\(^2\) Sensitivity for 1 viable cyst
### Rationale for selecting rT24

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sens(^1)</th>
<th>Spec</th>
<th>Assay format</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native gp42</td>
<td>94%</td>
<td>ND</td>
<td>LLGP-EITB (Tsang, 1989)</td>
</tr>
<tr>
<td>Native gp24</td>
<td>92%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rT24H</td>
<td>94%</td>
<td>98%</td>
<td>Immunoblot (Hancock, 1999)</td>
</tr>
<tr>
<td>rT24H</td>
<td>98%</td>
<td>100%</td>
<td>Immunoblot</td>
</tr>
</tbody>
</table>

\(^1\) Sensitivity for 2+ viable cysts
T24 ELISA design

• A portion of rT24 (T24H), the large, extracellular loop domain, was expressed in Tni insect cells.
• Assay employs a standard curve—results are expressed as Units/uL, calculated using 4-parameter curve fit analysis
• Optimized rT24, serum, and conjugate concentrations and incubation times
• Reportable range is 0-40 units/uL
• Established acceptance range for internal positive control
• Established a cut-off value using the J-index was 2.55 Units/uL
Evaluation of the T24 ELISA using defined cysticercosis sera

Clinically positive sera

- 2+ viable cysts
- 1 viable
- Degenerating cysts
- Calcified cysts
Evaluation of the T24 ELISA using defined cross-reactor sera

Negative and potentially cross reactive sera

US residents

Other parasitic infections

Non \( T.s \) endemic areas

\( T. solium \) endemic
Evaluation of the T24 ELISA using defined NCC serum battery

<table>
<thead>
<tr>
<th></th>
<th>2+ cysts</th>
<th>1 cyst</th>
<th>Neg *</th>
<th>All Neg**</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24 Pos</td>
<td>99</td>
<td>9</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>T24 Neg</td>
<td>4</td>
<td>6</td>
<td>161</td>
<td>280</td>
</tr>
<tr>
<td>Totals</td>
<td>103</td>
<td>15</td>
<td>171</td>
<td>335</td>
</tr>
</tbody>
</table>

Sensitivity = 96%
Specificity = *94% in sera collected in areas expected to be *T. solium* free; **84% if all presumed negative sera are used for calculation
Further evaluation of rT24 ELISA

• Evaluation of T24 using sera collected in community surveys
• Found a poor correlation of T24 ELISA results with LLGP-EITB, using kappa statistic, $k = 0.26$
• Discrepancies: LLGP+, T24- AND LLGP- T24+
• Results suggested that the T24 ELISA was not a viable assay
rProtein Blot Test

- Evaluated sera from community survey
- Agreement with LLGP-EITB using kappa statistic, k= 0.52
- Most (117/120) discordant specimens were LLGP-EITB +, T24 blot – due to gp50 only reactivity in 80 samples
- Advantages: easy to perform, no special equipment needed
- Disadvantages: qualitative results, subjective, lower throughput than ELISA, water quality is important
T24 ELISA v2

- Repurified baculovirus expressed T24 using MonoQ
- Re-tested a subset of samples from the Ecuador survey—53 discordant samples
- Kappa value of this subset using old T24 = .067
- Kappa value of repurified T24 = .73

- Suggests that prior poor assay performance was related to antigen purity
- STATUS—retesting the samples from the Ecuador study
**E. coli expressed rT24 ELISA**

Expressed in pGEX 4T-2 with a 6His tag

<table>
<thead>
<tr>
<th></th>
<th>rT24H</th>
<th>rT24HNS Factor Xa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos 40 Units/ul</td>
<td>2.269</td>
<td>2.923</td>
</tr>
<tr>
<td>NCC 1479</td>
<td>1.035</td>
<td>1.408</td>
</tr>
<tr>
<td>NCC 1481</td>
<td>0.512</td>
<td>0.560</td>
</tr>
<tr>
<td>NCC 1478</td>
<td>0.177</td>
<td>0.220</td>
</tr>
<tr>
<td>Echino +</td>
<td>0.093</td>
<td>0.102</td>
</tr>
<tr>
<td>NHS Pool #4</td>
<td>0.074</td>
<td>0.078</td>
</tr>
<tr>
<td>Normal 532</td>
<td>0.083</td>
<td>0.085</td>
</tr>
<tr>
<td>Normal</td>
<td>0.084</td>
<td>0.128</td>
</tr>
</tbody>
</table>

STATUS—preliminary, but would greatly simplify availability of the antigen
T24 ELISA—Conclusions

• Developed standardized methods for purification of baculovirus expressed rT24 from *Tni* insect cells and *E. coli*

• Preliminary data suggest we can develop a rT24 ELISA
  — quantitative
  — easy to perform and transfer to laboratories in endemic regions
  — *E. coli* expressed protein will facilitate technology transfer to commercial partners and researchers
The rT24 ELISA may be a valuable tool for epidemiologic studies and for estimates of the burden of cysticercosis

- Do results correlate to LLGP-EITB results?

More validation needed for use in community settings

Utility for detecting specific antibodies in pigs has not been done
Immunodetection of the tapeworm carrier

- Classic stool exam
- Coproantigen detection
- Serologic detection
Serodiagnosis of Taeniasis

- Original test: Used native ES antigen from *in vitro* cultured adult tapeworms collected from infected hamsters
- Production of the native antigens is labor intensive, time consuming and expensive
- Our goal: serological test using Recombinant proteins

Identification and Purification of Taeniasis Diagnostic Antigens

2-D gel electrophoresis

Western blot

Evaluation of rES38

Sensitivity = 99% (80/81)
Specificity 99.7% (299/300)

What is needed to be tool ready?

- Develop field ready reagents and assays for detection of cysticercosis cases
  - To determine if a single protein can be used for detection of cysticercosis (in humans and pigs?)
  - To determine if a single protein can be used for detection of taeniasis
  - Combine the 2 proteins into a single assay for simultaneous identification of both diseases
Rapid laboratory tests—MAPIA

- Multi-antigen printing line assay
  - Used to compare antigens
  - Antigens are sprayed onto nitrocellulose
  - Precursor to lateral flow test development
  - Optimum concentration of antigens is variable

MAPIA with cysticercosis and taeniasis antigens.
Cysticercosis/taeniasis-positive serum pool (lane 1),
Echinococcosis positive serum (lane 2),
Negative serum pool (lane 3)
The optimum concentration of each antigen is shown.

Handali et al, 2010 Clin Vac Immunol17:68-72
Rapid laboratory tests—MICT

Lateral flow tests

- rT24 Sens/Spec = 94%, 99%
- rES33 Sens/Spec = 95%, 96%

Advantages:
- Rapid
- Can be quantitative

Disadvantages:
- Difficult to develop
- Dry storage
- Subjective if visually read

Standardized collection method for fingerstick blood

- Method collects a measured amount of blood (100ul)
- Filter paper is stored in a storage buffer –Stabilzyme and is never dried
- Not compatible with freezing
- Each specimen is stored separately
Conclusions

- Serum Ab tests for human cysticercosis, and blood/stool tests for tapeworm infections in humans exist
- Utility for porcine cysticercosis still needed
- Commercial partner is needed
- Further evaluation is needed to optimize format
Luminex based assays

- Allows responses to multiple antigens to be determined in a single test
- Each antigen is attached to a different bead with an individual signature
- We coupled beads with rGP50, rT24H, sTS14, sTS18, sTSRS1, sTSRS2
- We also prepared beads with rES33 and rES38, but these assays did not work
## Luminex based assays for NCC

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<th>Proteins (s)</th>
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<th>Sens(^2)</th>
<th>Spec</th>
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<tr>
<td>Gp50 + rT24H + sTS18</td>
<td>99</td>
<td></td>
<td>91</td>
</tr>
<tr>
<td>Gp50</td>
<td>94</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>rT24H</td>
<td>91</td>
<td></td>
<td>94</td>
</tr>
<tr>
<td>sTS18</td>
<td>99</td>
<td></td>
<td>96</td>
</tr>
</tbody>
</table>

\(^1\) Sensitivity for 2+ viable cysts  
\(^2\) Sensitivity for 1 viable cyst
What are the 8kDa proteins?
Immunoreactive LLGP proteins

SDS PAGE separation of fractions collected from preparative gel; Immunoblot probed with cysticercosis + serum pool
TS14 and TS18 contain related peptide sequences

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS14</td>
<td>EKNKPKDVAASTKKGIEYVHEFFE</td>
</tr>
<tr>
<td>TS18</td>
<td>KNKPKDVAASTKKEIEYIWHNFFED</td>
</tr>
<tr>
<td>TS14</td>
<td>IAQLAK</td>
</tr>
<tr>
<td>TS18</td>
<td>IAQLAK</td>
</tr>
</tbody>
</table>

PCR cloning of TS14 and TS18 reveals related cDNAs

+1
TS14  TGAACAAACCTGTAGAATGCCGTCCTACATTGTGCTTTCTCCTCCTCACAATGTTGACGTGGTGGCCGAG
TS18  --------------------------------------------------TATTCGTAGTGCGGTTTCGGCCGAG
                    *  ************  ************

AAAAACAAACCCGAAAGATGTGCAAAATAGTACGAAAAGGATAGAATATGTCCACGAAT---TCTTCCACGAAGACCCGA
AAAAACAAACCCGAAAGATGCTGATGCAAAATAGTACTAAGAAAGAGATAGAATATATCCACAATTGGGTTTCATGATGACCCGA
                    **************  **************  **  ****  ************  ***  *  *  *  *  *  **********

TTGGTAAACAAATTGCTCAAATCAGCAAAGGAATGGAAGGAAGCAATGTTGGAAGACAAAGGCAAATACGGACGTCACTGGT
TTGGAAAACAAATTGCTCAAATCAGCAAAGGAATGGAAGGAAGCAATGTTGGAAGACAAAGGCAAATGGGCGTCACTGGC
                    ****  ***  ***  *******************  *****  **  **  ***  *****  **  *  **********

TTGAGCACTGCAAGGTCCTAAGAAGAAAACCTGTAAAACTTTGCACTTTTGTGCTCTTCTCTCTACATAATGCTCA
TTGAGTACTGCAAGGTCCTGAAAGCAAAACCTGTAAAACCTTTGCACTTTTGTGCTCTTCTCTCTACATAATGCTGA
                    ***** ******  *****  *****  *******************************************  **********

TTAATAAGAAAAAAAAAAAAAAAAAAAAA 343
TTAACCAAAAAA AAAAAAAAAAAAAA 297
                    ****  **************

TS14, Ts18 and Ts21 constitute a family of proteins

8-kDa gene family
- 32 nucleic acid sequences
- 26 unique nt sequences
- 23 unique protein sequences
- 18 unique mature protein sequences
- Encode mature peptides of 66-67 aa
- All have at least 1 N-glycosylation site
- Can be chemically synthesized
Phylogenetic tree 8-kDa proteins

Hancock, et al, 2003 JCM, 41: 2577–2586
LLGP Immunoreactive proteins consist of 8kDa proteins

Fig. 1. DTT reduction of preparative gel fractions and the crude EITB antigen preparation. Immunoblot showing DTT reduction of three selected fractions from preparative gel electrophoresis. Three parent fractions (lanes 1, 3, 5) were reduced with DTT (lanes 2, 4, 6) and separated by SDS-PAGE before immunoblotting and probing with anti-\textit{T. solium} pooled sera.

Multiple 8kDa protein complexes in the LLGP fraction

- Polyclonal rabbit anti-TS14 reacts with proteins larger than the 24 kDa (lane 2)
Other diagnostic proteins share similarities with 8kDa antigens

### Table 1. Identification of subunit proteins of the native TsM 120 kDa protein complex by MALDI-TOF MS and N-terminal sequencing

<table>
<thead>
<tr>
<th>Spot no.(s)</th>
<th>N-terminal sequence</th>
<th>Protein description (Accession number)</th>
<th>Matched peptide mass (m/z)</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EKNKPKDV</td>
<td>14 kDa glycoprotein (AF257776)</td>
<td>—*</td>
<td></td>
</tr>
<tr>
<td>2, 5</td>
<td>EKNKP</td>
<td>14 kDa glycoprotein (AF257776)</td>
<td>1916·92, 2045·01</td>
<td>20</td>
</tr>
<tr>
<td>7, 8</td>
<td>—</td>
<td>18 kDa glycoprotein (AF350070)</td>
<td>1302·62, 1487·73, 2160·02</td>
<td>34</td>
</tr>
<tr>
<td>10, 11</td>
<td>EKNKP</td>
<td>14 kDa glycoprotein (AF257776)</td>
<td>1255·65, 1916·92, 2045·01</td>
<td>32</td>
</tr>
</tbody>
</table>

* Not determined.

Lee et al, 2005 *Parasitology* 131, 867–879
8 kDa proteins are soluble in cyst fluid

- Nanobodies recognize 2 proteins in cyst fluid 50 and 32 kDa
- Both contained the same N-terminal aa sequence: EKNPKKDVA; TS14
8 kDa proteins are soluble in cyst fluid

Immunoreactivity of nanobodies with 8 kDa peptides

a = TS14, b = TS18, c = TSRS1, d = TSRS2

What are the 8kDa proteins?

- 8 kDa proteins have been identified by a number of groups
- Seem to form heteromeric complexes with other 8 kDa proteins and other proteins?
- May be the targets of the Ag detection assay
- What is their biological role?
- Where are they localized?
The team........

**CDC, Atlanta**
- John Noh
- Sukwan Handali
- Keith Levert
- Paul Anderson
- Patricia Lee
- Isabel McAuliffe
- Pete Harris

**CDC, Atlanta Alumni**
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- Anne Boyer
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- Marita Silvia

**ITM, Antwerp**
- Pierre Dorny
- Sarah Gabriel
- Nicholas Praet
- Nynke Deckers
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