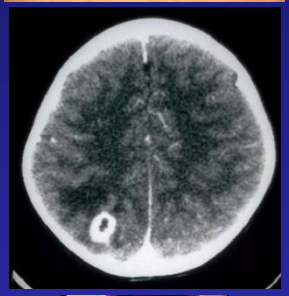


Development of Diagnostics for Cysticercosis and Taeniasis—CDC Research

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

Patricia Wilkins, PhD
Division of Parasitic Diseases & Malaria

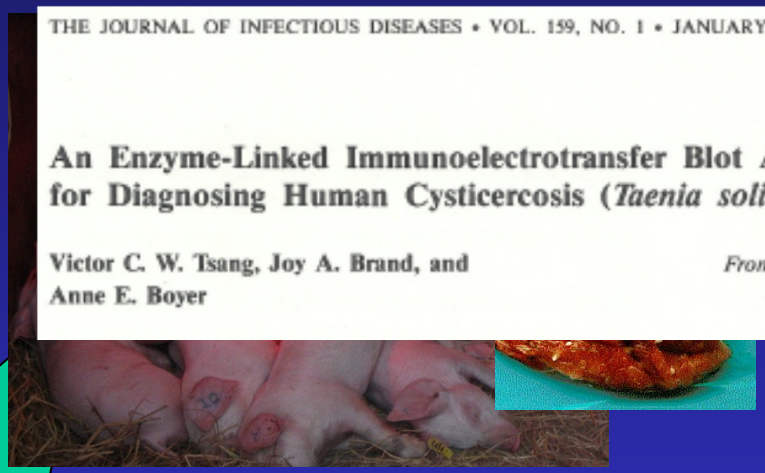
CDC
Centers for Disease Control and Prevention

Laboratory Objectives

Review progress to develop improved methods for

- Diagnosis of neurocysticercosis
- Detection of cysticercosis and taeniasis cases

LLGP Immunoblot for Cysticercosis

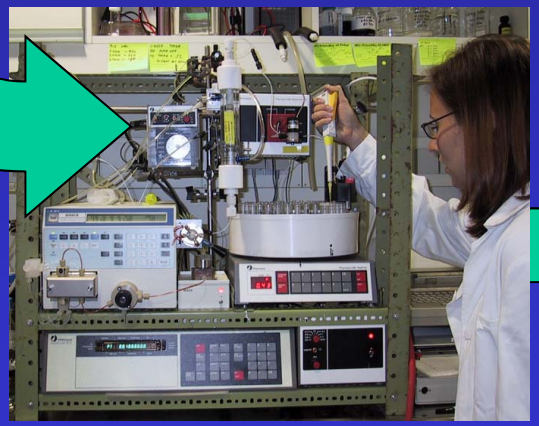


THE JOURNAL OF INFECTIOUS DISEASES • VOL. 159, NO. 1 • JANUARY 1989

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

Victor C. W. Tsang, Joy A. Brand, and Anne E. Boyer

From the Parasitic Diseases Branch, Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia

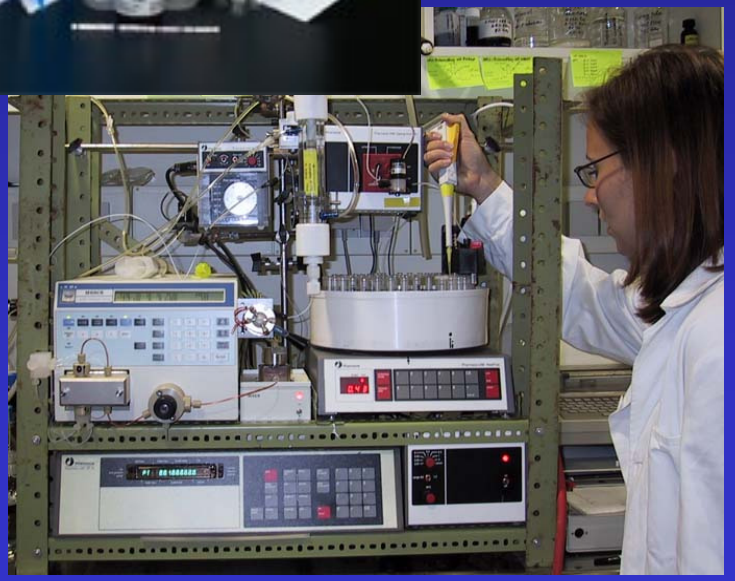


Assay performance of the LLGP Immunoblot

Patient type	*Specificity*	*Sensitivity*
2 or more cysts	100%	98%
Single cyst (USA)	100%	~60%
Single cyst (Peru)	100%	~80%
Single cyst (India)	100%	~79%

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

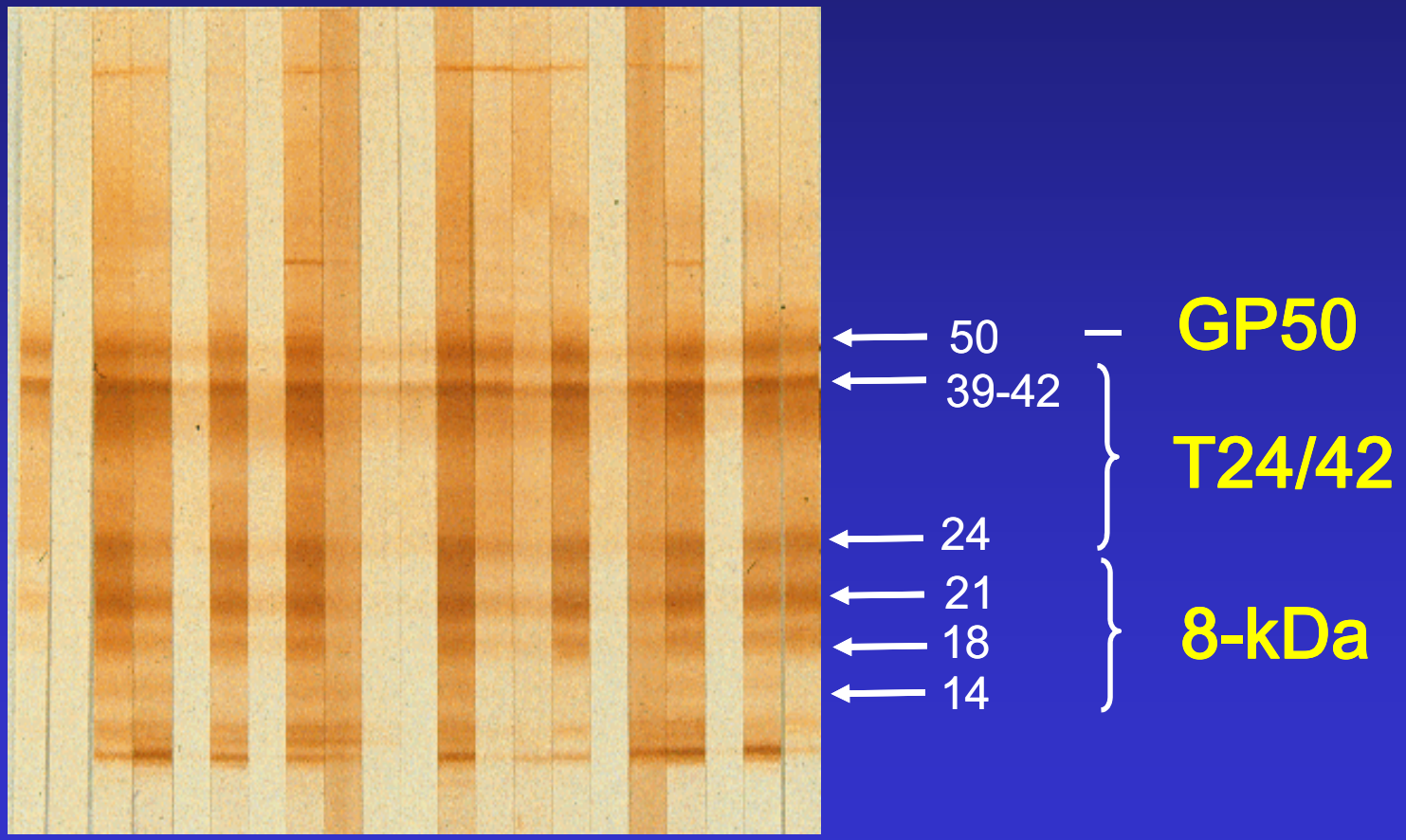
Proteomics Approach

- 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

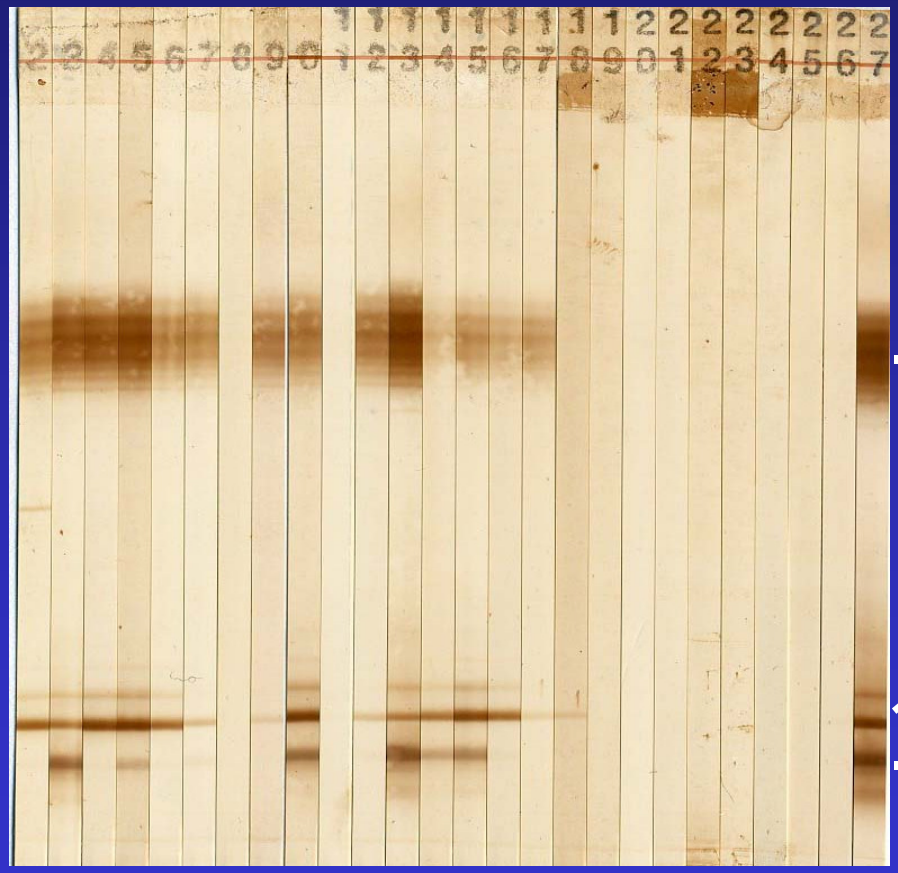
Isolation of cyst LLGP proteins by preparative gel electrophoresis

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



Recombinant proteins

Evaluation of recombinant proteins in immunoblot

Proteins (s)	Sens[1]	Sens[2]	Spec	J-Index
Gp50 + rT24H + TSRS1	99	83	98	.99
Gp50 + rT24H	99	83	99	.98
Gp50 + TSRS1	97	80	98	.96
rT24H+ TSRS1	99	81	99	.99
Gp50	96	79	99	.95
rT24H	99	80	100	.99
TSRS1	75	57	99	.75

[1] Sensitivity for 2+ viable cysts
[2] Sensitivity for 1 viable cyst

Rationale for selecting rT24

Protein	Sens[1]	Spec	Assay format
Native gp42	94%	ND	LLGP-EITB (Tsang, 1989)
Native gp24	92%		
rT24H	94%	98%	Immunoblot (Hancock, 1999)
rT24H	98%	100%	Immunoblot

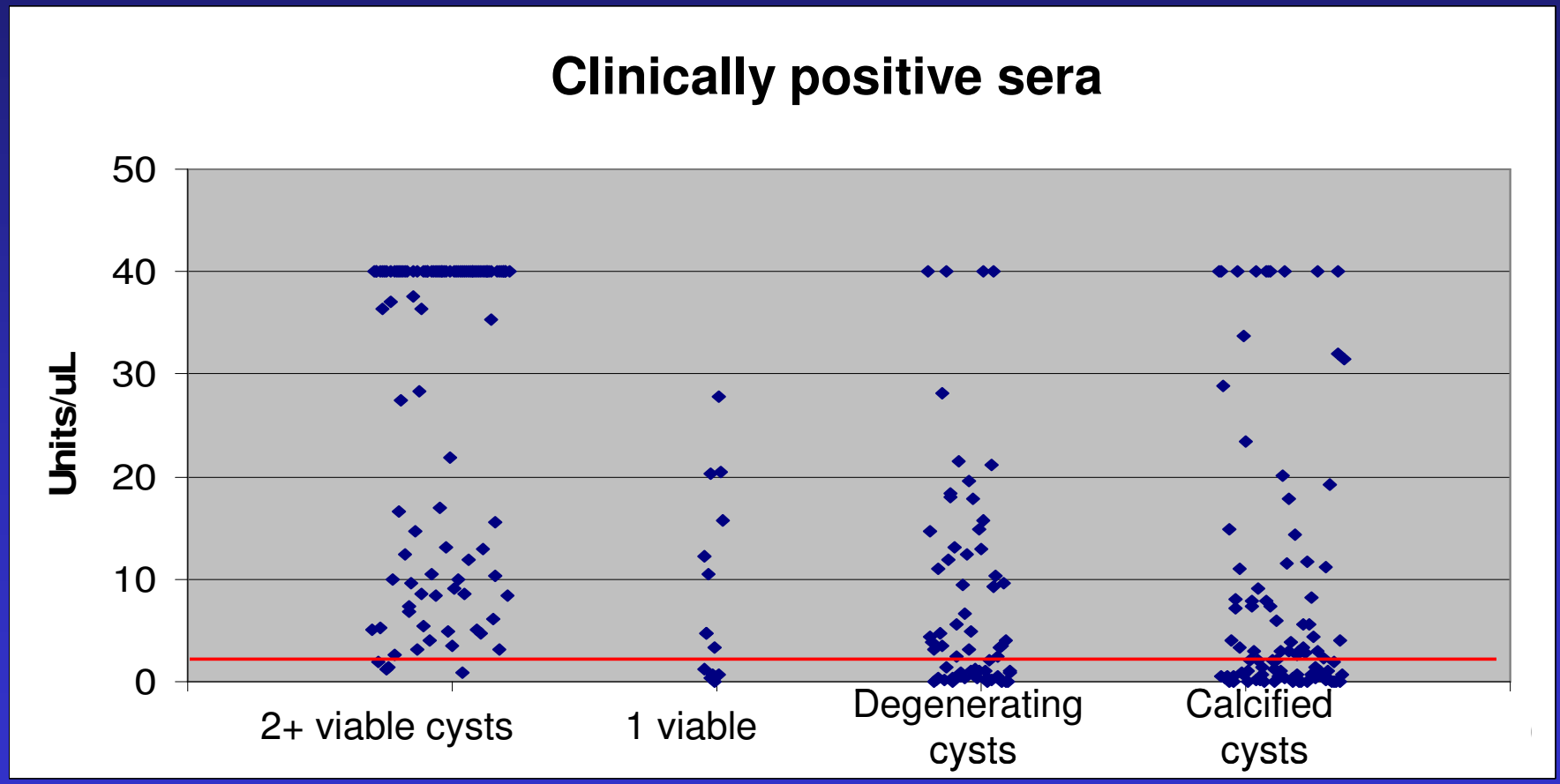
[1] Sensitivity for 2+ viable cysts

CDC

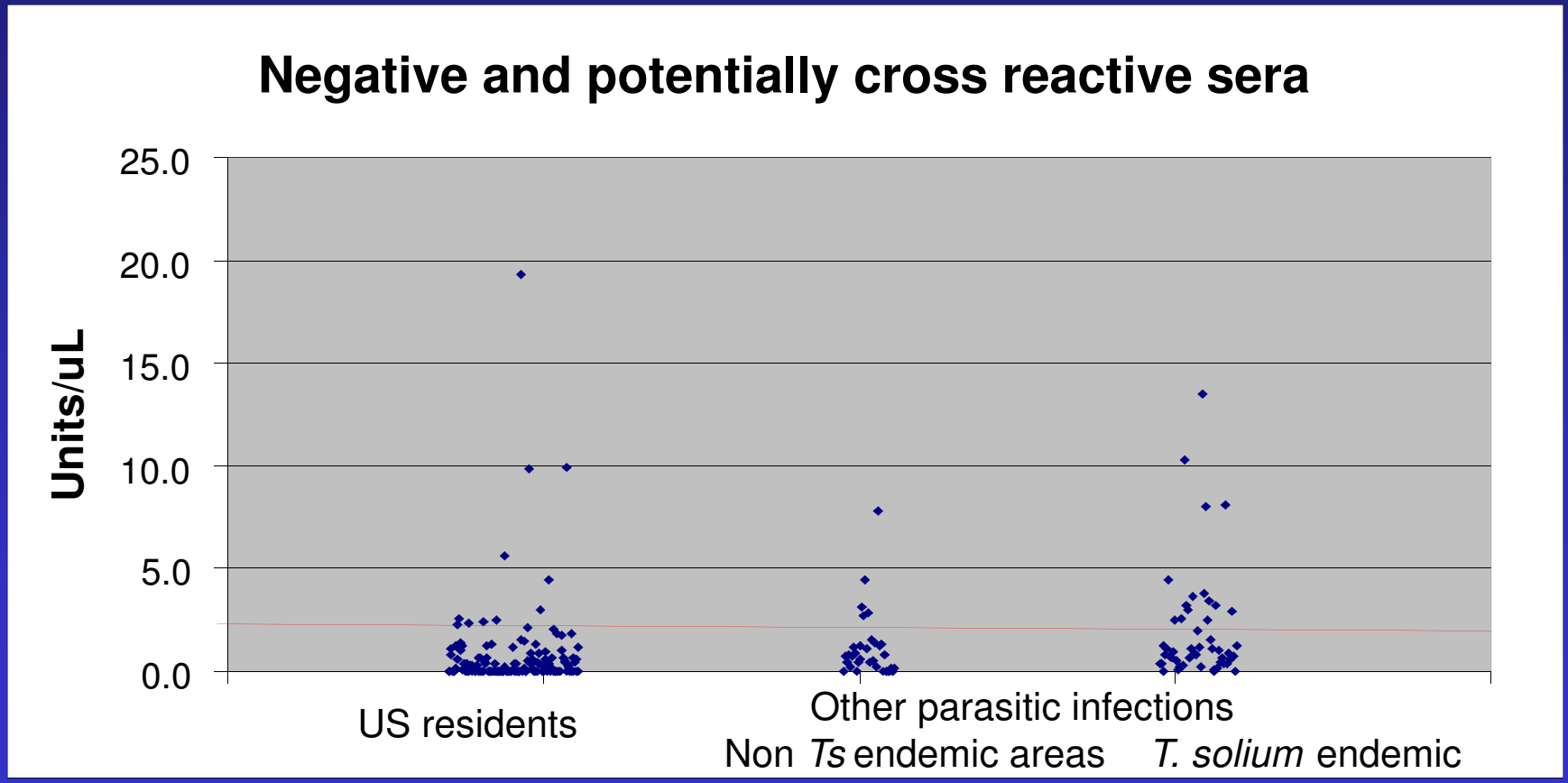
T24 ELISA design

- 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



Evaluation of the T24 ELISA using defined cross-reactor sera



Evaluation of the T24 ELISA using defined NCC serum battery

	2+ cysts	1 cyst	Neg *	All Neg**
T24 Pos	99	9	10	55
T24 Neg	4	6	161	280
Totals	103	15	171	335

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

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

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

CDC

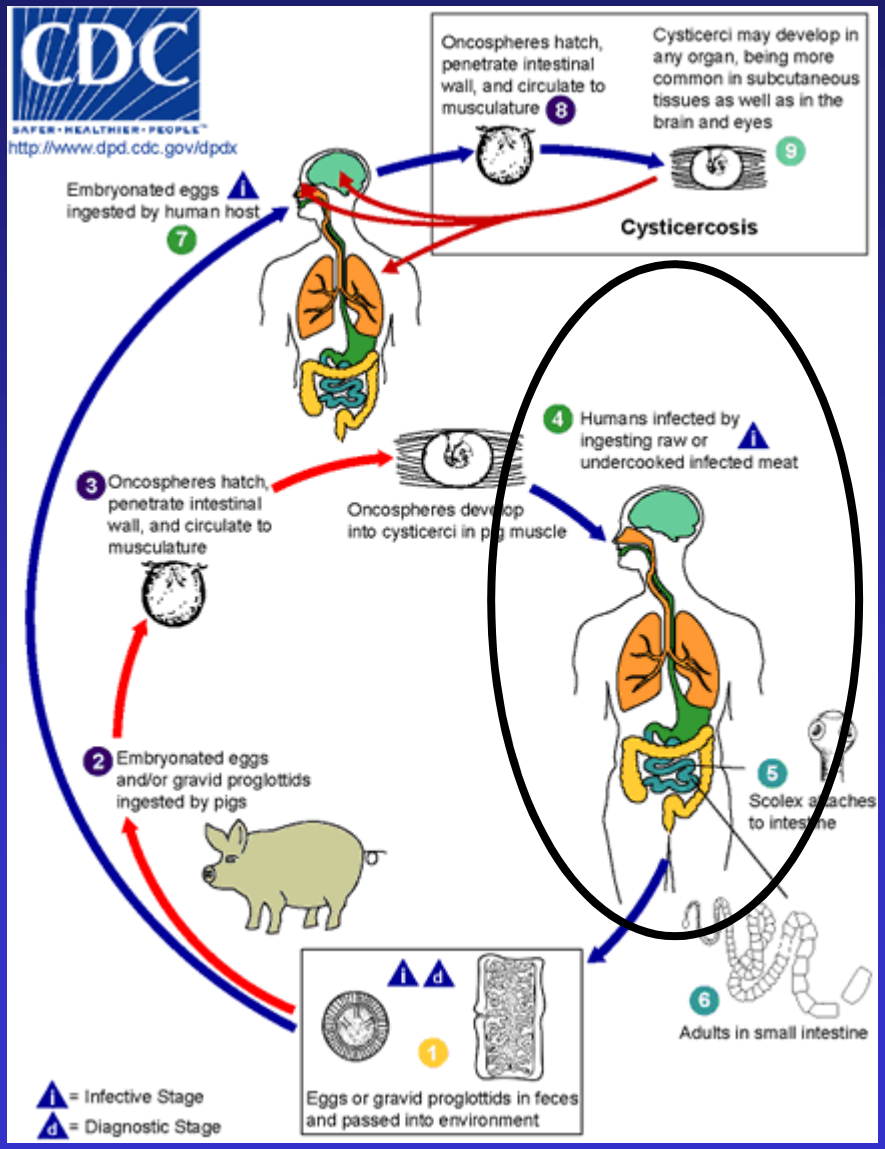
T24 ELISA—Conclusions

- 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

T24 ELISA—Conclusions 2

- 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

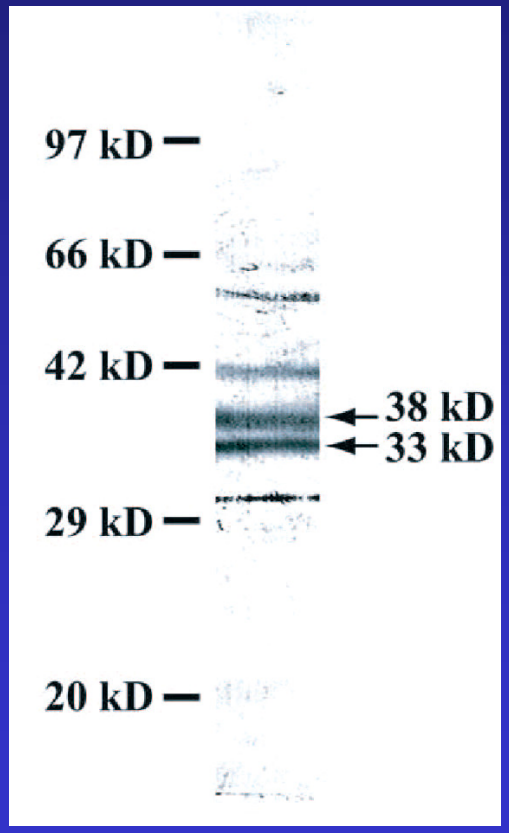
Immunodetection of the tapeworm carrier



- Classic stool exam
- Coproantigen detection
- Serologic detection

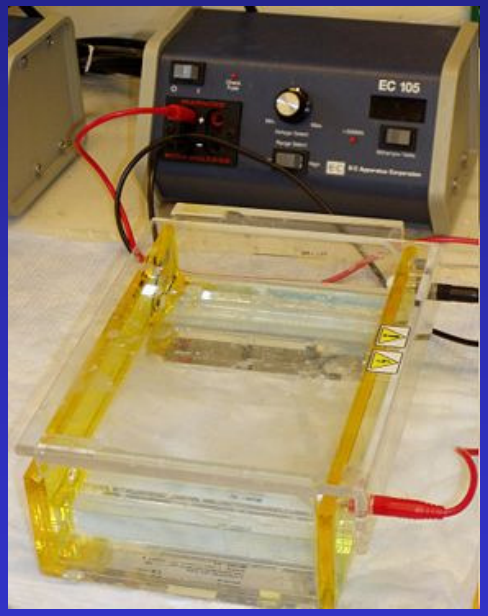
Serodiagnosis of Taeniasis

- 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

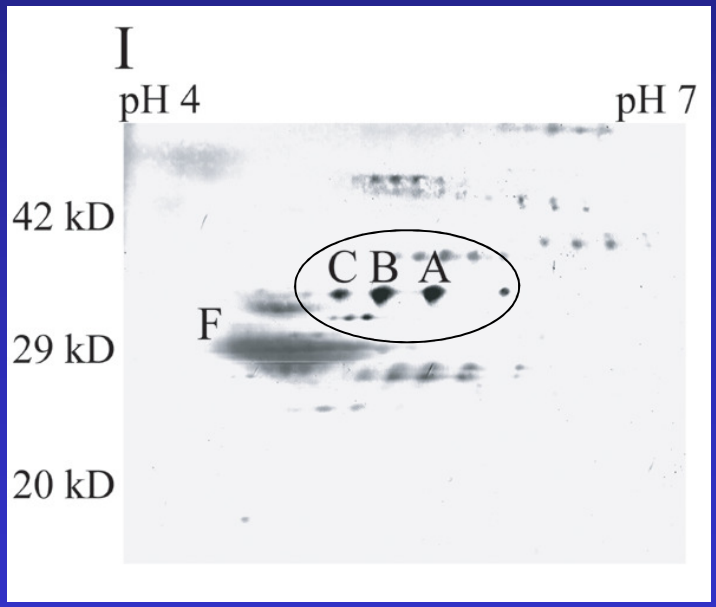


Wilkins et al, 1999 *Am. J. Trop. Med. Hyg.,* 60: 199–204

Identification and Purification of Taeniasis Diagnostic Antigens



2-D gel electrophoresis



Western blot

Levine et al, J. Parasitol., 90(3), 2004, pp. 631–638

Evaluation of rES38

rES38 →

Sensitivity = 99% (80/81)

Specificity 99.7% (299/300)

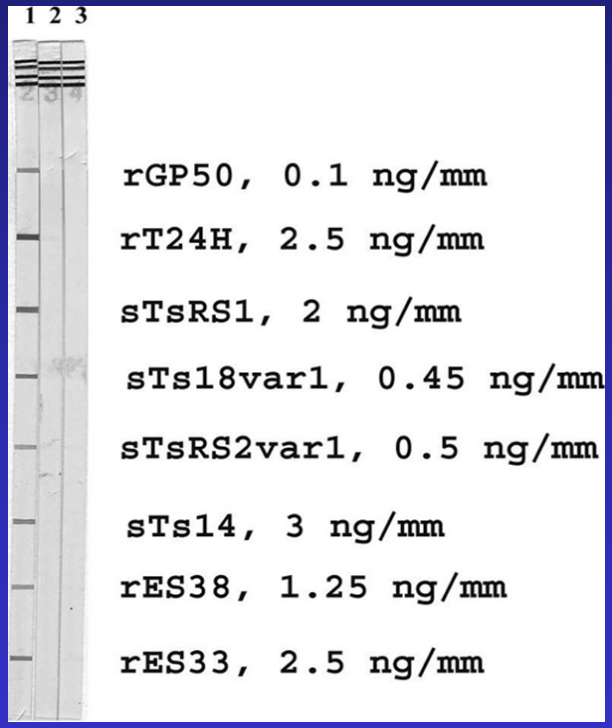
Levine et al, J. Parasitol., 90(3), 2004, pp. 631–638

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



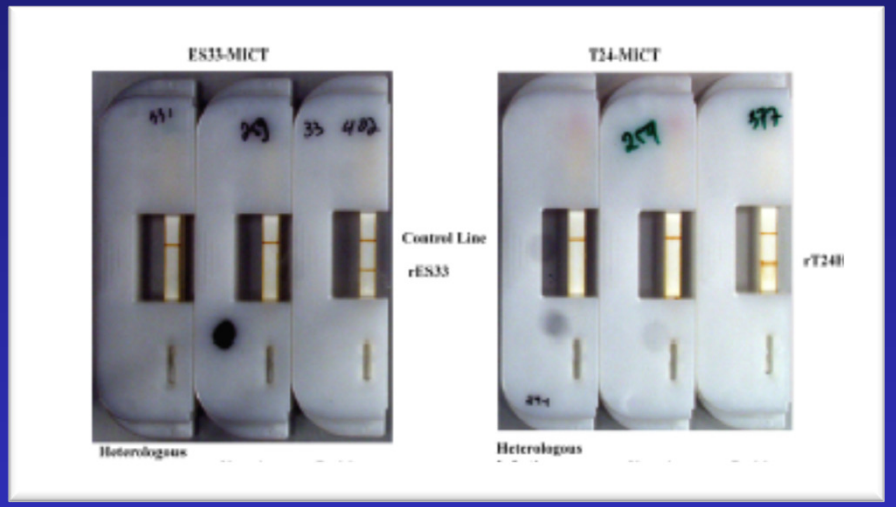
MAPIA with cysticercosis and taeniasis antigens.
Cysticercosis/taeniasis-positive serum pool (lane 1),
Echinococcosis positive serum (lane 2),
Negative serum pool (lane 3)
The optimum concentration of each antigen is shown.

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

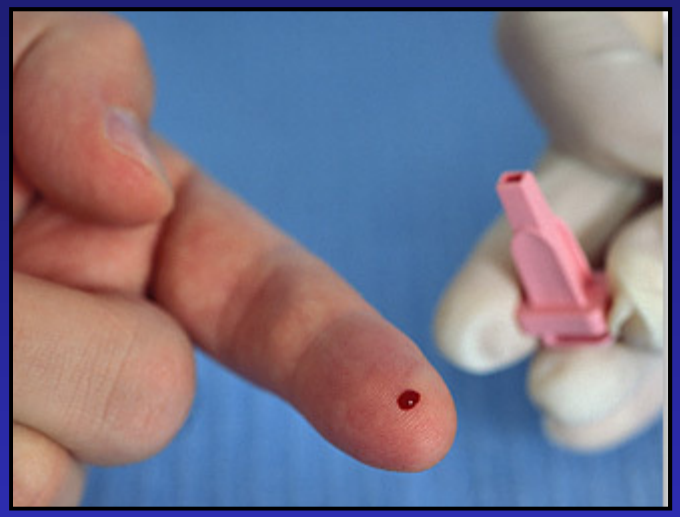



Bench top reader


Handheld reader

Handali et al, 2010 Clin Vac Immunol17:631–637

Standardized collection method for fingerstick blood



ELSEVIER

Journal of Immunological Methods 320 (2007) 164–171

JIM Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

A simple method for collecting measured whole blood with quantitative recovery of antibody activities for serological surveys

Sukwan Handali [a,b,*], Silvia Rodriguez [c], John Noh [a], Armando E. Gonzalez [d,e], Hector H. Garcia [c,e,f], Robert H. Gilman [e], Jacquelin M. Roberts [g], Kathy Hancock [a], Victor C.W. Tsang [a]

- 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

Proteins (s)	Sens[1]	Sens[2]	Spec
Gp50 + rT24H + sTS18	99		91
Gp50	94		96
rT24H	91		94
sTS18	99		96

[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

CDC

TS14 and TS18 contain related peptide sequences

Peptide	Amino acid sequence
TS14	EKNKPKDVAASTKKGIEYVHEFFE
TS18	KNKPKDVAASTKKEIEYIWHNFFED
TS14	IAQLAK
TS18	IAQLAK

Greene et al, 1999, *Mol Biochem Parasitol* 99:257

PCR cloning of TS14 and TS18 reveals related cDNAs

```
                      +1
TS14 TGAACAACCTGTAGAATGCGTGCCTACATTGTGCTTCTCGCTCTCACTGTTTTCGTAGTGACGGTGTCGGCCGAG   75
TS18 ----------------------------------------------TATTCGTAGTGGCGGTTTCGGCCGAG   26
                                                    * ********* **************

AAAAACAAACCGAAAGATGTTGCAAATAGTACGAAAAAAGGGATAGAATATGTCCACGAAT---TCTTCCACGAAGACCCGA 154
AAAAACAAACCGAAGTGTGATGCAAATAGTACTAAGAAAGAGATAGAATATATCCACAATTGGTTTTTCCATGATGACCCGA 109
***************   *************** ** **** ********** ***** * *   * ***** ** *******

TTGGTAAACAAATTGCTCAACTCGCAAAGGAATGGAAGGAAGCAATGTTGGAAGACAAAGGCAAAATACGGACGTCACTGGT 235
TTGGAAAACAAATTGCTCAACTCGCAAAGGACTGGAATGAAACAGTGCAGGAAGCCAAAGGCAAATTTTGGGCGTCACTGGC 190
**** *** **** ***************** ***** *** ** **  ***** ** ******* *  ** *********

TTGAGCACTGCAAAGGTCCTAAGAAAAAAACTGCTTAACTTGTCAACTTTCATGCGTTCTTCTCTTCACTAATAAATGCTCA 318
TTGAGTACTGCAGAGGTCTGAAGAACAAAACTGCTTAACTTGTCAACTTTCATGCGTTCTTCTCTTCACCAATAAATGCTGA 271
***** ****** *****  ***** ****************************************** ********** *

TTAATAAGAAAAAAAAAAAAAAAAA 343
TTAACAAGAAAAAAAAAAAAAAAAA 297
**** **********************
```

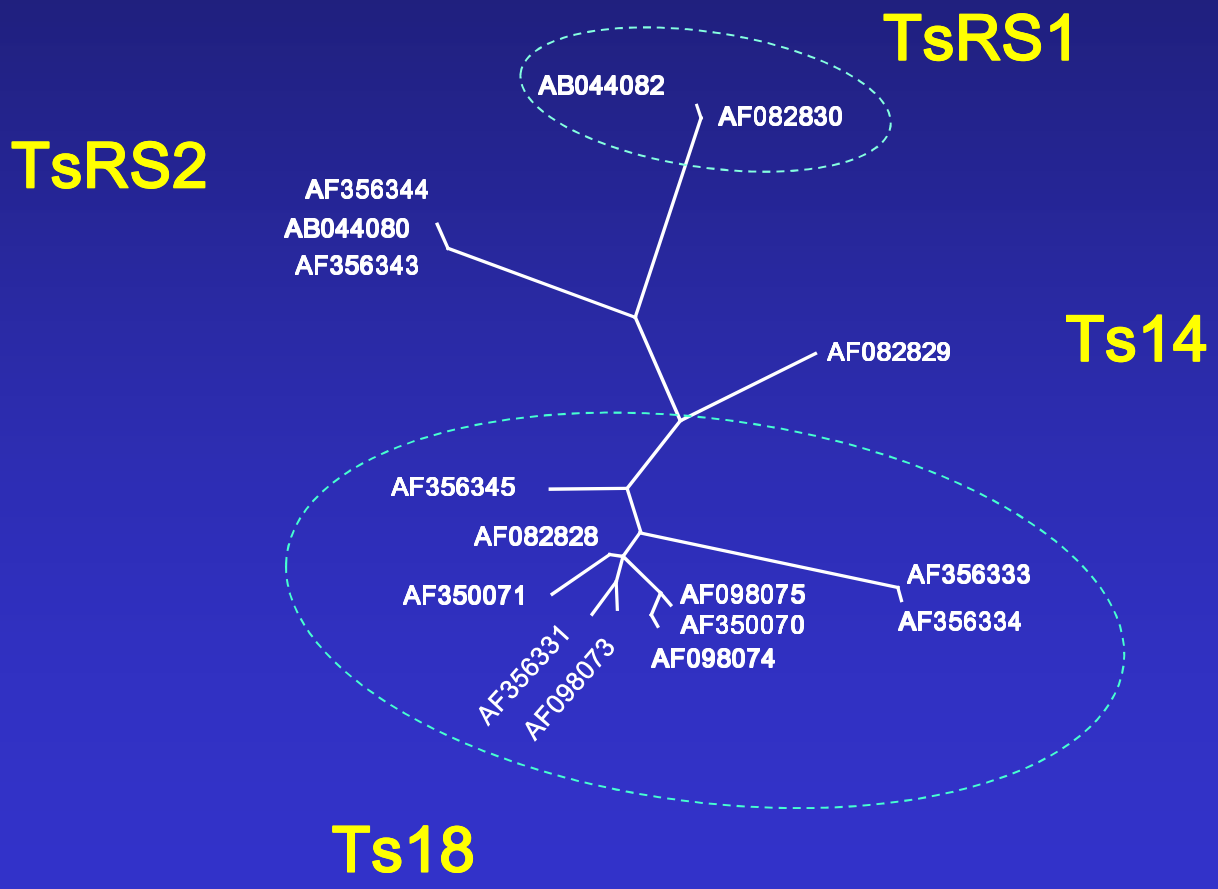
Greene et al, 2000. J Parasitol., 86: 1001–1007

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

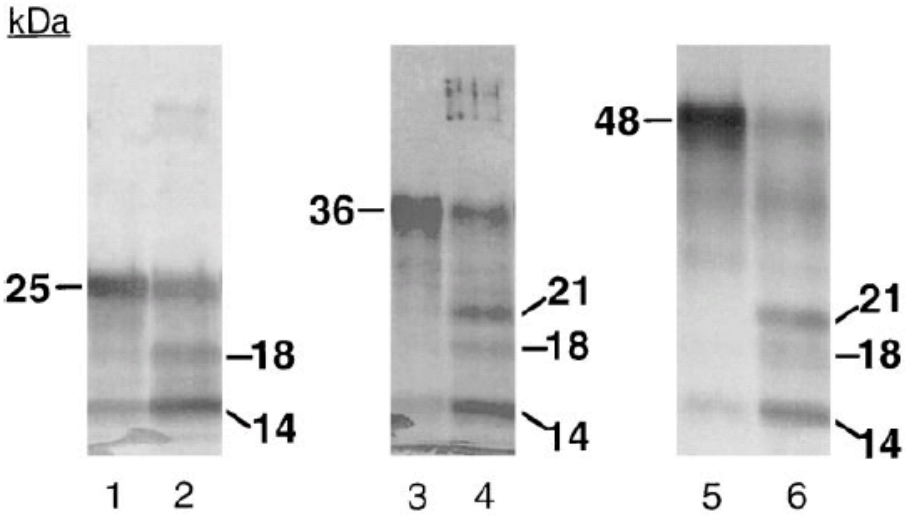


Fig. 1. DTT reduction of preparative gel fractions and the crude EITB antigen preparation. Immunoblot showing DTT reduction of three selected fractions from preparative gel electrophoresis. Three parent fractions (lanes 1, 3, 5) were reduced with DTT (lanes 2, 4, 6) and separated by SDS-PAGE before immunoblotting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

Greene et al, 1999, *Mol Biochem Parasitol* 99:257

Multiple 8kDa protein complexes in the LLGP fraction

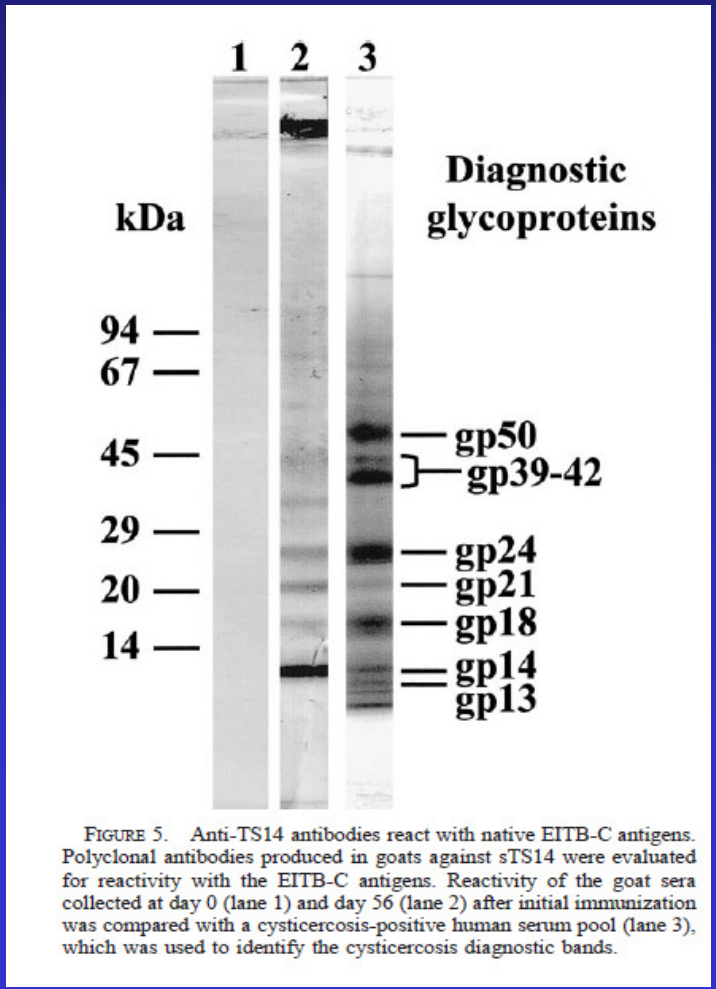


FIGURE 5. Anti-TS14 antibodies react with native EITB-C antigens. Polyclonal antibodies produced in goats against sTS14 were evaluated for reactivity with the EITB-C antigens. Reactivity of the goat sera collected at day 0 (lane 1) and day 56 (lane 2) after initial immunization was compared with a cysticercosis-positive human serum pool (lane 3), which was used to identify the cysticercosis diagnostic bands.

- 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

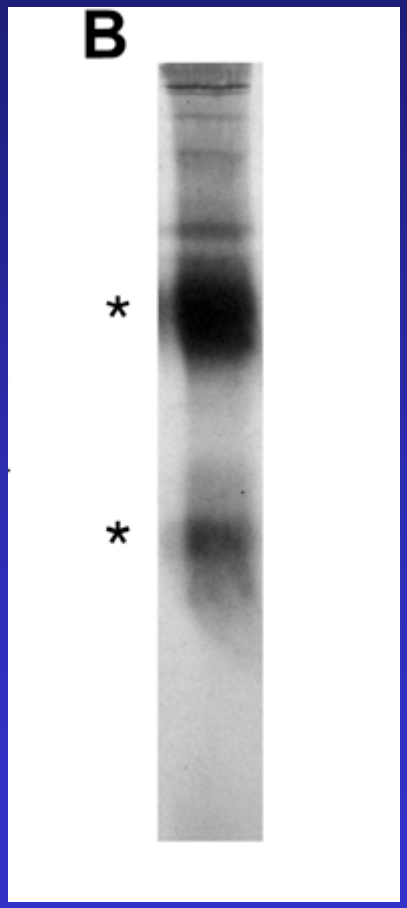
Spot no.(s).	N-terminal sequence	Protein description (Accession number)	Matched peptide mass (m/z)	Sequence coverage (%)
1	EKNKPKDV	14 kDa glycoprotein (AF257776)	—*	
2, 5	EKNKP	14 kDa glycoprotein (AF257776)	1916·92, 2045·01	20
7, 8	—	18 kDa glycoprotein (AF350070)	1302·62, 1487·73, 2160·02	34
10, 11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

* Not determined.

Lee et al, 2005 *Parasitology* 131, 867–879

CDC

8 kDa proteins are soluble in cyst fluid



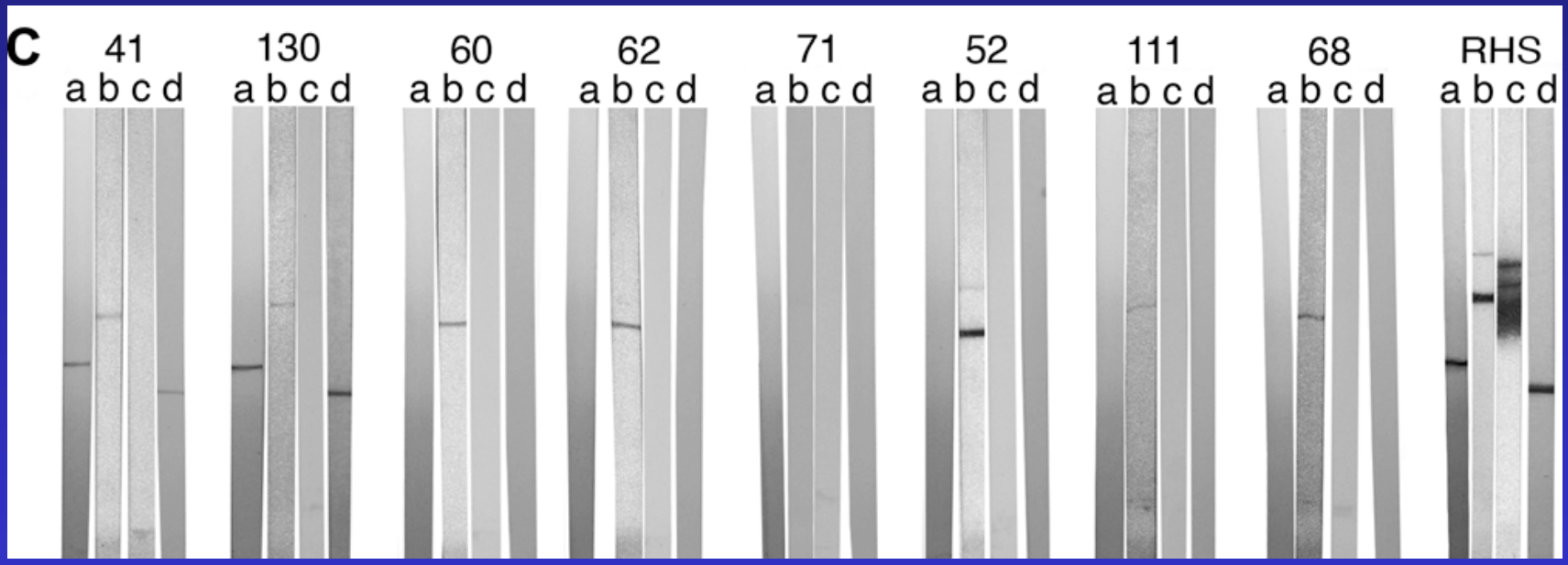
- Nanobodies recognize 2 proteins in cyst fluid 50 and 32 kDa
- Both contained the same N-terminal aa sequence: EKNKPKDVA; TS14

Deckers et al, Int J Parasitol. 2009 Apr;39(5):625-33

CDC

8 kDa proteins are soluble in cyst fluid

Immunoreactivity of nanobodies with 8 kDa peptides



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

Deckers et al, Int J Parasitol. 2009 Apr;39(5):625-33

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……..

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Nicholas Praet
Nynke Deckers

Thank You

CDC
Centers for Disease Control and Prevention