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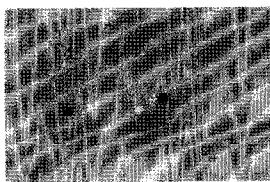
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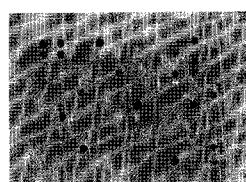
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(57) Resumo: MÉTODO E KIT PARA TESTE IMUNODIAGNÓSTICO DE DOENÇA DE CHAGAS. A presente invenção descreve método e kit para um imunodiagnóstico diferenciado da doença de chagas. Mais particularmente, a invenção trata do uso de quatro peptídeos derivados de epitopos de célula B, na identificação de diferentes cepas do parasito *Trypanossoma cruzi* em pacientes chagásicos, visando o desenvolvimento de um método diagnóstico com maior especificidade e sensibilidade e permitindo a sorotipagem do parasito, este último método ainda não disponível para a doença de chagas.

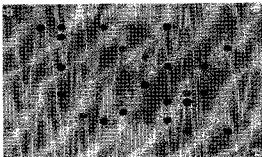
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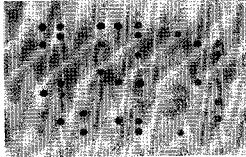
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C)



D)



## MÉTODO E KIT PARA TESTE IMUNODIAGNÓSTICO DE DOENÇA DE CHAGAS

A presente invenção descreve um método e Kit para imunodiagnóstico diferenciado da doença de chagas. Mais particularmente, a invenção trata do uso de quatro peptídeos derivados de epitopos de célula B, na identificação de diferentes cepas do parasito *Trypanossoma cruzi* em pacientes chagásicos, visando o desenvolvimento de um método de diagnóstico com maior especificidade e sensibilidade e permitindo a sorotipagem do parasito, este último método ainda não disponível para a doença de Chagas.

Doenças infecciosas causadas por protozoários continuam a ser uma das maiores causas de mortalidade em países pobres e em desenvolvimento (WHO. 2007. Disponível em: <<http://www.who.int/mediacentre/news/notes/2007/np16/en/>>. Acesso em: 9 Feb. 2011.). A doença de Chagas, causada pelo parasito *Trypanosoma cruzi*, está presente em 18 países da América Latina, do norte do México ao sul da Argentina. Segundo estimativas, 10 milhões de pessoas estão infectadas, 21.000 mortes ocorrem anualmente e são diagnosticados cerca de 50-200 mil novos casos por ano.

Estudos epidemiológicos, bioquímicos e moleculares têm demonstrado que o táxon *T. cruzi* é extremamente heterogêneo tanto genotipicamente quanto fenotipicamente. Baseado em vários marcadores moleculares, o táxon *T. cruzi* foi dividido inicialmente em duas linhagens geneticamente distintas denominadas *T. cruzi* I e *T. cruzi* II ( Souto RP et al. Mol Biochem Parasitol. 1996 Dec 20;83(2):141-52.). Posteriormente foi proposto que a linhagem II fosse subdividida em cinco sub-linhagens nomeadas IIa-IIe (Brisse S, Verhoef J, Tibayrenc M. Int J Parasitol. 2001 Sep;31(11):1218-26). Evidências foram então reportadas para a existência de uma terceira linhagem denominada *T. cruzi* III ( De-Freitas JM et al. PLoS Pathog. 2006 Mar;2(3):e24.). Recentemente, houve uma reclassificação das cepas de *T. cruzi*, agora divididos em seis linhagens nomeadas Tc I-VI, com os grupos Tc V e VI englobando cepas híbridas (Zingales B et al. Mem Inst Oswaldo Cruz. 2009 Nov;104(7):1051-4).

A principal metodologia, para identificação das diferentes linhagens de *T. cruzi*, baseia-se na genotipagem, onde é necessário obtenção do material genético do parasito (Devera R et al. Rev Soc Bras Med Trop. 2002 Jul-Aug;35(4):323-30; Freitas JM et al. Int J Parasitol. 2005 Apr 1;35(4):411-7; 5 D'Avila DA et al. J Clin Microbiol. 2009 Jun;47(6):1718-25). Na fase crônica, devido à baixa parasitemia, conseguir DNA suficiente para o trabalho é muito difícil e algumas metodologias tem sido usadas para facilitar essa obtenção, onde destaca-se a hemocultura (Chiari E et al. Rev Soc Bras Med Trop. 1989 Jan-Mar;22(1):19- 23; Miyamoto et al. Exp Parasitol. 2006 Mar;112(3):198-201) 10 e a obtenção de material genético diretamente do tecido (Freitas JM et al. Int J Parasitol. 2005 Apr 1;35(4):411-7). A hemocultura pode ser negativa quando a parasitemia é baixa e também fornece chance de seleção de populações de parasitos, comprometendo a identificação de co-infecções (Marcelo AM e Pena SD. Parasitol Today. 1998 Mar;14(3):119-24; Andrade Lo et al. Mem Inst 15 Oswaldo Cruz. 2010 Sep;105(6):834-7). A biopsia de tecido infectado é um método muito invasivo que necessita de cuidados médicos e por isso, inviabiliza estudos que requerem um grande número de amostras (Lescure FX et al. Lancet Infect Dis. 2010 Aug;10(8):556-70). No estado da arte apenas Bhattacharyya T et al (Int J Parasitol. 2010 Jul;40(8):921-8) sugeriu a utilização 20 de um antígeno nomeado TSSA ("trypomastigote small surface antigen"), pertencente a família TcMUC III, para discriminar pacientes infectados com TcI de pacientes infectados com TcII-VI (Di Noia JM et al. J Exp Med. 2002 Feb 18;195(4):401-13). Porém estudos moleculares posteriores mostraram que este antígeno seria capaz de agrupar TcI com TcIII e TcIV, separando estas 25 linhagens de TcII, V e VI (Bhattacharyya T et al. Int J Parasitol. 2010 Jul;40(8):921-8). Por isso, identificar epitopos linhagem-específica ainda é um desafio e teria um grande impacto para o diagnóstico da doença de Chagas. Assim, entre os fatores relacionados à dificuldade de desenvolvimento de um bom teste de diagnóstico para a Doença de Chagas, a variabilidade genética 30 do parasito pode ser responsável por casos de falso-negativos (Bhattacharyya T et al Int J Parasitol. 2010 Jul;40(8):921-8).

Neste contexto, na presente invenção foram identificados peptídeos, conservados e polimórficos entre as diferentes cepas do parasito, derivados de epitopos de célula B, visando contribuir para melhorar o sorodiagnóstico e realizar sorotipagem dos parasitos por ELISA, respectivamente. O teste para imunodiagnóstico de doença de chagas pode ser selecionado do grupo compreendendo ELISA, Western blot, dot blot, imunodifusão e imunocromatografia. A técnica de ELISA foi escolhida na presente invenção por ser menos invasiva, de fácil execução e automação, permitindo estudos e aplicação em grande número de amostras.

O uso de antígenos recombinantes e peptídeos sintéticos no diagnóstico da doença de chagas têm sido preferencialmente usados em substituição a lisados do parasito. Essas novas metodologias possuem maior reproduzibilidade dos resultados e eliminação de etapas que envolvem a manutenção e processamento de parasitos vivos (Frasch AC *et al.* Parasitol Today. 1991 Jun;7(6):148-51; Da-Silveira JF *et al.* Trends Parasitol. 2001 Jun;17(6):286-91; Meira WS *et al.* J Clin Microbiol. 2002 Oct;40(10):3735-40).

No estado da técnica foram encontrados documentos de patente que sugerem o uso de peptídeos no diagnóstico da doença de chagas, como exemplos, o documento PI9507182-2 A descreve o uso de polipeptídeos derivados da proteína TCR27 no diagnóstico de infecção pelo *Trypanossoma cruzi*, o documento PI9708188-4 A refere-se a histonas substancialmente isoladas de tripanosomatidae para uso no diagnóstico e como antígenos protetores contra infecção tripanosômica, o documento PI0904827-8 A2 descreve peptídeos recombinantes e motivos proteicos que mimetizam regiões antigênicas do *Trypanosoma cruzi* e suas aplicações no diagnóstico para as três principais formas da doença de Chagas crônica, o documento US6228372 descreve compostos e métodos para diagnosticar infecção por *T. cruzi*. Os compostos são polipeptídeos ou anticorpos que contêm um ou mais epitopos de antígenos de *T. cruzi*. O documento US2008/0096232 A1 descreve métodos de identificar e diagnosticar infecção por *Trypanosoma cruzi* usando uma combinação de quatro polipeptídeos recombinantes. No entanto, todos os documentos encontrados no estado da técnica, dentre os quais se encontram

os acima citados, apresentam peptídeos diferenciados dos apresentados nessa invenção e, ainda, não permitem um diagnóstico tão específico, através do qual se faz a distinção das diferentes cepas de *T. cruzi* no sangue contaminado, como na presente tecnologia. Assim, apesar dos avanços obtidos nos últimos 5 anos, os métodos de diagnóstico disponíveis da doença de Chagas ainda possuem diversas limitações. Além disso, na ausência de métodos de diagnóstico efetivos, a eficiência do tratamento e de qualquer iniciativa de bloqueio da transmissão ou vacinação fica comprometida (Ministério da Saúde. Doenças Infecciosas e Parasitárias: guia de bolso. Brasília. 2006).

## 10 BREVE DESCRIÇÃO DAS FIGURAS

**FIGURA 1 – A figura 1 mostra o padrão de reconhecimento da membrana por soro de animais infectados por diferentes cepas do parasito.** A) “Pool” de soros de animais não-infectados (controle negativo). B) “Pool” de soros de animais infectados com a cepa CL Brener. C) “Pool” de soros de animais infectados com a cepa Y. D) “Pool” de soros de animais infectados com a cepa Colombiana.

**FIGURA 2 – A figura 2 mostra a validação e caracterização da reatividade do peptídeo conservado C6\_30\_cons.** A) Soro de camundongos C57BL/6 infectados com diferentes cepas de *T. cruzi*. Painel à esquerda, ELISA convencional; Painel à direita, ELISA de afinidade. B) Soro de camudongos C57BL/6 infectados com diferentes inóculos da cepa CL Brener. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de “cutoff”. C-, controle negativo.

**25 FIGURA 3 – A figura 3 mostra a avaliação de reação cruzada do peptídeo C6\_30\_cons com outros tripanossomatídeos.** A) Soro de camudongos C57BL/6 infectados com *T. rangeli*. B) Soro de pacientes infectados com *L. brasiliensis* e não infectados por *T. cruzi*. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de “cutoff”. C-, controle negativo. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

**FIGURA 4 – A figura 4 mostra o mapeamento dos aminoácidos de importância para interação com anticorpos de animais infectados com diferentes cepas de *T. cruzi* com o peptídeo conservado C6\_30\_cons.** A) “pool” de soro de camundongos C57BL/6 infectados com a cepa Colombiana.

5 B) “pool” de soro de camundongos C57BL/6 infectados com a cepa Y. C) “pool” de soro de camundongos C57BL/6 infectados com a cepa CL Brener. No eixo das abscissas, 1 representa a sequência original do peptídeo C6\_30\_cons e os demais símbolos de aminoácidos (conforme IUPAC) indicam que o respectivo aminoácido foi substituído por alanina na sequência do epitopo. A intensidade  
10 relativa (IR) foi calculada pela razão do valor de densitometria do peptídeo alterado pelo valor de densitometria do peptídeo nativo. Em vermelho estão os aminoácidos que apresentaram IR menor que 0.5. Em azul estão destacados os aminoácidos de importância para interação com anticorpos produzidos por animais infectados pelas três cepas de *T. cruzi*.

15 **FIGURA 5 – A figura 5 mostra a reatividade do peptídeo conservado C6\_30\_cons contra soro de pacientes chagásicos.** A) Soro de pacientes chagásicos infectados com a linhagem TcII. B) Soro de pacientes chagásicos com diferentes formas clínicas da doença. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de  
20 “cutoff”. C-, controle negativo. Indet, pacientes com forma clínica indeterminada. CCC1, pacientes com cardiopatia chagásica crônica de grau 1. CCCG, pacientes com cardiopatia chagásica crônica grave. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

**FIGURA 6 – A figura 6 mostra a validação e caracterização da reatividade do peptídeo A6\_30\_col.** A) Soro de camundongos C57BL/6 infectados com diferentes cepas de *T. cruzi*. B) Soro de camundongos C57BL/6 infectados com diferentes inóculos da cepa CL Brener. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de “cutoff”. C-, controle negativo.

30 **FIGURA 7 – A figura 7 mostra a avaliação de reação cruzada do peptídeo A6\_30\_col com outros tripanossomatídeos.** A) Soro de camudongos C57BL/6 infectados com *T. rangeli*. B) Soro de pacientes infectados com *L.*

*braziliensis* e não infectados por *T. cruzi*. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de “cutoff”. C-, controle negativo. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

5 **FIGURA 8 – A figura 8 mostra o mapeamento dos aminoácidos de importância para interação com anticorpos de animais infectados com *T. cruzi* com o peptídeo A6\_30\_col.** A) mensuração de densitometria da interação de anticorpos do “pool” de soro de camundongos C57BL/6 infectados com a cepa Colombiana com o peptídeo por Ala scan. B) Comparação dos  
10 aminoácidos importantes para interação com anticorpo entre as sequência dos pares de alelos dos dois haplótipos de *T. cruzi*. No eixo das abscissas, 1 representa a sequência original do peptídeo A6\_30\_col e os demais símbolos de aminoácidos (conforme IUPAC) indicam que o respectivo aminoácido foi substituído por alanina na sequência do epitopo. A intensidade relativa (IR) foi  
15 calculada pela razão do valor de densitometria do peptídeo alterado pelo valor de densitometria do peptídeo nativo. Em vermelho estão os aminoácidos que apresentaram IR menor que 0.5.

**FIGURA 9 – A figura 9 mostra a reatividade do peptídeo A6\_30\_col contra soro de pacientes chagásicos.** A) Soro de pacientes chagásicos infectados  
20 com a linhagem TcII. B) Soro de pacientes chagásicos com diferentes formas clínicas da doença. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de “cutoff”. C-, controle negativo. Indet, pacientes com forma clínica indeterminada. CCC1, pacientes com cardiopatia chagásica crônica de grau 1. CCCG, pacientes com cardiopatia  
25 chagásica crônica grave. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

**FIGURA 10 – A figura 10 mostra a validação e caracterização da reatividade do peptídeo B2\_30\_Y.** A) Soro de camundongos C57BL/6 infectados com diferentes cepas de *T. cruzi*. B) Soro de camundongos C57BL/6  
30 infectados com diferentes inóculos da cepa CL Brener. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de

"cutoff". C-, controle negativo. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

**FIGURA 11 – A figura 11 mostra a avaliação de reação cruzada do peptídeo B2\_30\_Y com outros tripanossomatídeos.** A) Soro de

5 camudongos C57BL/6 infectados com *T. rangeli*. B) Soro de pacientes infectados com *L. braziliensis* e não infectados por *T. cruzi*. Linhas pretas contínuas representam a média de cada grupo. Linhas tracejadas representam o valor de "cutoff". C-, controle negativo. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

10 **FIGURA 12 – A figura 12 mostra o mapeamento dos aminoácidos de importância para interação com anticorpos de animais infectados com *T.***

***cruzi* com o peptídeo B2\_30\_Y.** A) mensuração de densitometria da interação de anticorpos do "pool" de soro de camundongos C57BL/6 infectados com a cepa Y com o peptídeo por Ala scan. B) Comparação dos aminoácidos

15 importantes para interação com anticorpo entre as sequência dos pares de alelos dos dois haplótipos de *T. cruzi*. No eixo das abscissas, 1 representa a sequência original do peptídeo B2\_30\_Y e os demais símbolos de aminoácidos (conforme IUPAC) indicam que o respectivo aminoácido foi substituído por alanina na sequência do epitopo. A intensidade relativa (IR) foi calculada pela

20 razão do valor de densitometria do peptídeo alterado pelo valor de densitometria do peptídeo nativo. Em vermelho estão os aminoácidos que apresentaram IR menor que 0.5.

**FIGURA 13 – A figura 13 mostra a reatividade do peptídeo conservado B2\_30\_Y contra soro de pacientes chagásicos.** A) Soro de pacientes

25 chagásicos infectados com a linhagem TcII. B) Soro de pacientes chagásicos com diferentes formas clínicas da doença. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de "cutoff". C-, controle negativo. Indet, pacientes com forma clínica indeterminada. CCC1, pacientes com cardiopatia chagásica crônica de grau 1.

30 CCCG, pacientes com cardiopatia chagásica crônica grave. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

**FIGURA 14 – A figura 14 mostra a validação e caracterização da reatividade do peptídeo B9\_30\_cl.** A) Soro de camundongos C57BL/6 infectados com diferentes cepas de *T. cruzi*. B) Soro de camundongos C57BL/6 infectados com diferentes inóculos da cepa CL Brener. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de “cutoff”. C-, controle negativo. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

**FIGURA 15 – A figura 15 mostra a avaliação de reação cruzada do peptídeo B9\_30\_cl com outros tripanossomatídeos.** A) Soro de camundongos C57BL/6 infectados com *T. rangeli*. B) Soro de pacientes infectados com *L. braziliensis* e não infectados por *T. cruzi*. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de “cutoff”. C-, controle negativo. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

**FIGURA 16 – A figura 16 mostra o mapeamento dos aminoácidos de importância para interação com anticorpos de animais infectados com *T. cruzi* com o peptídeo B9\_30\_cl.** A) mensuração de densitometria da interação de anticorpos do “pool” de soro de camundongos C57BL/6 infectados com a cepa CL Brener com o peptídeo por Ala scan. B) Comparação dos aminoácidos importantes para interação com anticorpo

entre as sequência dos pares de alelos dos dois haplótipos de *T. cruzi*. No eixo das abscissas, 1 representa a sequência original do peptídeo B9\_30\_cl e os demais símbolos de aminoácidos (conforme IUPAC) indicam que o respectivo aminoácido foi substituído por alanina na sequência do epitopo. A intensidade relativa (IR) foi calculada pela razão do valor de densitometria do peptídeo alterado pelo valor de densitometria do peptídeo nativo. Em vermelho estão os aminoácidos que apresentaram IR menor que 0.5.

**FIGURA 17 – A figura 17 mostra a reatividade do peptídeo B9\_30\_cl contra soro de pacientes chagásicos.** A) Soro de pacientes chagásicos infectados com a linhagem TcII. B) Soro de pacientes chagásicos com diferentes formas clínicas da doença. Linhas pretas contínuas representam a média de cada grupo. |Linhas tracejadas representam o valor de “cutoff”. C-, controle negativo.

Indet, pacientes com forma clínica indeterminada. CCC1, pacientes com cardiopatia chagásica crônica de grau 1. CCCG, pacientes com cardiopatia chagásica crônica grave. Painéis à esquerda, ELISA convencional; Painéis à direita, ELISA de afinidade.

## 5 DESCRIÇÃO DETALHADA DA TECNOLOGIA

Na presente tecnologia foram identificados epitópos de células B, eficientes no diagnóstico sorológico da doença de chagas e na sorotipagem dos parasitos que infectam cada paciente, através dos dados gerados no projeto genoma de *T. cruzi*, de ferramentas de bioinformática e ensaios experimentais de validação, com um objetivo de identificar epitópos conservados e polimórficos em diferentes cepas do parasito.

Foram utilizadas neste estudo as cepas Colombiana (pertencentes à linhagem TcI), cepas Y (TcII) e cepas CL Brener (TcVI).

Para os testes imunodiagnósticos, camundongos machos C57BL/6 com duas a quatro semanas de vida foram infectados intraperitonealmente com diferentes cepas de *T. cruzi* e *T. rangeli*. Cada grupo experimental foi composto de seis animais. Um grupo foi infectado com 50 triatomastigotas da cepa Colombina, um com 500 triatomastigotas da cepa Y. Para o clone CL Brener, foram utilizados três inóculos diferentes de parasitos, sendo um grupo de animais infectados com 50 triatomastigotas, outro grupo com 100 triatomastigotas e um último grupo com 500 triatomastigotas. Para *T. rangeli*, os animais foram infectados com  $1 \times 10^5$  parasitos. Um grupo contendo seis animais não infectados foi utilizado como controle negativo dos experimentos.

Para *T. cruzi*, 10 dias após a inoculação, a infecção foi confirmada através da observação triatomastigotas por exame a fresco de sangue obtido da cauda destes animais. Para *T. rangeli*, a infecção foi acompanhada de dois em dois dias através da observação triatomastigotas por exame a fresco. O sangue dos animais infectados foi coletado por punção cardíaca três meses após a infecção e após a coagulação, o soro foi obtido por centrifugação a 4000 G por 15 minutos. A presença de IgG anti-parasito foi confirmada pela reatividade com antígeno bruto de *T. cruzi*.

Para melhor compreensão da tecnologia seguem os exemplos abaixo, não limitantes:

**Exemplo 1- Identificação *in silico* de epitopos lineares de células B**

5 Inicialmente, epitopos lineares de célula B foram preditos em todo o proteoma da cepa CL Brener de *T. cruzi* utilizando o programa BepiPred (Larsen JE, Lund O e Nielsen M. Immunome Res. 2006 Apr 24;2:2). Nesta análise foi utilizada uma abordagem conservativa com valor de corte igual a 1.3 que fornece 96% de especificidade e 13 % de sensibilidade, minimizando a  
10 seleção de muitos epitopos falso-positivos. Utilizando também o proteoma predito de *T. cruzi*, os pares de alelos de genes cópias simples Esмо e Non-  
esмо foram alinhados utilizando o programa ClustalW (Chenna R et al. Nucleic  
Acids Res. 2003 Jul 1;31(13):3497-500). Cada aminoácido alinhado recebeu  
uma pontuação, de acordo com a interpretação do seu alinhamento, conforme  
15 a TABELA 1. Um script em Perl utilizando a metodologia de janela deslizante  
percorreu sequências de 15 aminoácidos ao longo de toda a proteína, a fim de  
correlacionar as pontuações de polimorfismo e de predição de epitopos  
lineares de célula B. Sequências com o somatório dos scores de polimorfismo  
acima de 6 e score médio de predição do Bepipred acima de 1.3 foram  
20 consideradas como epitopos polimórficos. Sequências de 15 aminoácidos com  
score médio de predição de epitopo igual entre as duas sequências e acima de  
1.3 e score de polimorfismo igual a zero foram considerados epitopos  
conservados. Para minimizar a chance de reação cruzada com outros parasitos  
filogeneticamente relacionados, sequências de peptídeos com alta similaridade  
25 com proteínas de outros tripanossomatídeos foram identificadas utilizando o  
algoritmo BLAST (Altschul SF et al. J Mol Biol. 1990 Oct 5;215(3):403-10) e  
descartadas. A versão do algoritmo denominada blastp foi utilizado para  
comparar epitopos polimórficos e conservados com o proteoma predito de *L. braziliensis*, *L. infantum*, *L. major* e *T. brucei*. Epitopos com mais de 70% de  
30 identidade ao longo de mais de 70% da sequência foram eliminados das  
análises subsequentes.

Ao final da seleção *in silico*, obteve-se 242 epitopos preditos polimórficos e 1086 epitopos preditos conservados, ambos *T. cruzi* específicos baseado nas análises *in silico*. Destas sequências, 49 peptídeos conservados e 98 peptídeos polimórficos (49 Esmo e 49 Non-esmo específicos) foram 5 selecionados utilizando o critério de maior valor de predição de Bepipred. Como controles positivos foram selecionados os peptídeos experimentalmente mapeados derivados das proteínas L7a (Pais FS et al., Microbes Infect. 2008 Jun;10(7):716-25) e trans-sialidase (Peralta JM et al. J Clin Microbiol. 1994 Apr;32(4):971-4). O controle negativo corresponde ao peptídeo 10 PIELVLWMPTLCRAN está presente na região da proteína L7a para a qual não é observada reatividade com o soro de indivíduos infectados (Pais FS et al., Microbes Infect. 2008 Jun;10(7):716-25). Os peptídeos foram sintetizados em membrana de celulose pela técnica spot síntese.

**TABELA 1 – Pontuação de alinhamento entre proteínas Esmo e Non-esmo de *T. cruzi*.**

Interpretação do alinhamento	Pontuação
Aminoácidos idênticos	0
Aminoácidos diferentes com propriedades químicas similares	1
Aminoácidos diferentes com propriedades químicas diferentes	2
Gap em sequência	3

### **Exemplo 2- Spot síntese**

Um total de 150 peptídeos foram sintetizados em membrana de celulose pré-ativada (Frank RJ. Immunol Methods. 2002 Sep 1;267(1):13-26) e derivatizada com um espaçador de 10 resíduos de polietilenoglicol (Intavis AG). 20 Duas membranas foram sintetizadas em duplicata, uma possuindo 30 peptídeos em um arranjo de 10 X 3 e a outra com 120, em um arranjo de 10 X 12 utilizando o equipamento MultiPep SPOT (Intavis AG). Inicialmente, os Fmoc-aminoácidos a 0,5 mM foram ativados por 20 minutos com HOBt 0,05 mM e DIC 0,1 mM. Após distribuição de cada Fmoc-aminoácido pré-ativado 25 nas membranas em sua respectiva posição, foi esperado um tempo de reação de 30 minutos. Sítios da membrana que não se ligaram a aminoácidos foram então bloqueados com anidrido acético 10%, seguido pela desproteção dos

Fmoc-aminoácidos com 4-metilpiperidina 25%. A desproteção dos aminoácidos foi confirmada corando os spots com solução de azul de bromofenol 2%. Um novo ciclo de distribuição de aminoácidos foi então realizado até que o último aminoácido seja incorporado ao peptídeo que está sendo sintetizado. Após o 5 término da síntese dos peptídeos, foi realizada a desproteção das cadeias laterais utilizando 25:25:1,5:1 de TFA, DCM, triisopropilsilano e água e posterior monitoramento da desproteção com azul de bromofenol 2%.

O mapeamento dos aminoácidos de epitopos que são importantes nas ligações com anticorpos foi realizada pela técnica de Ala scan (Frank RJ. Immunol 10 Methods. 2002 Sep 1;267(1):13-26). Nesta técnica de Ala scan, o aminoácido alanina é usado para substituir sequencialmente cada resíduo de aminoácido do peptídeo original. A substituição de um resíduo crítico no reconhecimento do anticorpo resulta em uma diminuição de reatividade. Durante a análise por densitometria utilizando o software ImageMaster™ 2D Platinum (GE Healthcare), os spots que deixaram de ser reconhecidos ou tiveram o nível de 15 reconhecimento diminuído foram identificados.

#### **Exemplo 4- Immunoblotting**

Os experimentos de reatividade das membranas contendo os arranjos de peptídeos contra diferentes soros foram realizados. Inicialmente, as 20 membranas foram bloqueadas com uma solução 5% BSA e 4% de sacarose em PBS pH 7,4 e incubadas por uma hora sob agitação. Em seguida, as membranas foram incubadas por uma hora sob agitação com soro de camundongo diluído 1:5000 em solução de bloqueio. Após lavagem com PBS, as membranas foram incubadas por uma hora sob agitação com anticorpos 25 secundários anti-IgG de camundongo conjugado à peroxidase e diluídos 1:10000 na solução de bloqueio. Novamente realizou-se uma etapa de lavagem com PBS, seguindo a revelação da membrana com *ECL Plus Western blotting* utilizando o fotodocumentador Kodak. Como controle negativo, membranas foram submetidas às mesmas condições experimentais usando 30 soro de camundongos não infectados. Em seguida, para cada cepa foi realizado análise densitométrica de cada “spot” utilizando o software

ImageMaster™ 2D Platinum (GE Healthcare).

Após revelação, as membranas foram regeneradas para retirar qualquer anticorpo ligado a elas. Inicialmente, as membranas foram lavadas três vezes com DMF por 10 minutos cada, seguida de uma lavagem com água deionizada 5 por 1 minuto. Em seguida, foram realizadas três lavagens de 30 minutos cada com uma solução desnaturante de uréia 8 M e SDS 10%, seguida por mais uma lavagem de 1 minuto com água deionizada. As membranas foram submetidas a três lavagens com uma solução ácida de etanol 55% e ácido acético 10%, seguida de uma última lavagem de 1 minuto com água deionizada. As membranas foram colocadas em etanol duas vezes, por 10 minutos cada, para retirar umidade e secas em ar frio. Após este protocolo de regeneração, estavam prontas para serem reutilizadas.

O perfil de reconhecimento dos peptídeos da membrana por cada cepa é mostrado na FIGURA 1. Para ser considerado reativo, um “spot” deveria ter 15 IR acima de 2, ou seja, ter duas vezes maior valor de densitometria para o “pool” de soro de animais infectados do que o controle negativo.

Dos 150 peptídeos analisados, 36 foram reativos com “pool” de soros de animais infectados com pelo menos uma cepa de *T. cruzi* (FIGURA 1). Os valores de IR dos peptídeos reativos com soro de animais infectados com cada 20 uma das cepas e anotação das proteínas que contém os epitopos são mostrados na TABELA 2.

#### **Exemplo 5- Síntese dos peptídeos solúveis**

Como a técnica de “*immunoblotting*” seguido de análise de densitometria é apenas semi-quantitativa é necessário validação dos resultados por uma 25 técnica quantitativa, como ELISA. Para tanto, 4 dos 36 epitopos reativos foram selecionados para a síntese solúvel e posterior análise de antigenicidade por ELISA (Destacados em negrito na TABELA 2).

**TABELA 2- Peptídeos reativos com soro de camundongos infectados com diferentes cepas de *T. cruzi***

<b>TABELA 2 – Peptídeos reativos com soro de camundongos infectados com diferentes cepas de <i>T. cruzi</i>.</b>						
<b>Membrana ID</b>	<b>Intensidade Relativa</b>		<b>Gene</b>	<b>Peptídeo</b>	<b>Anotação</b>	
	<b>Colombia na</b>	<b>CL Brener</b>	<b>Y</b>			
A1_120	1,67	2,09	2,15	Tc00.1047053 506401.320	AAKTAAKPAAKSAAKPAAKP	60S ribosomal protein L7a, putative
A5_120	1,23	1,23	2,83	Tc00.1047053 508831.140	PSPFGIAAAGDKPSPFGQA	surface antigen 2 (CA-2), putative
C9_120	1,02	4,80	1,85	Tc00.1047053 511529.150	KENESEGASGPDDSAG	hypothetical protein, conserved
C10_120	2,24	1,26	1,79	Tc00.1047053 506531.40	GGSNEEKDPGETPAC	hypothetical protein, conserved
D6_120	2,09	0,93	1,60	Tc00.1047053 503813.10	SPSESKTPGQPSLE	hypothetical protein, conserved
E2_120	2,00	0,96	1,02	Tc00.1047053 506825.150	VGEPSNNGTPDEERE	hypothetical protein, conserved
E7_120	2,10	1,30	1,48	Tc00.1047053 508539.50	EANGSCSDYAQSAADD	hypothetical protein, conserved
F10_120	2,31	2,22	2,64	Tc00.1047053 506825.30	PHAASPTGTGANRST	hypothetical protein, conserved
G2_120	1,10	2,90	2,47	Tc00.1047053 505807.120	SLSSSPSPLEPKKPR	lysyl-tRNA synthetase, putative
G10_120	1,28	1,03	2,86	Tc00.1047053 503853.10	TPNDTANETTNELED	hypothetical protein, conserved
H2_120	2,02	1,08	0,81	Tc00.1047053 504431.10	SDRDRDQRSREGPEE	hypothetical protein, conserved
I4_120	2,14	1,36	1,46	Tc00.1047053 511907.190	PADPGGEDATDSSFR	hypothetical protein
I8_120	2,50	0,88	0,95	Tc00.1047053 508857.90	PAGKTHTAERGEEKT	hypothetical protein, conserved
J3_120	3,35	1,26	2,21	Tc00.1047053 511859.50	SRGESGGVAPSDGVA	hypothetical protein, conserved
J10_120	1,52	1,64	2,00	Tc00.1047053 509937.220	STGDAPQGAPSAPPMM	protein kinase, putative

K3_120	1,32	2,64	0,93	Tc00.1047053 510219.10	KRTTSPQISPEKSAS	hypothetical protein, conserved
K4_120	2,41	1,67	1,26	Tc00.1047053 511291.40	QNRDSGVLEGPTATT	protein kinase, putative
K5_120	1,53	1,07	3,23	Tc00.1047053 508153.300	ERTSSTEGSQPHLPG	hypothetical protein, conserved
L3_120	1,80	1,50	2,03	Tc00.1047053 506509.50	AGGTSAGPAAGNSTQ	hypothetical protein, conserved
L4_120	1,37	1,03	2,63	Tc00.1047053 508175.20	KGGTTKSAVGTPFSS	hypothetical protein, conserved
L6_120	2,13	2,21	3,00	Tc00.1047053 506303.150	ALAEPDPGGERNASD	hypothetical protein, conserved
L8_120	2,07	1,61	1,37	Tc00.1047053 511907.220	VSRRVSGDGDDGGVG	hypothetical protein, conserved
A2_30	1,39	1,00	2,95	Tc00.1047053 507221.30	NGSGKGGTDREGDDDFN	hypothetical protein, conserved
A5_30	1,09	2,73	3,30	Tc00.1047053 510857.40	FEGPSPGDPDKDF	RNA-editing complex protein, putative
A6_30	2,61	1,71	0,73	Tc00.1047053 511837.129	ENSANPPPPDRSLPTP	RNA-binding protein, putative
A8_30	1,10	1,10	2,83	Tc00.1047053 506401.330	GDDNDDDDGEEGDNTAL	hypothetical protein, conserved
B1_30	1,26	1,45	3,92	Tc00.1047053 510307.284	AEPKSAEPKPAEPKS	trans-sialidase, putative
B2_30	1,39	1,77	4,26	Tc00.1047053 511589.70	FFQPQPQPQPQPQQF	ADP-ribosylation factor GTPase activating protein, putative
B3_30	1,64	1,06	2,38	Tc00.1047053 506251.40	ATAASGGGGGGGRNPSE	hypothetical protein
B4_30	1,68	2,87	2,89	Tc00.1047053 508675.20	KVDDDDDDDKN	DNA excision repair protein, putative
B6_30	2,50	1,33	1,50	Tc00.1047053 511041.20	FSDGAHGSGASRGSGR	hypothetical protein, conserved
B9_30	0,78	2,43	1,30	Tc00.1047053 510359.320	MDDDDDDERTGGG	DNA-directed

						RNA polymerase III subunit, putative
B10_30	0,92	2,20	1,25	Tc00.1047053 506859.150	VGAEGEGEKNNDVPSEEQ	hypothetical protein
C6_30	2,59	2,70	2,31	Tc00.1047053 510421.310	QRMSNASGGGGGMRQNE	hypothetical protein, conserved
C7_30	1,34	2,11	2,97	Tc00.1047053 511153.100	ARKNEDVNKADEEREI	RNA-binding protein, putative

Os peptídeos selecionados foram sintetizados em fase sólida pelo método de N-9-fluorenilmeticarbonil (Wellings DA e Atherton E. Methods Enzymol. 1997;289:44- 67) na escala de 30 umol. Toda síntese foi realizada no equipamento PSSM8. Fmoc-aminoácidos foram ativados por 30 minutos com 1 equivalente de HObt e 2 equivalentes de DIC. Após este tempo, os aminoácidos ativados foram colocados em contato com a resina Rink amidada com grau de substituição de 0,61 por 45 minutos. Os grupos Fmoc foram então removidos com solução de 4-metilpiperidina 25%. A ativação, incorporação na resina e desproteção do grupo Fmoc foram então repetidas até síntese completa da cadeia dos peptídeos. Após a síntese, os peptídeos foram clivados da resina juntamente com a desproteção da cadeia lateral com uma mistura contendo 9,4% de TFA, 2,4% de água e 0,1% de triisopropilsilano. O peptídeo foi precipitado do material bruto com éter diisopropílico por 16 horas a 4 °C, seguido de centrifugação a 3000 G por 5 minutos. Os peptídeos sintetizados foram purificados por cromatografia em fase reversa utilizando-se a coluna Sephasil Peptide C18 (Shimadzu) acoplada a sistema de HPLC (Shimadzu) utilizando-se um gradiente de 0 a 25% de acetonitrila durante 75 minutos com 0 –10 minutos até 10% de acetonitrila. Após a purificação, os peptídeos foram submetidos à espectrometria de massa em equipamento Autoflex Speed MALDI/TOF (Bruker) para confirmação de sua massa molecular.

Os peptídeos A6\_30\_col, B2\_30\_Y e B6\_30\_cl foram escolhidos por ter a maior reatividade preferencial com o “pool” de soros de camundongos

infectados com a cepa Colombiana, Y ou CL Brener, respectivamente (**Figura 1**).

#### **Exemplo 6- Validação dos peptídeos frente a soro de camundongos e de pacientes infectados**

5 Ensaios de ELISA foram realizados para validar e caracterizar a reatividade dos peptídeos frente às cepas de *T. cruzi*. Foram testados soros de camundongos infectados e foi feita a sorotipagem de pacientes infectados também. Os ensaios foram realizados em placas de PVC flexível de 96 poços (BD Falcon). Ensaios de ELISA de avidez foram baseados no método  
10 dissociativo usando uréia como agente desnaturante. Cada poço foi sensibilizado com 2 ug de peptídeo sintético ou de antígeno bruto. O bloqueio foi realizado com uma solução 5% de soro bovino fetal em PBS por 1 hora a 37°C, seguido de três lavagens com PBS-0,05% Tween 20 e adição de soros diluídos 1:100 e incubação por 1 hora a 37°C. Para ELISA de afinidade, após a  
15 incubação foi adicionado uréia 6 M incubadas por 5 minutos a 37°C. As placas foram novamente lavadas por quatro vezes e anticorpo anti-IgG humano ou murino conjugado a peroxidase diluído 1:2000 foi adicionado. Após incubação das placas por 1 hora a 37°C, um novo ciclo de quatro lavagens foi realizado e solução substrato contendo ácido cítrico 0,1 M, Na<sub>2</sub>PO<sub>4</sub> 0,2 M, OPD 0,05% e  
20 H<sub>2</sub>O<sub>2</sub> 0,1% foi adicionada. As placas foram incubadas à temperatura ambiente ao abrigo de luz por cerca de 15 minutos, a reação parada com H<sub>2</sub>SO<sub>4</sub> 4N e a absorbância mensurada em leitor de ELISA a 492 nm. Para ELISA, os valores de cutoff (C) foram calculados para cada placa como sendo: C=m+3SD, onde  
25 m é a média da absorbância e SD é o desvio padrão de resultados obtidos com soro de indivíduos ou camundongos não infectados. Para ELISA de avidez (Hedman et al. J Med Virol. 1989 Apr;27(4):293-8), os resultados foram apresentados como índice de avidez (IA), determinados pela razão entre os valores de absorbância das amostras tratadas com uréia e os valores de absorbância das amostras não tratados e expressos em porcentagem. Um IA  
30 menor que 40% foi considerado de baixa avidez, entre 41 e 70% de média avidez e maior que 70% de alta avidez.

O peptídeo C6\_30\_cons, originado de uma proteína hipotética de *T. cruzi* (Tc00.1047053510421.310), durante a etapa de triagem apresentou intensidade relativa de reatividade acima de 2 com “pools” de soros de animais infectados por todas as cepas de *T. cruzi* utilizadas (TABELA 2). Validação 5 deste peptídeo contra soros individuais de camundongos C57BL/6 por ELISA mostraram que para as três cepas houve reconhecimento acima do “cutoff” e sem diferença estatística (FIGURA 2A). Além disso, para a maioria das amostras, os anticorpos possuíam média afinidade pelo peptídeo, com algumas amostras possuindo anticorpos de alta afinidade (FIGURA 2A). Este 10 perfil de reatividade dos soros e afinidades dos anticorpos foram independentes da quantidade de inóculo utilizado na infecção dos animais, uma vez que não foi observado diferença estatística entre os soro de camundongos C57BL/6 infectados com diferentes quantidades de inóculos da cepa CL Brener (FIGURA 2 B).

15 Nenhuma amostra de soro de camundongos C57BL/6 infectados com *T. rangeli* apresentou reatividade acima do “cutoff” com o peptídeo conservado C6\_30\_cons (FIGURA 3A). Em relação às 14 amostras de pacientes infectados com *L. braziliensis* e não infectados com *T. cruzi*, apenas uma apresentou reatividade acima do valor de “cutoff” e esta reatividade é associada a 20 anticorpos de alta afinidade (FIGURA 3B).

Como o peptídeo apresentou reatividade similar para as três cepas de *T. cruzi*, identificou-se quais os aminoácidos seriam importantes para interação com anticorpos produzidos por camundongos infectados com cada uma destas 25 cepas utilizando a técnica de Ala scan. Observou-se um padrão conservado GXXXXMRQNE na região carboxi-terminal do peptídeo que sugere ser a principal sequência à qual os anticorpos provenientes das três cepas se ligariam (FIGURA 4).

Além de modelos animais, a reatividade do peptídeo C6\_30\_cons foi 30 avaliada utilizando soro de pacientes chagásicos. Oito de dez soros de pacientes chagásicos com parasitos genotipados pertencentes a linhagem TcII apresentaram reatividade acima com valor de “cutoff” (FIGURA 5A). Os anticorpos destas amostras possuíam de média a alta afinidade. Além destas

amostras, a reatividade e afinidade de anticorpos presentes em soro de pacientes chagásicos com diferentes formas clínica da doença foram também mensurados. Dos 20 soros de pacientes chagásicos, 18 possuíram reatividade acima do valor de “cutoff” (FIGURA 5B). Soro de pacientes sintomáticos com 5 cardiopatia chagásica crônica apresentaram significativamente maiores níveis de reatividade do que pacientes com a forma clínica indeterminada. Porém, para todas as amostras, os anticorpos possuíam afinidades baixas e médias. O peptídeo solúvel conservado C6\_30\_cons apresentou uma especificidade de 93% (13/14) e sensibilidade de 87% (27/31) para soros humanos.

10 **Exemplo 7- Validação e caracterização dos peptídeos com reatividade específica para a cepa Colombiana baseado nos experimentos de imunoblotting**

O peptídeo polimórfico A6\_30\_col presente em uma proteína anotada como “*RNA-binding protein*” de *T. cruzi* (Tc00.1047053511837.129) foi 15 selecionado por ter intensidade relativa de reatividade acima de 2 apenas com “pools” de soros de animais infectados com a cepa Colombiana de *T. cruzi* durante a fase de triagem (TABELA 2). Validação deste peptídeo contra soros individuais de camundongos C57BL/6 por ELISA mostraram que a reatividade 20 do peptídeo com amostras de animais infectados com a cepa Colombiana foi significativamente maior que a reatividade com soro de animais infectados com a cepa Y (FIGURA 6A). A afinidade dos anticorpos das amostras de Colombiana foram classificadas como alta e significativamente maior que aquelas de Y e CL Brener (FIGURA 6A). Entre amostras de camundongos infectados com diferentes inóculos de tripomastigotas de CL Brener não foi 25 observado diferença significativa no reconhecimento do peptídeo A6\_30\_col (FIGURA 6B).

Metade das amostras de soro de camundongos C57BL/6 infectados com *T. rangeli* apresentaram reatividade acima do “cutoff” com o peptídeo A6\_30\_col e os anticorpos possuíam em sua maioria média afinidade (FIGURA 30 7A). Para amostras de pacientes infectados com *L. braziliensis* e não infectados com *T. cruzi*, três dos quatorzes soros também apresentaram

reatividade positiva com este peptídeo e os anticorpos apresentavam baixa afinidade (FIGURA 7B).

O mapeamento dos aminoácidos importantes para interagir com anticorpos de soro de animais infectados com a cepa Colombiana de *T. cruzi* identificou o padrão PPDXSLXXP (FIGURA 8A). Este padrão está presente na sequência sintetizada obtida do alelo pertencente ao haplótipo Non-esmo do genoma da cepa CL Brener de *T. cruzi* e quando comparados com o alelo pertencente ao haplótipo Esmo observa-se a ausência de aminoácidos importantes para o reconhecimento pelo anticorpo (FIGURA 8B).

- 10 Apenas dois soros de pacientes chagásicos com parasitos genotipados pertencentes a linhagem TcII apresentaram reatividade positiva acima do “cutoff” com o peptídeo solúvel conservado A6\_30\_col e a maioria das amostras possuíam anticorpos de baixa afinidade (FIGURA 9A). Um perfil semelhante de reatividade e afinidade foi observado para soro de pacientes 15 com diferentes formas clínicas da doença e não houve diferença estatística entre as amostras de pacientes com a forma indeterminada e os dois graus de cardiopatia chagásica crônica analisados (FIGURA 9B).

**Exemplo 8- Validação e caracterização do peptídeo com reatividade específica para a cepa Y**

- 20 O peptídeo polimórfico B2\_30\_Y identificado de uma sequência protéica anotada como “ADP-ribosylation factor GTPase activating protein” de *T. cruzi* (Tc00.1047053511589.70) foi escolhido por ter intensidade relativa de reatividade acima de 2 somente com “pool” de soros de animais infectados com a cepa Y de *T. cruzi* durante o “immunoblotting” dos peptídeos ligados a 25 membrana de celulose (TABELA 2). A reatividade do peptídeo por ELISA com o soro de camundongos C57BL/6 infectados com a cepa Y foi estatisticamente maior que a reatividade com soros de animais infectados com a cepa Colombiana (FIGURA 10A). A afinidade dos anticorpos de todas amostras de Y foi alta e significativamente maior que aquela para Y e CL Brener, que 30 possuíram anticorpos em suas amostras classificadas como de média afinidade (FIGURA 10A). Entre amostras murinas infectadas com diferentes quantidades

de tripomastigotas não foi observado diferença significativa no reconhecimento do peptídeo (FIGURA 10B).

Os soro de camundongos C57BL/6 infectados com *T. rangeli* apresentaram pouca reatividade com o peptídeo conservado B2\_30\_Y com apenas um soro positivo e estes anticorpos possuíam baixa afinidade pelo epitopo (FIGURA 11A). Apenas uma amostra de pacientes infectados com *L. braziliensis* e não infectados com *T. cruzi* foi reativa com este peptídeo (FIGURA 11B). Os anticorpos da maioria destes pacientes apresentaram média afinidade.

O mapeamento dos aminoácidos importantes para interagir com anticorpos de soro de animais infectados com a cepa Y de *T. cruzi* utilizando a técnica de Ala scan identificou o padrão QPQPXPQXXXQP (FIGURA 12A). Este padrão está presente na subsequência sintetizada do alelo pertencente ao haplótipo Esмо do genoma da cepa CL Brener de *T. cruzi*. Quando comparado com o alelo pertencente ao haplótipo Non-esмо, observa-se a ausência de aminoácidos importantes para o reconhecimento pelo anticorpo (FIGURA 12B).

O peptídeo B2\_30\_Y foi capaz de identificar sete dos dez soros de pacientes chagásicos com parasitos genotipados pertencentes a linhagem TcII e os anticorpos destes soros apresentaram de média a alta afinidade pelo epitopo (FIGURA 13A). Seis de dez, quatro de cinco e cinco de seis soros de pacientes com as respectivas formas clínicas indeterminadas, cardiopatia crônica chagásica de grau 1 e de grau grave apresentaram reatividade positiva (FIGURA 13B). Houve diferença estatisticamente significativa entre os pacientes com cardiopatia grau 1 e com forma clínica indeterminada com estes últimos possuindo menor valor médio de reatividade. Os anticorpos dos pacientes com a forma indeterminada apresentaram em média menor afinidade do que os anticorpos dos pacientes sintomáticos que foram classificados como de alta afinidade (FIGURA 13B).

### **Exemplo 9- Validação e caracterização do peptídeo com reatividade específica para a cepa CL Brener**

O peptídeo polimórfico B9\_30\_cl identificado de uma sequência protéica

anotada como “*DNA-directed RNA polymerase III subunit*” de *T. cruzi* (Tc00.1047053510359.320) foi escolhida por ter intensidade relativa de reatividade acima de 2 somente com “pool” de soros de animais infectados com a cepa CL Brener de *T. cruzi* durante a etapa de triagem (TABELA 2). Os soros individuais de camundongos C57BL/6 infectados com a cepa CL Brener foram significativamente mais reativos com o peptídeo do que soros de animais infectados com as outras duas cepas (FIGURA 14A). Os anticorpos das amostras das três cepas possuíam em sua maioria de média a alta, sem diferença estatística entre elas (FIGURA 14A). Para amostras de soros de camundongos infectados com diferentes quantidades de tripomastigotas de CL Brener não foi observado diferença significativa na reatividade com o peptídeo, bem como entre as afinidades dos anticorpos, que também possuíam de média a alta afinidade (FIGURA 14B).

Todas as amostra de soro de camundongos C57BL/6 infectados com *T. rangeli* apresentaram reatividade negativa (FIGURA 15A). Dois dos dez soros de pacientes infectados com *L. braziliensis* e não infectados com *T. cruzi* apresentaram reatividade positiva com este peptídeo e maioria das amostras possuíam anticorpos com média ou baixa afinidade ao epitopo (FIGURA 15B).

O mapeamento dos aminoácidos importantes para interagir com os anticorpos de soro de animais infectados com a cepa CL Brener de *T. cruzi* identificou o padrão DEXXXG (FIGURA 16A). O padrão está presente na sequência sintetizada obtida do alelo pertencente ao haplótipo Esmo do genoma da cepa CL Brener de *T. cruzi* e quando comparados com o alelo pertencente ao haplótipo Non-esmo observa-se a existência de expansão de repetição do aminoácido aspartato nas regiões onde estes importantes aminoácidos estão presentes (FIGURA 16B).

Apenas um soro de pacientes chagásicos com parasitos genotipados pertencentes a linhagem TcII apresentaram reatividade positiva acima do “cutoff” com o peptídeo solúvel B9\_30\_cl (FIGURA 17A).. Para soro de pacientes com diferentes formas clínicas da doença, dez amostras foram reativas com o epitopo e não houve diferença estatística entre as amostras de pacientes com a forma indeterminada e os dois graus de cardiopatia chagásica

crônica analisados (FIGURA 17B). Os anticorpos destas amostras possuíam de baixa a média afinidade ao peptídeo.

Os peptídeos identificados apresentaram especificidade e sensibilidade alta para o uso no diagnóstico do parasito e após estudos mais aprofundados, 5 principalmente com grandes quantidades de amostras de diferentes regiões geográficas, teriam o potencial para ser incorporados na rotina laboratorial.

### Análise estatística

A análise estatística foi realizada utilizando software Prism 5.0 e como todos os dados seguiam distribuição gaussiana, a comparação entre dois 10 grupos experimentais foi feita usando o teste T de Student não-pareado enquanto que o ANOVA foi utilizado para comparações de mais de dois grupos experimentais. Valores  $p < 0,05$  foram considerados como diferenças estatisticamente significativas. Para os peptídeos conservados entre as diferentes cepas de *T. cruzi*, foram realizados cálculos de especificidade e 15 sensibilidade para peptídeos conservados com potencial de serem usados para o diagnóstico de *T. cruzi*. A sensibilidade foi calculada pela expressão:  $S = VP/(VP + FN)$ , onde VP é o número de soros de indivíduos infectados com *T. cruzi* que possuem reatividade acima do cutoff para um determinado peptídeo e FN é o número de soros de indivíduos infectados que possuem reatividade 20 abaixo do cutoff para o mesmo peptídeo. A especificidade foi calculada pela seguinte equação:  $E = VN/(VN + FP)$ , onde VN é o número de soros de indivíduos infectados com *L. braziliensis* e não infectados *T. cruzi* que não possuem reatividade acima do cutoff para um determinado peptídeo e FP é o 25 número de soros de indivíduos infectados com *L. braziliensis* e não infectados *T. cruzi* que possuem reatividade acima do cutoff para o mesmo peptídeo.

## REIVINDICAÇÕES

**1- Método para teste imunodiagnóstico de doença de chagas**

characterizado por compreender os seguintes passos:

- 5 a) ligação de anticorpos antitripanossomais de uma amostra a um ou mais polipeptídios, ligados a um suporte sólido ou um carreador, consistindo das sequências de aminoácidos SEQ ID N<sup>o</sup>s 1, 2, 3 e 4.
- 10 b) contactar os anticorpos do passo (a) com um anticorpo secundário ou uma proteína, conjugados a uma enzima ou a um marcador e que se ligam aos anticorpos do passo (a)
- c) detectar os anticorpos antitripanossomais na amostra supracitada pela detecção do anticorpo secundário ou proteína especificamente ligados ao dito anticorpo anti-tripanossomal.

**2- Método para teste imunodiagnóstico de doença de chagas, de**

15 acordo com a reivindicação 1, caracterizado pelos polipeptídeos SEQ ID N<sup>o</sup>s 1, 2, 3 e 4 poderem ser modificados em suas extremidades.

**3- Método para teste Imunodiagnóstico de doença de chagas, de**

20 acordo com a reivindicação 1, caracterizado pelas amostras serem selecionadas do grupo consistindo de sangue, soro, plasma e outro fluído corporal.

**4- Método para teste imunodiagnóstico de doença de chagas, de**

acordo com a reivindicação 1, caracterizado pelo carreador ser uma partícula de ouro.

**5- Método para teste imunodiagnóstico de doença de chagas, de**

25 acordo com a reivindicação 1, caracterizado pela proteína ser selecionada do grupo consistindo de proteína A e proteína G.

**6- Método para teste Imunodiagnóstico de doença de chagas, de**

acordo com a reivindicação 1, caracterizado pela enzima ser selecionada do grupo consistindo de fosfatase alcalina, peroxidase, β-galactosidase, urease, xantina oxidase, glicose oxidase e penicilinase.

**7- Método para teste Imunodiagnóstico de doença de chagas**, de acordo com a reivindicação 1, caracterizado pelo marcador ser selecionado do grupo consistindo de enzimas, radioisótopos, biotina, cromóforos, fluoróforos e quimioluminescentes.

5       **8- Kit para teste imunodiagnóstico de doença de chagas**, caracterizado por compreender:

a) os peptídeos consistindo das sequências SEQ ID N<sup>os</sup> 1, 2, 3 e 4 associados ou isolados;

b) Um anticorpo secundário ou uma proteína, onde o anticorpo secundário ou a proteína está conjugado a um enzima ou marcador, e um reagente para detectar a dita enzima ou marcador.

10      **9- Kit para teste imunodiagnóstico de doença de chagas**, de acordo com a reivindicação 8, caracterizado pelas proteínas recombinantes ou os peptídeos estarem ligados a uma suporte sólido ou carreador.

15      **10-Kit para teste imunodiagnóstico de doença de chagas**, de acordo com a reivindicação 8, caracterizado pelo suporte sólido ser selecionado do grupo de material consistindo de nitrocelulose, nylon, látex, polipropileno e polistireno.

20      **11-Kit para teste imunodiagnóstico de doença de chagas**, de acordo com a reivindicação 8, caracterizado pelo carreador consistir de partículas de ouro.

25      **12-Kit para teste imunodiagnóstico de doença de chagas**, de acordo com a reivindicação 8, caracterizado pela proteína ser selecionada do grupo consistindo de proteína A e proteína G.

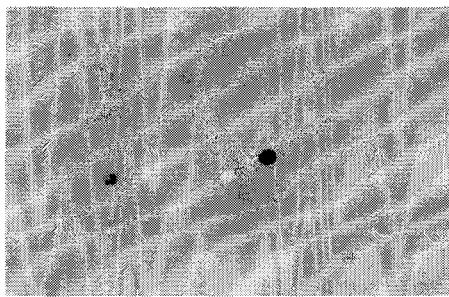
**13-Kit para teste imunodiagnóstico de doença de chagas**, de acordo com a reivindicação 8, caracterizado pelo dito marcador ser selecionado do grupo consistindo de enzimas, radioisótopos, biotina, cromóforos, fluoróforos e quimioluminescentes.

30      **14-Kit para teste imunodiagnóstico de doença de chagas**, de acordo com a reivindicação 8, caracterizado pela enzima ser selecionada do grupo compreendendo fosfatase alcalina, peroxidase, β-

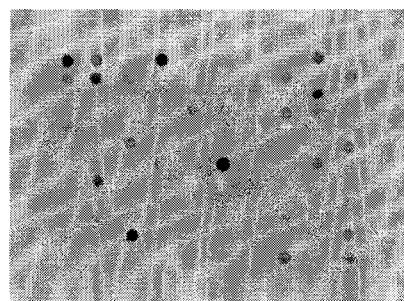
galactosidase, urease, xantina oxidase, glicose oxidase e penicilinase.

**FIGURAS**

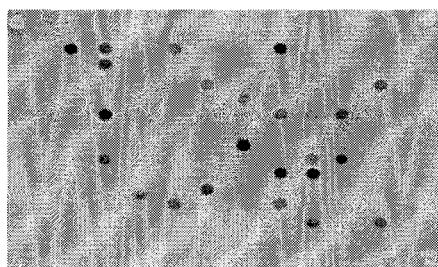
**A)**



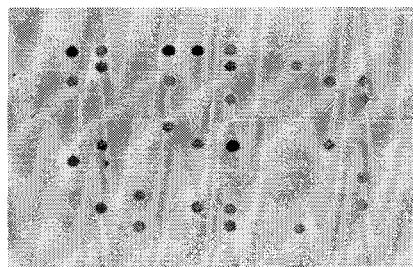
**B)**



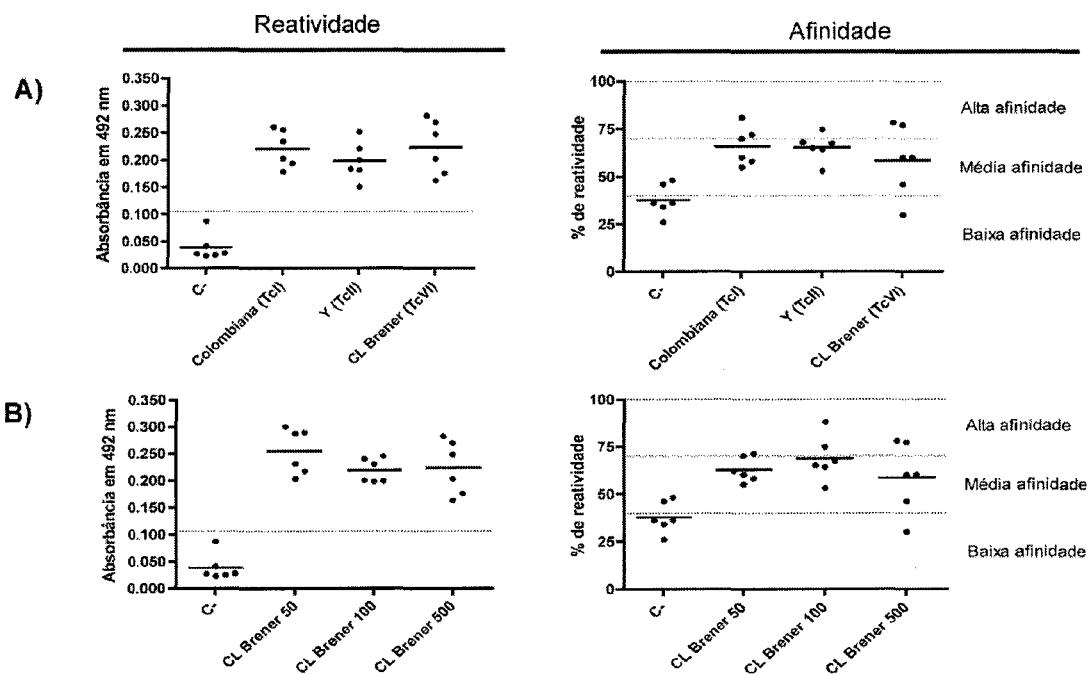
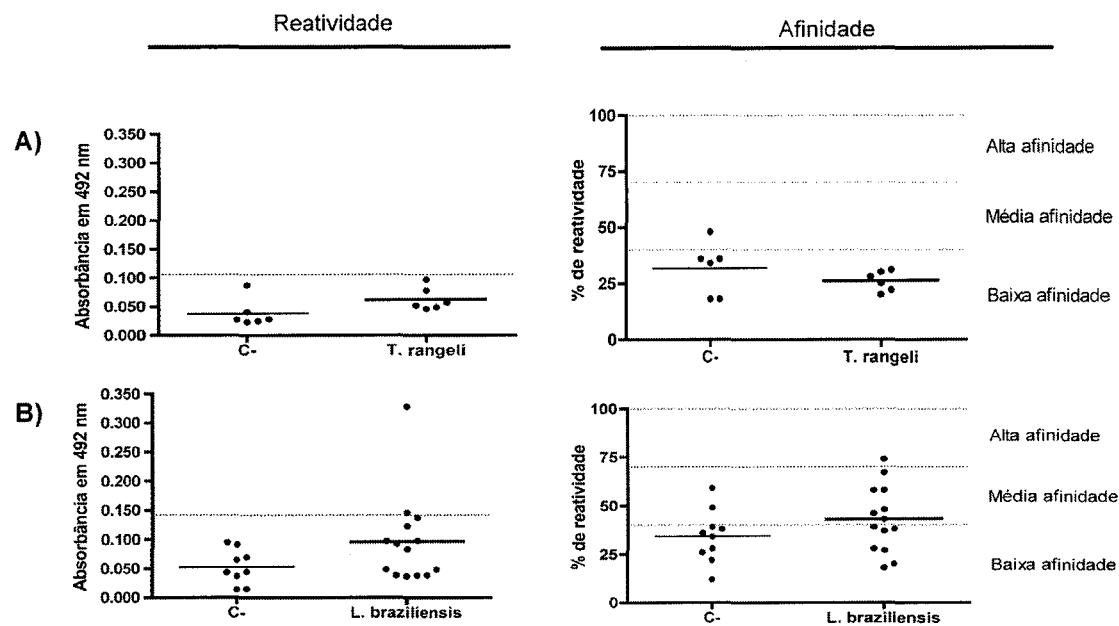
**C)**

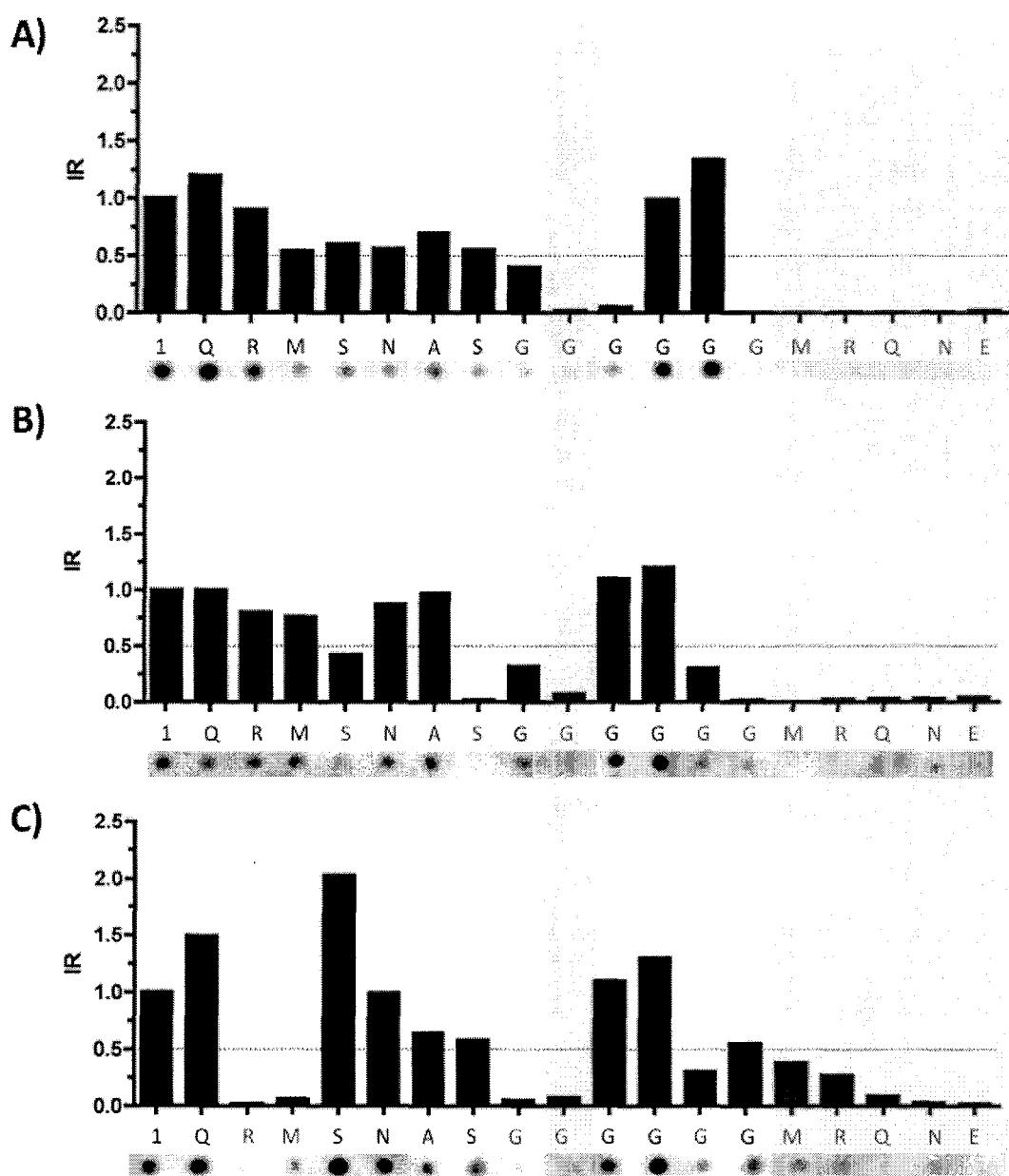


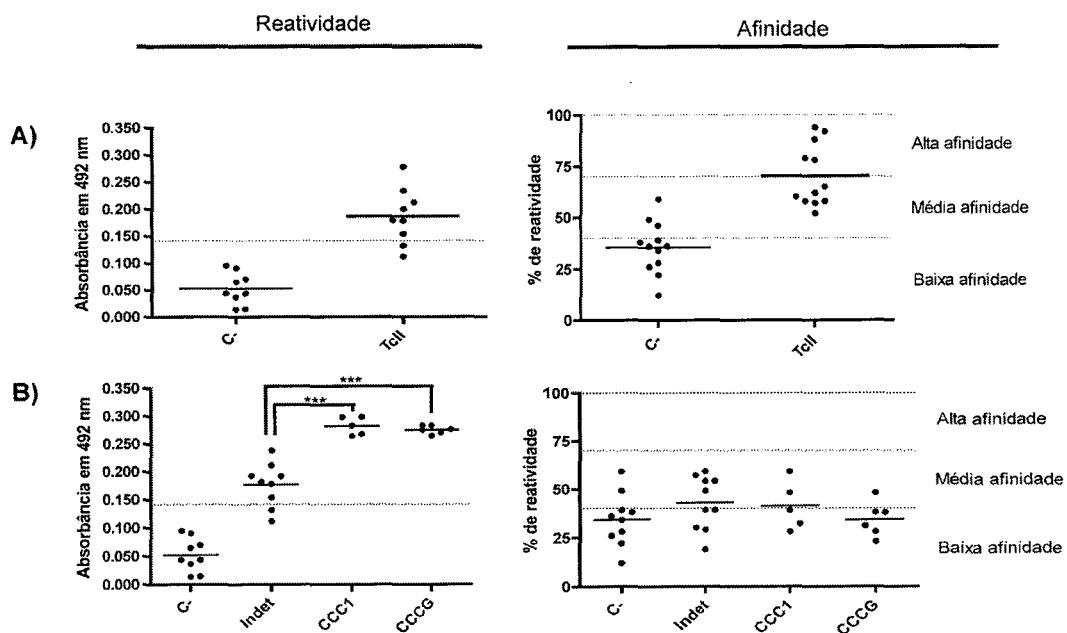
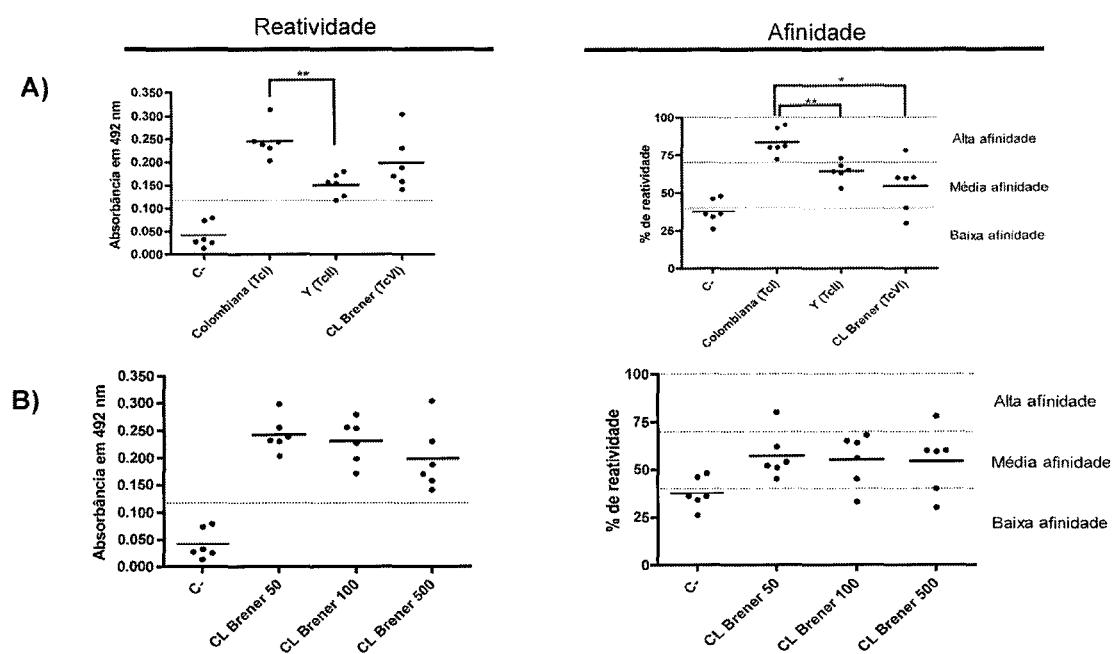
**D)**

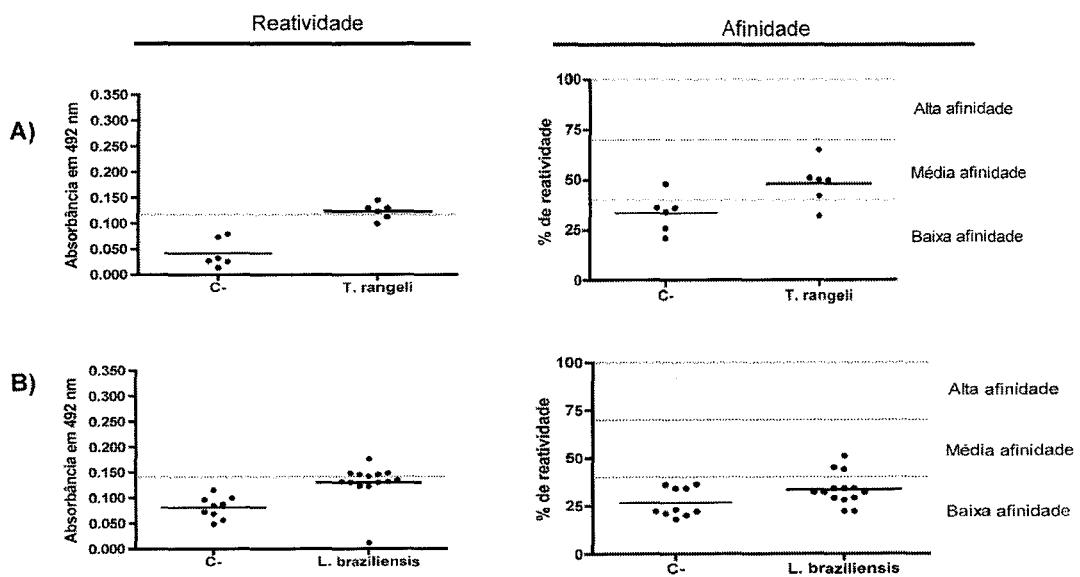
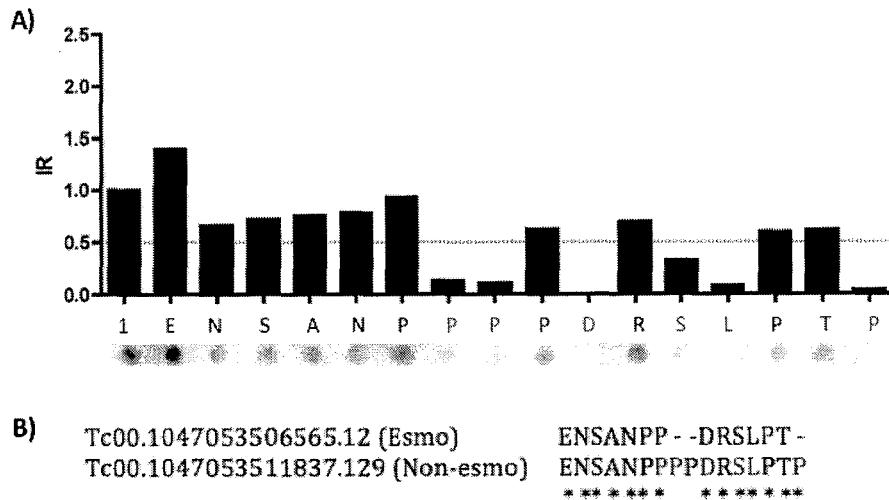


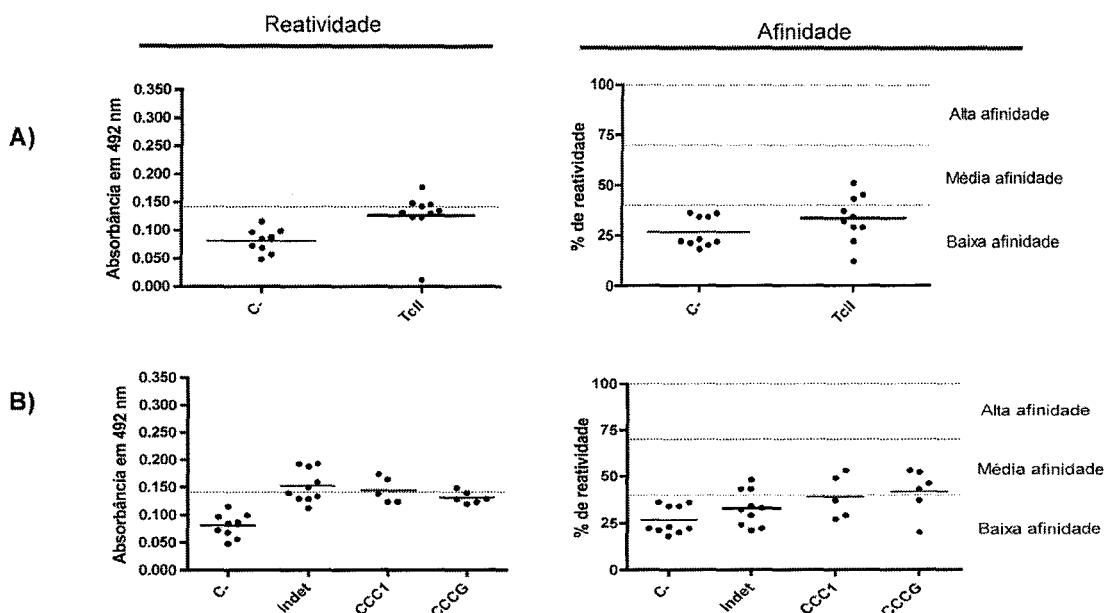
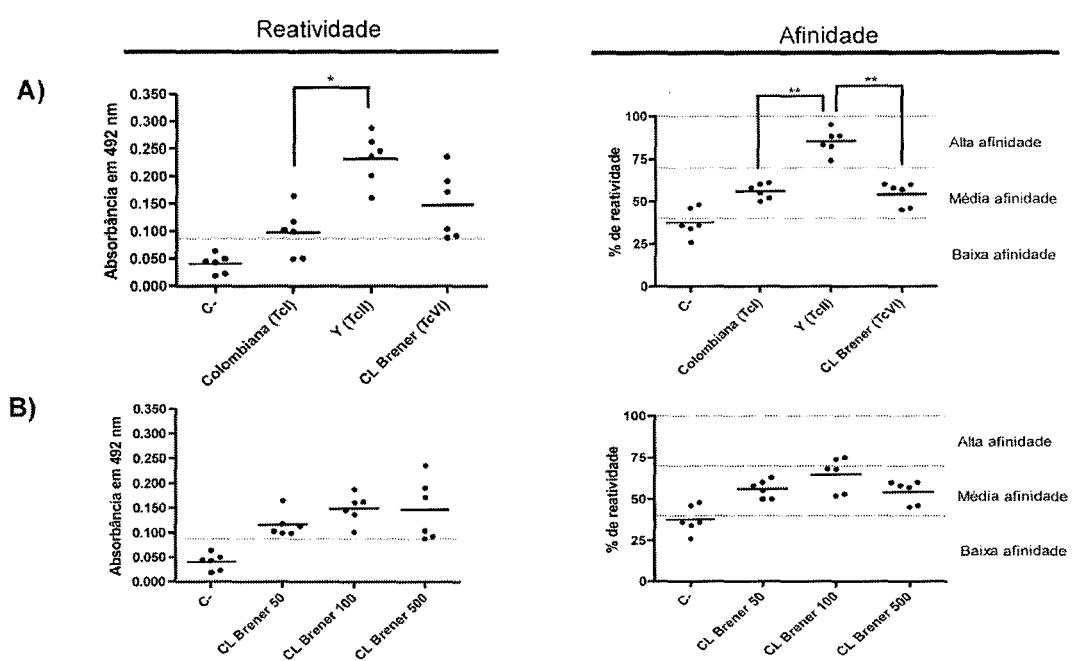
**FIGURA 1**

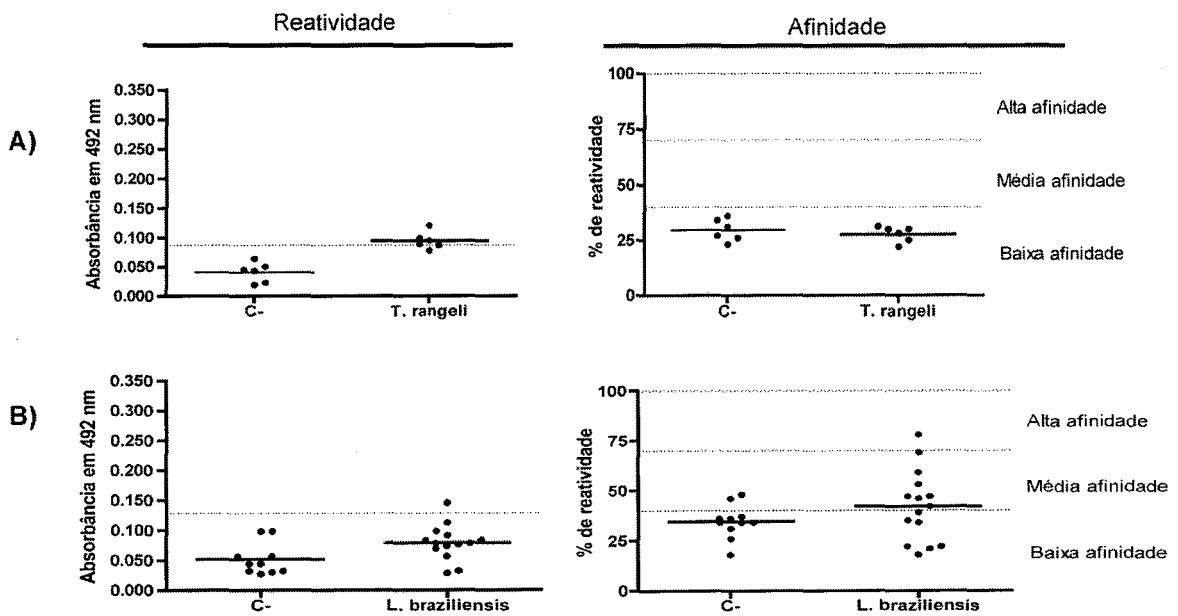
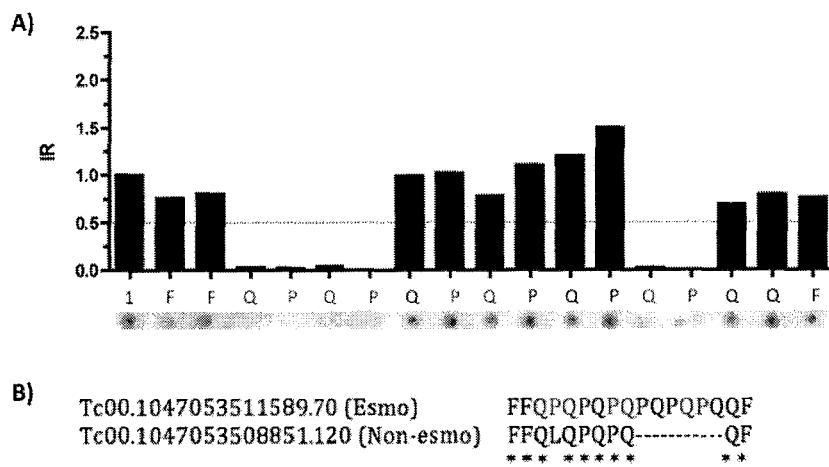
**FIGURA 2****FIGURA 3**

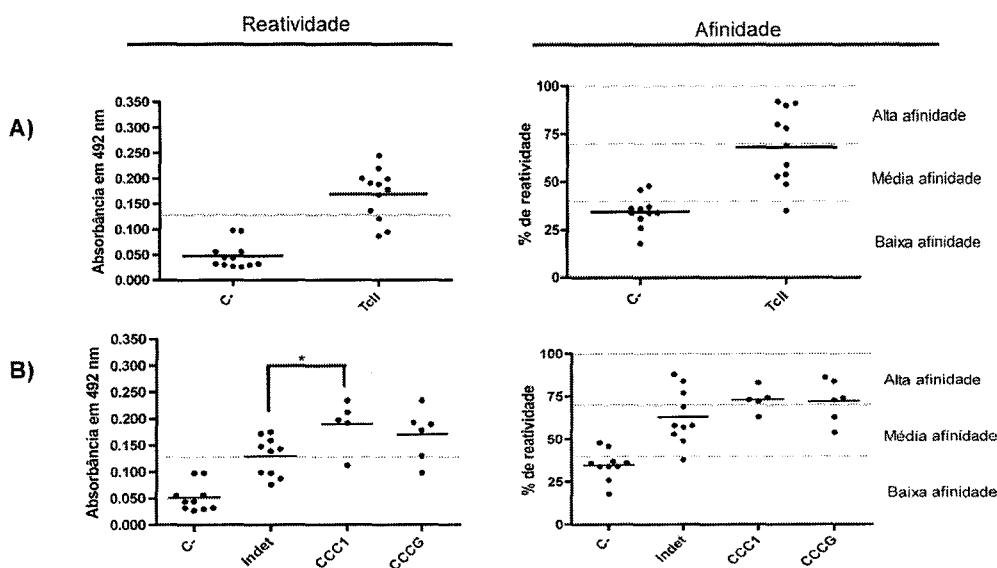
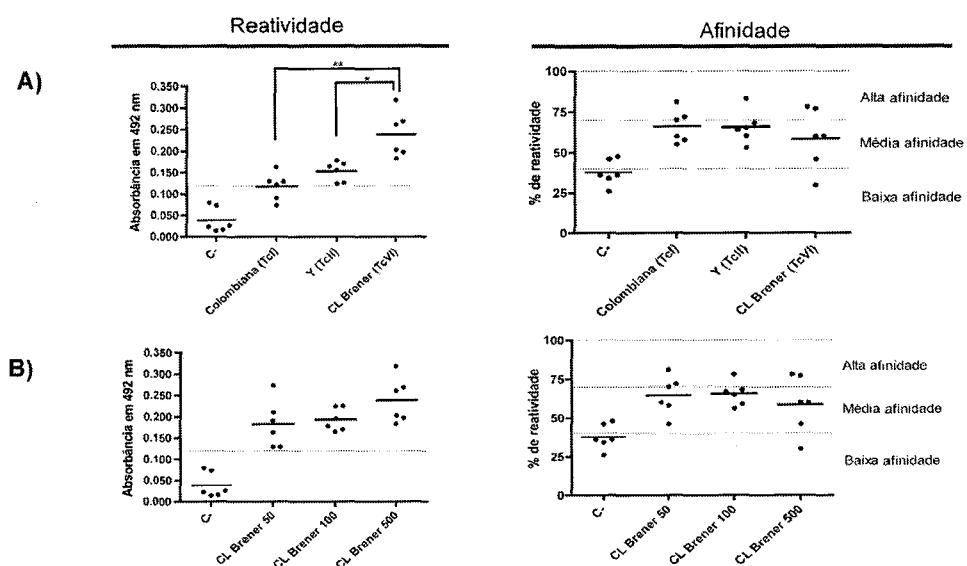
**FIGURA 4**

**FIGURA 5****FIGURA 6**

**FIGURA 7****FIGURA 8**

**FIGURA 9****FIGURA 10**

**FIGURA 11****FIGURA 12**

**FIGURA 13****FIGURA 14**

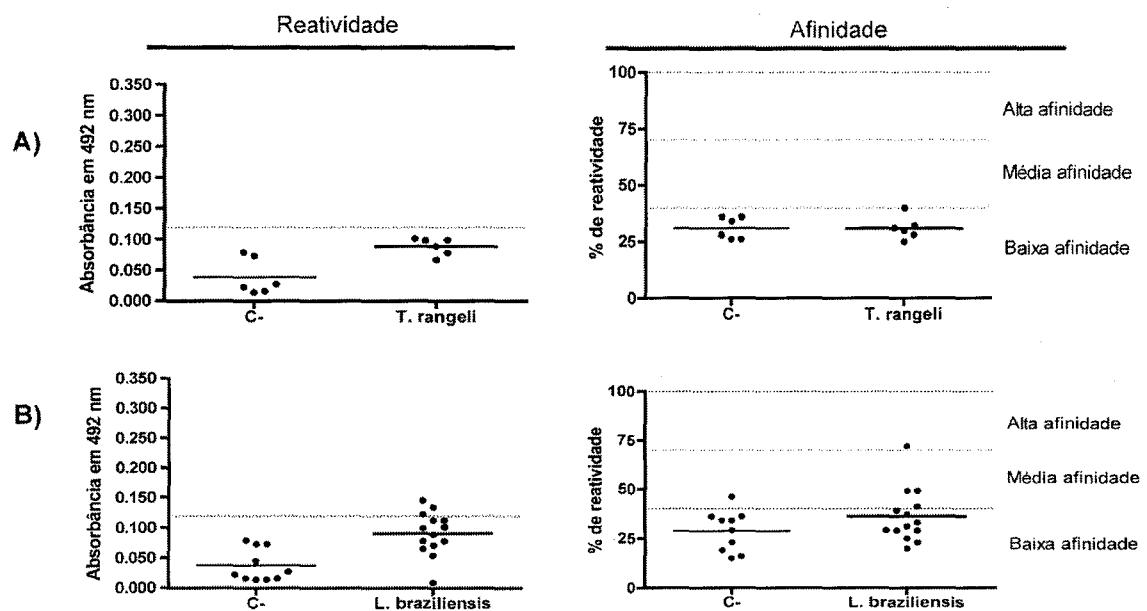


FIGURA 15

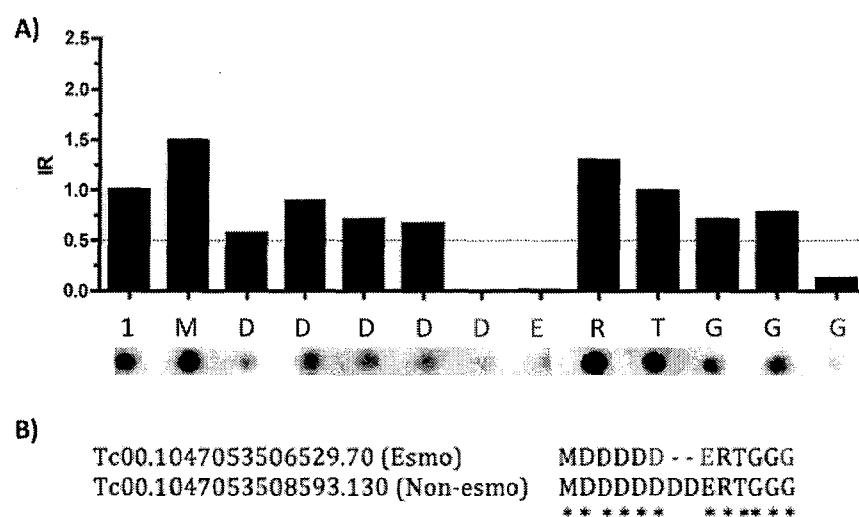


FIGURA 16

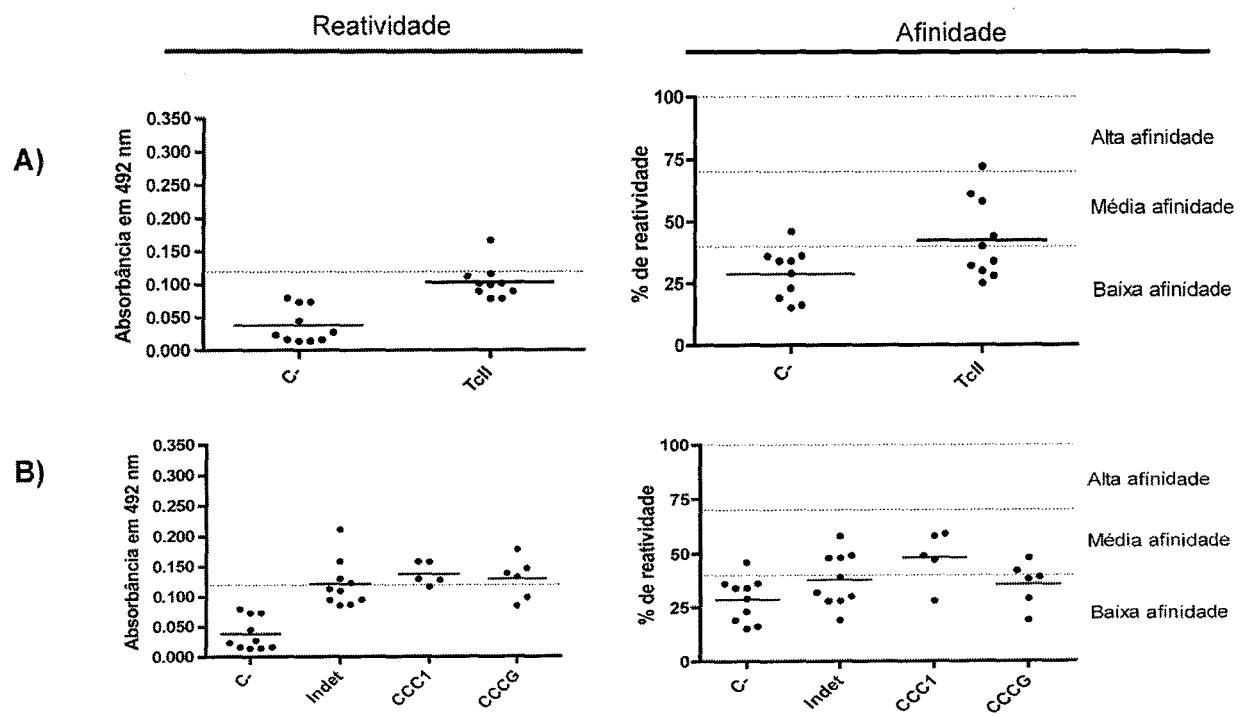
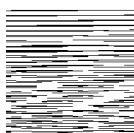


FIGURA 17

## RESUMO

### MÉTODO E KIT PARA TESTE IMUNODIAGNÓSTICO DE DOENÇA DE CHAGAS

A presente invenção descreve método e Kit para um imunodiagnóstico diferenciado da doença de chagas. Mais particularmente, a invenção trata do uso de quatro peptídeos derivados de epitopos de célula B, na identificação de diferentes cepas do parasito *Trypanossoma cruzi* em pacientes chagásicos, visando o desenvolvimento de um método diagnóstico com maior especificidade e sensibilidade e permitindo a sorotipagem do parasito, este último método ainda não disponível para a doença de Chagas.

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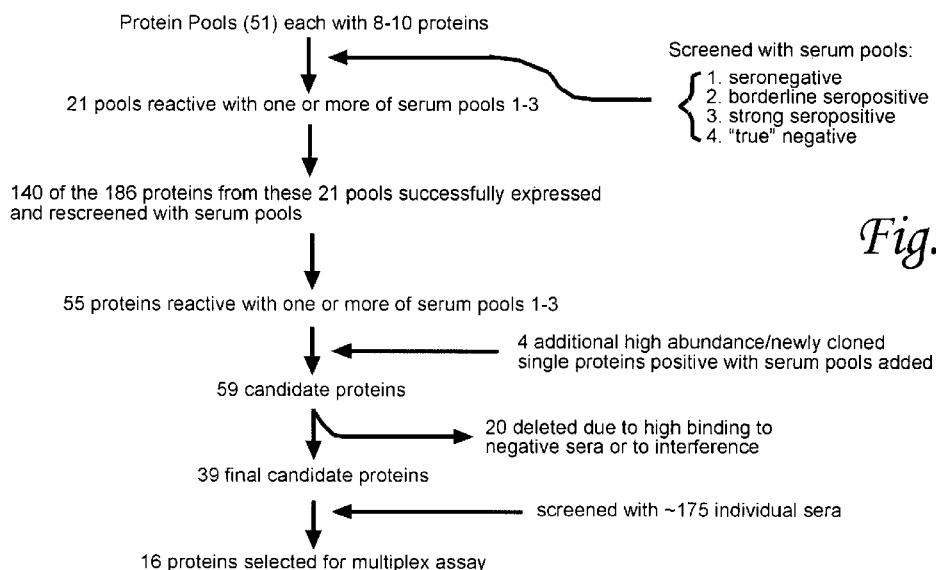
**Selection of Diagnostic Proteins**

Fig. 7

(57) Abstract: A sensitive, multicomponent diagnostic test for infection with *T. cruzi*, the causative agent of Chagas disease, including methods of making and methods of use. Also provided is a method for screening *T. cruzi* polypeptides to identify antigenic polypeptides for inclusion as components of the diagnostic test, as well as compositions containing antigenic *T. cruzi* polypeptides.

WO 2009/017736 A1

DIAGNOSTIC ASSAY FOR *TRYPANOSOMA CRUZI* INFECTION

5

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/962,498, filed July 30, 2007, and is a continuation-in-part application of U.S. Serial No. 11/587,283, filed October 23, 2006, which is a 10 U.S. National Stage Application of international application PCT/US2005/013777, filed April 22, 2005, which claims the benefit of U.S. Provisional Application Serial No. 60/564,804, filed 23 April 2004, and U.S. Provisional Application Serial No. 60/623,299, filed 29 October 2004, each of which is incorporated herein by reference in its entirety.

15

## STATEMENT OF GOVERNMENT RIGHTS

This invention was made with government support under Program Project P01 AI0449790 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

20

## BACKGROUND OF THE INVENTION

*Trypanosoma cruzi* is an obligate intracellular protozoan parasite. In mammalian hosts *T. cruzi* cycles between a trypomastigote stage which circulates in the blood and the amastigote stage which replicates in the 25 cytoplasm of infected host cells (primarily muscle).

*T. cruzi* is the etiological agent of Chagas disease and is ranked as the most serious parasitic disease in the Americas, with an economic impact far outranking the combined effects of other parasitic diseases such as malaria, schistosomiasis, and leishmania (Dias et al., Mem. Inst. Oswaldo Cruz , 1999, 30 94:Suppl.1:103). Chagas Disease affects up to 20 million individuals primarily in the Americas where the insect vectors are present and where zoonotic transmission cycles guarantee a steady source of parasites. *T. cruzi* infection has its greatest human impact in areas of Latin America where housing conditions bring people, infected animals, and vector insects into close

proximity. More than 90 million are at risk of infection in endemic areas, and roughly 50,000 children and adults die of chronic Chagas disease every year due to lack of effective treatments. Additionally, 2-5% of fetus carried by infected mothers in endemic areas are either aborted or born with congenital 5 Chagas disease. Loss of revenue in terms of productivity lost due to sickness and medical costs have an overwhelming effect on economic growth of these countries.

Recently, increasing travel and immigration have brought *T. cruzi* infection into the spotlight globally, even in areas where transmission has 10 previously been absent or very low. *T. cruzi* has spread beyond the borders of Latin America and has been detected in Europe, Asia, and the United States (Ferreira et al., J. Clin. Micro., 2001, 39:4390). In the U.S., 50-100 thousand serologically positive persons progressing to the chronic phase of Chagas disease are present, and the number of infected immigrants in developed 15 countries is increasing. It is expected that, due to the exponential increase in emigration from Latin America, Chagas disease may become a serious health issue in North America and Europe in the next decade.

Congenital and transfusion/transplantation-related transmissions are thus becoming increasingly recognized as significant threats. As the number of 20 infected individuals grows, transmission of *T. cruzi* to non-infected individuals through blood transfusion and organ transplants from the infected immigrant donors is emerging as a route for *T. cruzi* transmission in more developed nations (Umezawa et. al. J. Clin. Micro., 1999, 37:1554; Silveira et. al. Trends Parasitol., 2001, 17; Chagas disease after organ transplantation--United States, 25 2001; MMWR Morb Mortal Wkly Rep. 2002 Mar 15;51(10):210-2). Each year, 15 million units of blood are transfused and approximately 23,000 organ transplants are performed in the United States alone, and presently almost none of the blood supply is tested for *T. cruzi*. A few cases of infection by *T. cruzi* through organ donation have already been reported to United States Centers for 30 Disease Control since 2001. It has therefore become apparent that the screening of blood and organ donors is necessary not only in Latin America but also in developed countries that receive immigrants from endemic areas.

Diagnosis of *T. cruzi* infection is challenging for a number of reasons. The initial infection is seldom detected except in cases where infective doses are high and acute symptoms very severe, as in localized outbreaks resulting from oral transmissions. Classical signs of inflammation at proposed sites of parasite entry (e.g. "Romahia's sign") or clinical symptoms other than fever, are infrequently reported. As a result, diagnosis is very rarely sought early in the infection, when direct detection of parasites may be possible. In the vast majority of human cases, *T. cruzi* infection evolves undiagnosed into a well-controlled chronic infection wherein circulating parasites or their products are difficult to detect even with the use of amplification techniques. A "conclusive" diagnosis of *T. cruzi* infection is often reached only after multiple serological tests and in combination with epidemiological data and (occasionally) clinical symptoms. Further complicating matters, some researchers have reported positive PCR and clinical disease in patients with negative serology. Salomone et al. *Emerg Infect Dis.* 2003 Dec;9(12):1558-62.

Unfortunately, multiple studies from geographically distinct areas and utilizing a wide range of tests and test formats have shown current diagnostics to be far from dependable (Pirard et al., 2005, *Transfusion* 45: 554-561; Salomone et al., 2003, *Emerg Infect Dis* 9: 1558-1562; Avila et al., 1993, *J Clin Microbiol* 31: 2421-2426; Castro et al., 2002, *Parasitol Res* 88: 894-900; Caballero et al., 2007, *Clin Vaccine Immunol.* 14:1045-1049; Silveira-Lacerda et al., 2004, *Vox Sang* 87: 204-207; Wincker et al., 1994, *Am J Trop Med Hyg* 51: 771-777; Gutierrez et al., 2004, *Parasitology* 129: 439-444; Marcon et al., 2002, *Diagn Microbiol Infect Dis* 43: 39-43; Picka et al., 2007, *Braz J Infect Dis* 11: 226-233; Zarate-Blades et al., 2007, *Diagn Microbiol Infect Dis* 57: 229-232). Many of the most widely employed serological tests, including one recently licensed by the United States Food and Drug Administration for use as a blood screening test in the U.S. (Tobler et al., 2007, *Transfusion* 47: 90-96), use crude or semi-purified parasite preparations, often derived from parasite stages present in insects but not in infected humans. The most widely accepted serological tests for *T. cruzi* infection utilize antigens from either whole to semi-purified parasite lysates from epimastigotes that react with anti-*T. cruzi* IgG antibodies. These tests show a degree of variability due to a lack of

standardization of procedures and reagents between laboratories, and a number of inconclusive and false positive results occur due to cross-reactivity with antibodies developed against other parasites (Nakazawa et. al. Clin. Diag. Lab. Immunol., 2001, 8:1024).

5        Other tests have incorporated more defined parasite components, including multiple fusion proteins containing epitopes from various parasite proteins, which, individually have shown some promise as diagnostics (Caballero et al., 2007, Clin Vaccine Immunol. 14:1045-1049; da Silveira JF et al., 2001, Trends Parasitol 17: 286-291; Chang et al., 2006, Transfusion 46: 10 1737-1744). Unfortunately, in the absence of a true gold standard, the sensitivity of new tests is generally determined using sera that have been shown to be unequivocally positive on multiple other serologic tests, but rarely with sera that are borderline or equivocal on one or more tests, an approach that assures only that the test being evaluated is no worse, but not necessarily any 15 more sensitive, than the existing tests.

#### SUMMARY OF THE INVENTION

The present invention provides new tools for diagnosing and treating *T. cruzi* infections in people and animals. In one aspect, the invention provides a 20 method of screening for antigenic *T. cruzi* polypeptides. First and second substrates are provided that each include a plurality of individually addressable candidate antigens derived from *T. cruzi*. The antigens present on the first and second substrate are substantially the same in order to facilitate comparison. The candidate antigens of the first substrate are contacted with a body fluid of a 25 first mammal known to be positive for *T. cruzi* infection. The candidate antigens of the second substrate are contacted with a body fluid from a second mammal known or reasonably believed to be unexposed to *T. cruzi* infection. At least one antigenic *T. cruzi* polypeptide is then identified using a process in which the antigenic *T. cruzi* polypeptide binds to an antibody present in the 30 body fluid of the first mammal but exhibits little or no binding to an antibody present in the body fluid of the second mammal. Optionally, the first and second mammals may be humans.

Positive evidence of *T. cruzi* infection in the first mammal may, for example, be based on a detection method such as a T cell assay, polymerase chain reaction (PCR), hemoculture or a xenodiagnostic technique. Evidence of negative serology in the second mammal is preferably shown by a negative 5 result when the mammal is tested for *T. cruzi* infection utilizing a conventional serodiagnostic test that relies on antigens from whole or semi-purified parasite lysates from *T. cruzi*, such as, for example, from a *T. cruzi* epimastigote lysate.

More than two substrates that include a plurality of individually addressable candidate antigens may be used. Each substrate is contacted with 10 the body fluid from a mammal which exhibits a different level of serological reaction to *T. cruzi* using a conventional serodiagnostic test that relies on antigens from whole or semi-purified parasite lysates from *T. cruzi*. The method optionally further includes the step of preparing the polypeptide antigens from an expression vector including a nucleotide sequence from *T. 15 cruzi*.

Optionally, the screening method may further include a preliminary screening step. The preliminary screening step includes providing a first and a second substrate comprising a plurality of individually addressable antigen pools derived from *T. cruzi* in which the antigen pools present on the first and 20 second substrate are substantially the same. The first substrate is contacted with a body fluid of a first mammal known to be positive for *T. cruzi* infection and the second substrate is contacted with a body fluid from a second mammal known or reasonably believed to be unexposed to *T. cruzi* infection. An antigen pool is then identified that binds to an antibody present in the body fluid of the 25 first mammal but exhibits little or no binding to an antibody present in the body fluid of the second mammal.

In another aspect, the present invention provides an article that includes a substrate and a plurality of individually addressable antigenic *T. cruzi* polypeptides. The antigenic polypeptides can be selected from the polypeptides 30 identified in Table 1, 2 and/or 4, and include antigenic analogs or subunits thereof. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a

polypeptide that is not listed in Table 1. The polypeptides are immobilized onto a surface of the substrate. Optionally, the article may include at least one antigenic *T. cruzi* polypeptide identified according to the screening method described above, or antigenic analogs or subunits thereof, immobilized onto the 5 surface of the substrate. In embodiment, the polypeptides are immobilized on the substrate surface to form a microarray. In another embodiment, the substrate includes at least one nanoparticle, with the polypeptides being immobilized on the surface of the nanoparticle.

The present invention also provides a kit for diagnosis of *T. cruzi* 10 infection that includes an article that includes a substrate and a plurality of individually addressable antigenic *T. cruzi* polypeptides selected from the polypeptides identified in Table 1, 2 and/or 4, in which the polypeptides are immobilized onto a surface of the substrate. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or 15 Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. The kit also includes packaging materials and instructions for use. Optionally, the kit may include at least one antigenic *T. cruzi* polypeptide identified by the screening 20 method described above and immobilized onto the surface of the substrate. The kit may be formulated for medical or veterinary use.

The present invention also provides a diagnostic method for obtaining information about a known or suspected *T. cruzi* infection in a mammal, or for determining whether a mammal is or has been infected by *T. cruzi*. Execution 25 of the method involves obtaining a biological sample from the mammal, contacting the biological sample with a plurality of individually addressable antigenic *T. cruzi* polypeptides selected from the polypeptides identified in Table 1, 2, and/or 4, or antigenic analogs or subunits thereof, and evaluating the presence, absence, intensity or pattern of interaction of components of the 30 biological sample with the antigenic *T. cruzi* polypeptides. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. Optionally, an antigenic *T. cruzi* polypeptide identified

according to the screening method described herein, or antigenic analogs or subunits thereof, can be included in the plurality of antigenic *T. cruzi* polypeptides. In a preferred embodiment, the biological sample is contacted with an article that includes a substrate and a plurality of individually addressable antigenic *T. cruzi* polypeptides immobilized onto a surface of the substrate. Information that can be obtained according to the method includes, for example, the presence or absence of *T. cruzi* infection, the identity of the infective strain, the length of the infection, the stage of the infection, whether the infection is still present or the mammal has been cured, the vaccination status of the mammal, the success of treatment, or any combination thereof.

The method can, for example, be a serodiagnostic method, wherein the biological sample component that interacts with an antigenic *T. cruzi* polypeptide is an antibody from the mammal. Alternatively, the method may be embodied by a cellular assay method where the biological sample component that interacts with an antigenic *T. cruzi* polypeptide is T cell from the mammal.

Like all diagnostic methods described herein, the method can be implemented as a multiplexed assay in which the biological sample is contacted simultaneously with the plurality of antigenic *T. cruzi* polypeptides. The biological sample can, for example, be obtained from a person suspected of having or being exposed to disease, or obtained from an actual or potential blood donor or transplant donor. Alternatively, the biological sample is obtained from a pooled blood product supply intended for use in transfusions or research.

Also provided by the invention is a method for detecting a *T. cruzi* infection, particularly a maternally transmitted *T. cruzi* infection, in an infant born to a mother who is known to have, or suspected of having, a *T. cruzi* infection. A biological sample is obtained from the infant and contacted with a plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof. The biological sample is preferably a bodily fluid, more preferably blood, plasma or serum. The timing for obtaining the sample from the infant is important, as enough time should have elapsed after the birth such that antibodies produced by the infant can be detected. The sample can be obtained from the infant at about two or three months after birth

but is preferably obtained about 4, 5 or 6 months after birth, or later. Preferably, at least one polypeptide is selected from the polypeptides listed in Table 1, Table 2 and/or Table 4. The presence, absence, intensity or pattern of interaction of components of the biological sample, particularly antibodies, with 5 the antigenic *T. cruzi* polypeptides is evaluated to determine whether the infant exhibits an antibody response that exceeds background levels.

The method optionally further includes comparing the infant's antibody response to the plurality of antigenic *T. cruzi* polypeptides with the antibody response of the infant's mother to the same or similar panel of *T. cruzi* 10 polypeptides. Comparison with the mother's antibody response is especially useful when the infant's antibody response is higher than background level. A biological sample is obtained from the infant's mother and contacted with the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof. The biological sample of the mother can 15 be obtained prior to birth, during birth, or after birth. The presence, absence, intensity or pattern of interaction of components of the mother's biological sample with the antigenic *T. cruzi* polypeptides is compared to the presence, absence, intensity or pattern of interaction of components of the infant's biological sample with the antigenic *T. cruzi* polypeptides, to determine whether 20 the infant's antibody response differs from the mother's antibody response. A difference in antibody responses, where the infant's response is above background levels, indicates that the infant may have a *T. cruzi* infection. Similar antibody responses for mother and infant indicate that maternal 25 antibodies may still be present in the infant's bodily fluids. In that event, the comparison is optionally repeated using a biological sample can be obtained from the infant at a later date.

Additionally or alternatively, the method further optionally includes comparing the infant's antibody response to the plurality of antigenic *T. cruzi* polypeptides with the infant's antibody response to the same or similar panel of 30 *T. cruzi* polypeptides as measured earlier, i.e., shortly after birth. Shortly after birth, the infant's antibody response is expected to mirror the mother's antibody response, reflecting the presence of maternal antibodies in the infant's bodily fluids. A biological sample is obtained from the infant shortly after birth,

contacted with the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof, and the presence, absence, intensity or pattern of interaction of components of the infant's earlier biological sample with the antigenic *T. cruzi* polypeptides is compared to the  
5 presence, absence, intensity or pattern of interaction of components of the infant's later biological sample, or of the mother's biological sample, or both, with the antigenic *T. cruzi* polypeptides, to determine whether the infant's later antibody response differs from the mother's antibody response, wherein a difference in antibody responses indicates that the infant may have a *T. cruzi*  
10 infection.

In instances wherein the method identifies an infant having or suspected of having a *T. cruzi* infection, the method further optionally includes treating the infant for a *T. cruzi* infection, for example by administering a therapeutic agent to the infant.

15 In another aspect, the present invention provides a method for detecting contamination of a blood product supply with *T. cruzi*. The method of detecting contamination includes selecting a sample from the blood supply, contacting the sample with a plurality of individually addressable antigenic *T. cruzi* polypeptides selected from the polypeptides identified in Table 1, 2 and/or 4, or  
20 antigenic analogs or subunits thereof, and evaluating the presence, absence, intensity or pattern of interaction of components of the sample with the antigenic *T. cruzi* polypeptides to determine whether the blood supply is contaminated with *T. cruzi*. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4,  
25 with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. Optionally, an antigenic *T. cruzi* polypeptide identified according to the screening method described herein, or antigenic analogs or subunits thereof, can be included in the plurality of antigenic *T. cruzi* polypeptides. In a preferred embodiment, the  
30 blood supply sample is contacted with an article that includes a substrate and a plurality of individually addressable antigenic *T. cruzi* polypeptides immobilized onto a surface of the substrate.

Blood products that can be tested include whole blood, a blood product, or a blood fraction. For example, a cellular blood component, a liquid blood component, a blood protein, or mixtures thereof, or a red blood cell concentrate, a leukocyte concentrate, a platelet concentrate, plasma, serum, a clotting factor, 5 an enzymes, albumin, plasminogen, or a immunoglobulin, or mixtures of thereof, can be tested for contamination according to the method.

The method of detecting contamination can be a serodiagnostic method, wherein the sample component that interacts with an antigenic *T. cruzi* polypeptide is an antibody. Alternatively, the method can take the form of a 10 cellular assay method, wherein the sample component that interacts with an antigenic *T. cruzi* polypeptide is T cell.

In yet another aspect, the present invention provides a multicomponent vaccine. In one embodiment, the vaccine includes a plurality of immunogenic *T. cruzi* polypeptides selected from the *T. cruzi* polypeptides listed in Table 1, 2, 15 and/or 4, or immunogenic subunits or analogs thereof. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. The multicomponent polypeptide vaccine optionally includes at least one 20 immunogenic *T. cruzi* polypeptide identified according to the screening method described herein, or immunogenic subunit or analog thereof. In another embodiment, the multicomponent vaccine includes one or more polynucleotides operably encoding a plurality of immunogenic *T. cruzi* polypeptides selected from the *T. cruzi* polypeptides listed in Table 1, 2, and/or 4 or immunogenic 25 subunits or analogs thereof. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. The multicomponent polynucleotide vaccine optionally includes a polynucleotide 30 operably encoding a polypeptide identified according to the screening method, or immunogenic subunit or analog thereof. The multicomponent vaccine may be a therapeutic or prophylactic vaccine.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a pictoral overview of the bio-plex array analysis method; A) shows the protein-antibody-microsphere complex used by the bio-plex method, B) shows multiple complexes in the well of a microplate substrate, 5 and C) shows laser excitation of the complexes as they flow through a flow cytometer.

Figure 2 provides a pictoral overview of the Gateway<sup>®</sup> cloning method used to provide an expression vector used for the preparation of *T. cruzi* polypeptide antigens in one embodiment of the invention.

10 Figure 3 shows assay development using varicella voster (VV)-ovalbumin sera; A, BioPlex assay; B, ELISA assay.

Figure 4 shows testing of protein pools for antigenic potential using A, negative control sera; B, very low positive sera; C, borderline positive sera; and D, strong positive sera. From left to right, in each panel at each of the sera 15 dilutions, the tested samples are: lysate control, ovalbumin, pool 1C, pool 2A, pool 2I, pool 3A, pool 3K and pool 6. Pool 3K reacted with antibodies from infected individuals and was a candidate for further testing.

Figure 5 shows testing of the component proteins of pool 3K for 20 antigenic potential using A, negative control sera; B, very low positive sera; C, borderline positive sera; and D, strong positive sera. From left to right, in each panel at each of the sera dilutions, the tested samples are: lysate control, ovalbumin, protein 3K-1, 3K-2, 3K-3, 3K-4, 3K-5 and 3K-6, and pool 3K. Proteins 3K-1, 3K-2, 3K-3 and 3K-5 demonstrated varying degrees of reactivity 25 to antibodies in sera from infected individuals.

Figure 6 shows testing of four different serum samples using a panel of serodiagnostic proteins; A, strong seropositive serum; B, Subject 58: T cell reactive/seronegative serum; C, Subject 44: T cell non-reactive/seronegative serum; D, Subject 60: T cell non-reactive/seronegative. From left to right, in 30 each panel at each of the sera dilutions, the test proteins are: lysate control, ovalbumin, protein 3K-1, 3K-3, 1A-1 and 4A-3, and pool 3K. Subject 58, declared seronegative by standard serological assay but exhibiting T cell reactivity to *T. cruzi* antigens, is of particular interest because antibodies are

detected that recognize the recombinant *T. cruzi* antigens but not the parasite lysate.

Figure 7 is a schematic showing a screening process for the high-throughput selection of diagnostic proteins for detection of *T. cruzi* infection.

5       Figure 8 shows an SDS-PAGE gel of production of pooled protein. Sets of 6-8 genes were moved in pools from pDONR entry plasmids into pDEST-PTD4 via a Gateway LR reaction (Invitrogen) and the resulting plasmids transformed into BL21(DE3)pLysS cells for protein production. Recombinant HIS-tagged proteins were purified on Co+2 affinity resin and the bound proteins  
10 analyzed by SDS-PAGE. Protein pools depicted in lanes 1-5 were generated from the pooling of 8 genes, while lanes 6, 7 and 8 were derived from 7, 6, and 6 genes respectively. Lane "S" contained molecular weight standards (BenchMark Prestained Protein Standard; Invitrogen). Overall, approximately 80% of genes yielded proteins when expressed as pools.

15       Figure 9 shows reactivity of a representative set of proteins tested with individual sera. A selection of 29 individual recombinant proteins was tested for the ability to bind IgGs present in the sera of 54 subjects. The sera are grouped as "uniformly positive" (reactive on all three conventional serological tests and a commercial assay kit), "inconclusive" (negative on at least one  
20 conventional serologic tests), "negative by conventional tests" (negative by all three conventional tests), and "known negative" (from residents of North America with very low chance of being infected based upon residency and travel history). Recombinant ovalbumin and *T. cruzi* lysate -coated beads were used as negative and positive controls, respectively. Horizontal bars in each box  
25 indicate mean fluorescence intensity (MFI) on a scale from 0 to 30,000 arbitrary light units. A number of the recombinant proteins either failed to discriminate between uniformly positive and known negative sera sets (e.g. 3, 16, 17, 22, 26, 27, 28) or showed no reaction with either set (e.g. 10). In contrast a number of proteins detected nearly the entire uniformly positive group, as well as some in  
30 the inconclusive and conventional negative groups but none in the known negative set (e.g. 4, 11, 19).

Figure 10 shows stability of serological responses over time. The MFI of sera to a panel of 16 recombinant proteins (top 16 in Table 2), GFP negative

control protein and *T. cruzi* lysate for a total of 18 measurements (bars) are shown for each serum. A) Reactivity of a set of 8 known negative sera. B) Stability of unique pattern of antigen activity for 6 seropositive subjects assayed at 4 time points over 12-21 months. Arrows in lower right panel (RD 07) 5 indicate that detection of protein “8” (paraflagellar rod protein) which distinguishes the pattern of reactivity of serum RD07 from that of the similar RD09.

Figure 11 shows the effect of benznidazole treatment on serological responses in chronically infected subjects. The MFI of sera to a panel of 16 10 recombinant proteins (top 16 in Table 2), GFP negative control protein and *T. cruzi* lysate for a total of 18 measurements (bars) are shown for each serum. A) Change in pattern of reactivity in 4 subjects over 36 months post-benznidazole treatment, measured using both the multiplex serologic assay (left) and conventional serology (right). B) Benznidazole-treated subjects exhibiting no 15 evidence of change in multiplex assay for 24 months post-treatment (left; PP044) and or only changes in reactivity to selected recombinant proteins (right; PP024).

Figure 12 shows the pattern of antigen-specific antibody responses in chronic Chagas disease over time (A, 0 months; B, 12 months) and among 20 different individuals.

Figure 13 shows the effects of benznidazole treatment over time on antibody titers to recombinant proteins; A, untreated; B, treated.

Figure 14 shows the effect of treatment with benznidazole on *T. cruzi*-specific memory T cell responses in chronic Chagas disease subjects. The 25 frequency of IFN-(gamma)-producing T cells specific for *T. cruzi* significantly decreased in the benznidazole-treated patient group vs non-treated, at 12 months after follow-up. ELISPOT responses became negative in 11/25 (44% ), 6 patients at 12 months post-treatment and 5 patients at 24 months post treatment.

Figure 15 shows the antibody profiles of four mothers (A, B, C, D) with 30 chronic Chagas disease and their infants at a time point relatively soon after birth, and again later when the infant is 6-7 months old.

## DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

In one aspect, the present invention is directed to the detection of *T. cruzi* infection in a mammal, particularly a human. A plurality of *T. cruzi* polypeptides, or subunits or analogs thereof, that are detectable by antibodies present in a bodily fluid, such as blood, plasma or serum, of at least some individuals that are infected with *T. cruzi* are included in a multicomponent panel for use in a diagnostic assay, which may be a serodiagnostic assay or a cellular assay. The term "serodiagnostic" is used because the assay is typically performed on a blood component such as whole blood, plasma or serum, but it should be understood that any bodily fluid that may provide evidence of an immune response to *T. cruzi* can be assayed using the serodiagnostic test of the invention.

The panel components are contacted with a bodily fluid of an individual such as blood, plasma, serum, urine, saliva or tears and the like, and the presence of absence of evidence of an immune response to *T. cruzi* in the individual is evaluated. The body fluid that is tested can be that of an individual patient to be screened, or it can be a body fluid that is part of a blood or plasma supply, for example, pooled or unpooled, that is available for transfusion and/or research.

An immune response indicative of *T. cruzi* infection may be evidenced by the binding of antibodies in the biological fluid to panel components. The panel components can likewise be used to assess the presence of a T cell response in the subject.

In a preferred embodiment, the diagnostic test is highly specific for *T. cruzi* infection and sufficiently sensitive to detect infection in subjects considered negative with conventional serological assays based on *T. cruzi* lysates due to a poor or inconsistent B cell response to infection. Optionally the test can include, as specificity controls, polypeptide antigens that are recognized when other infections are present.

The diagnostic test can detect the presence or absence of *T. cruzi* infection. In some embodiments, and depending on the antigenic polypeptides selected for including in the multicomponent panel, the pattern of antigen recognition may provide additional information such as the stage of infection or

the severity of disease. The antigen recognition pattern may also be useful to discriminate among patients with active or latent infections, and those who have been cured or vaccinated.

The multicomponent diagnostic assay (also referred to herein as a multiplexed assay) has advantages over conventional serodiagnostic methods. For example, the multiplexed assay of the invention consistently detects infection, whereas conventional assays are plagued by high failure rates and inconsistent performance. The Examples below show that infected subjects produce individual patterns of antibody responses that differ from one another, rendering serodiagnostics based on a single antigen a less effective diagnostic than a multiplexed assay. There are numerous examples of the failure of conventional serology to detect infection, and parasitological tests are also unreliable. Individuals who are seropositive in the multiplex assay of the invention are likely to be infected with *T. cruzi*, particularly if they exhibit antibodies to at least 4, more preferably 6, and even more preferably 8 different recombinant *T. cruzi* proteins, and/or were born in endemic areas and/or have evidence of heart disease. Such individuals are likely to be infected with *T. cruzi* even if they exhibit negative results with conventional serologic assays.

Additionally, the multiplexed serodiagnostic assay of the invention provides a better measure of the efficacy and effectiveness of therapeutic treatment than conventional serological or parasitological assays. Most subjects are negative by parasitological assays prior to treatment and remain positive by conventional serology for long periods of time after treatment. However a multiplexed assay using a selected set of recombinant proteins as described herein can detect changes in antibody levels upon completion of treatment. The use of multiple targets allows serologic changes to be detected following treatment when similar changes are not consistently observed using conventional serologic tests.

Also, the *T. cruzi* proteins identified herein for use in the multiplexed assays of the invention (both serodiagnostic and cellular) are expected to be effective diagnostics for different *T. cruzi* strains in different regions, since many of them are unique to *T. cruzi* and/or highly abundant proteins, such as

"housekeeping" proteins, that are expected to show little variation among the different strains.

The invention is applicable to human disease but also has veterinary applications. For example, a diagnostic assay developed according to the 5 invention can be used to diagnose *T. cruzi* infection in farm animals or pets, such as dogs.

#### *Antigenic polypeptides*

A *T. cruzi* polypeptide, or subunit or analog thereof, that is suitable for 10 inclusion in the panel is one that reacts to antibodies in the sera of individuals infected with *T. cruzi*. Such a polypeptide is referred to herein as an antigenic polypeptide or a polypeptide antigen.

A preferred antigenic polypeptide, or antigenic subunit or analog thereof, is one that detectably binds antibodies in a bodily fluid of a subject who 15 is known to be infected or to have been infected by *T. cruzi*, but whose bodily fluid is seronegative when assayed by conventional means. A bodily fluid that is seronegative when assayed by conventional means is one that, for example, does not show a positive reaction (antibody binding) when exposed to antigens from either whole or semi-purified parasite lysates, for example those from 20 epimastigotes, in conventional diagnostic tests. A subject who shows evidence of *T. cruzi* infection using, for example, a T cell assay, polymerase chain reaction (PCR), hemoculture, or xenodiagonistic techniques, is considered to known to be infected or to have been infected by *T. cruzi*, even if the subject 25 shows a negative response to a conventional serodiagnostic test.

Another preferred polypeptide, or subunit or analog thereof, is one that 30 detectably binds antibodies in a bodily fluid of a subject who is seropositive when assayed by conventional means, regardless of whether the polypeptide also exhibits detectable binding to antibodies in a bodily fluid of a subject who is known to be infected or to have been infected by *T. cruzi*, but whose bodily fluid is seronegative when assayed by conventional means.

The antigenic *T. cruzi* polypeptides, and antigenic subunits and analogs thereof, bind antibodies in a bodily fluid of a subject, such as blood, plasma or sera, thereby providing evidence of exposure to *T. cruzi*. These antigenic

polypeptides, and antigenic subunits and analogs thereof, may also be immunogenic; i.e., they may also, when delivered to a subject in an appropriate manner, cause an immune response (either humoral or cellular or both) in the subject. Immunogenic *T. cruzi* polypeptides, as well as immunogenic subunits and analogs thereof, are therefore expected to be useful in vaccines, as described below.

It should be understood that the term “polypeptide” as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, and protein are included within the definition of polypeptide.

An antigenic *T. cruzi* polypeptide according to the invention is not limited to a naturally occurring antigenic *T. cruzi* polypeptide; it can include an antigenic subunit or antigenic analog of a *T. cruzi* polypeptide. Likewise the antigenic polypeptide can be a multivalent construct that includes epitopes obtained from different antigenic polypeptides of *T. cruzi*. An antigenic analog of an antigenic *T. cruzi* polypeptide is a polypeptide having one or more amino acid substitutions, insertions, or deletions relative to an antigenic *T. cruzi* polypeptide, such that antigenicity is not entirely eliminated. Substitutes for an amino acid are preferably conservative and are selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH<sub>2</sub>. Antigenic subunits of an antigenic *T. cruzi* polypeptide are antigenic *T. cruzi* polypeptides that are truncated at either or both of the N-terminus or C-terminus, without eliminating their ability to detect serum antibodies against *T. cruzi*. Preferably, an antigenic subunit contains an epitope recognized by a host B cell or T cell. Fragments of an antigenic *T. cruzi* protein

contain at least about eight amino acids, preferably at least about 12 amino acids, more preferably at least about 20 amino acids.

Examples of antigenic *T. cruzi* polypeptides suitable for inclusion in the multicomponent panel of the invention are listed in Tables 1, 2 and 4 in the Examples, below. The "Gene ID Numbers" represent gene numbers assigned by annotators of the *T. cruzi* genome and are accessed via the *T. cruzi* genome database on the worldwide web at "TcruziDB.org".

Furthermore, as described below, the present invention also includes a method for identifying additional antigenic polypeptides indicative of *T. cruzi* infection. The use of the additional *T. cruzi* polypeptides thus identified, or antigenic subunit or analog thereof, alone or in combination with each other, with the antigenic *T. cruzi* polypeptides of Table 1, 2 and 4, and/or with other known antigens, in diagnostic and therapeutic applications relating to *T. cruzi* infection as described is also envisioned. It should be understood that the antigenic *T. cruzi* polypeptides described herein or identified using the screening method described herein are generally useful in any of diagnostic and/or therapeutic applications relating to *T. cruzi* infection.

Antigenic polypeptides used in the multicomponent panel of the invention preferably include polypeptides that are abundant during the two stages (amastigote and trypomastigote) that are prevalent in the life cycle of the parasite in mammals. In a mammalian host, *T. cruzi* cycles between a dividing intracellular stage (the amastigote) and a non-replicative extracellular trypomastigote form which circulates in the blood. The presence of two developmental stages of *T. cruzi* in mammalian hosts provides two anatomically and (to some degree) antigenically distinct targets of immune detection - the trypomastigotes in the bloodstream and the amastigotes in the cytoplasm of infected cells. The intracellular location of amastigotes of *T. cruzi* has long been considered a "hiding place" for the parasite wherein it is not susceptible to immune recognition and control. Notably, most current serological tests for *T. cruzi* are based upon antigens from epimastigotes, the form of *T. cruzi* present in insects but not humans. Thus, in a preferred embodiment, an antigenic polypeptide for use in a *T. cruzi* diagnostic test or vaccine according to the invention can be one that is expressed by *T. cruzi* in the extracellular

(tryomastigote) stage, in the intracellular (amastigote) stage, or during both stages of the life cycle.

*Diagnostic method*

5        The diagnostic of the invention utilizes a multicomponent panel to assess the presence of an immune response (e.g., the presence of antibodies or reactive T cells) in the subject to multiple antigenic *T. cruzi* polypeptides, or antigenic subunits or analogs thereof. The panel may contain a number of antigenic *T. cruzi* polypeptides, or antigenic subunits or analogs thereof, 10 wherein said number is between 5 and 50 or even more, depending on the embodiment and the intended application. For example, the panel may contain 5, 8, 10, 12, 15, 18, 20, 25, 30, 40 or more antigenic *T. cruzi* polypeptides. A typical multicomponent panel may contain 10 to 20 antigenic *T. cruzi* polypeptides. Preferably, some or all of the antigenic *T. cruzi* polypeptides used 15 in the multicomponent panel are selected from those listed in one or more of Tables 1, 2 or 4. Conveniently, the *T. cruzi* polypeptides that are used in the multicomponent diagnostic test can be recombinant polypeptides; however they can be naturally occurring polypeptides or polypeptides that have been chemically or enzymatically synthesized, as well.

20       In one embodiment, the diagnostic test takes the form of a serodiagnostic assay, which detects a humoral (antibody) immune response in the subject. The binding of an antibody that is present in a biological fluid, such as a serum antibody, to any of the various components of the panel is determined. The threshold for a diagnosis of *T. cruzi* infection can be readily 25 determined by the scientist, medical personnel, or clinician, for example based upon the response of known infected and control sera to the particular panel being used. For example, diagnosis criteria can be based on the number of "hits" (i.e., positive binding events) or they can represent a more quantitative determination based, for example, on the intensity of binding and optional subtraction of background. As an illustrative example, the multicomponent panel could contain 15 to 20 antigenic polypeptides, or antigenic analogs or 30 subunits thereof, and a positive diagnosis could be interpreted as, say, 5 or more positive responses. Optionally, the serodiagnostic test could be further refined

to set quantitative cutoffs for positive and negative based upon the background response to each individual panel component. So, for example, the response to each polypeptide could be set to be >2 standard deviations above the response of "pooled normal," sera and an individual would have to have responses to a 5 minimum of 5 out of 20 polypeptides.

The serodiagnostic assay of the invention can take any convenient form. For example, standard immunoassays such as indirect immunofluorescence assays (IFA), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), fluorescent bead technology and Western blots can 10 be employed. Detection can be by way of an enzyme label, radiolabel, chemical label, fluorescent label, chemiluminescent label, a change in spectroscopic or electrical property, and the like.

In another embodiment, the diagnostic method can take the form of a cellular assay. In this embodiment, a multicomponent panel of antigenic *T. cruzi* 15 polypeptides as described herein is used to assess T cell responses in a mammalian subject, thereby providing another method for evaluating the presence or absence (or stage, etc.) of *T. cruzi* infection. Individuals are known who are serologically negative (based upon conventional tests) but who have T cells reactive with parasite antigens (usually a lysate of trypomastigotes and 20 epimastigotes - but in some cases also against specific *T. cruzi* polypeptides). This suggests that T cell responses may be a sensitive way to assess infection, or to determine the stage of infection or exposure.

Recombinant antigenic *T. cruzi* polypeptides can be readily produced, for example, as histidine-tagged polypeptides. These His-tagged polypeptides 25 can be purified onto a nickel-coated substrate, then added to a blood fraction comprising peripheral blood lymphocytes (e.g., a peripheral blood mononuclear cell, PBMC, fraction). The ability of the T cells to make IFN-gamma is then assessed, for example using an ELISPOT assay (e.g., Laucella et al., J Infect Dis. 2004 Mar 1;189(5):909-18). As another example, antigenic *T. cruzi* 30 polypeptides, or antigenic analogs or subunits thereof, can be bound to major histocompatibility complex (MHC) tetramers and presented to T cells, for example in a composition of peripheral blood lymphocytes, in a microarray format. In this assay, smaller polypeptides, for example antigenic peptide

subunits of antigenic *T. cruzi* polypeptides described herein, are preferred as they are more readily bound to the MHC tetramers and recognized by the T cells. Antigenic subunits of antigenic *T. cruzi* polypeptides can be predicted using various computer algorithms, and are amenable to chemical synthesis.

- 5 Binding of T cells to the spots containing MHC-polypeptide complexes indicates recognition and hence *T. cruzi* infection. See, for example, Stone et al (Proc. Nat'l. Acad. Sci. USA, 2005, 102:3744) and Soen et al. (PLoS. Biol, 2003, 1:429) for a description of the general technique.

In a multiplexed assay, multiple analytes are simultaneously measured.

- 10 Each polypeptide antigen is positioned such that it is individually addressable. For example, the polypeptide antigens can be immobilized in a substrate. In a preferred embodiment, the multiplexed serodiagnostic assay of the invention is performed using a bioassay such as the Luminex system (Luminex Corporation, Austin, TX). The Luminex system, which utilizes fluorescently labeled 15 microspheres, allows up to 100 analytes to be simultaneously measured in a single microplate well, using very small sample volumes. However, other multiplex platforms such as protein microarrays can also be used, and the invention is not intended to be limited by the type of multiplex platform selected.

- 20 The panel components can be assembled on any convenient substrate, for example on a microtiter plate, on beads, or in a microarray on a microchip. A microarray format is advantageous because it is inexpensive and easy to read using a standard fluorescence microscope. In this format, one might just use the total number of spots (proteins) positive for each test patient to make a positive 25 or negative diagnosis. In addition, the diagnostic test of the invention is well-suited to adaptation for use with commercially available high-throughput devices and immunoassay protocols, for example those available from Abbott Laboratories and Applied Biosystems, Inc. The serodiagnostic assay can also take the form of an immunochromatographic test, in the form of a test strip 30 loaded with the panel components. The bodily fluid can be wicked up onto the test strip and the binding pattern of antibodies from the fluid can be evaluated.

*Detection of *T. cruzi* infection in infants*

In another aspect, the invention provides method for determining whether an infant has a *T. cruzi* infection. In situations where the infant's mother is infected with *T. cruzi*, the method facilitates early detection of a 5 maternally transmitted infection. A maternally transmitted infection can be transmitted prior to or during birth (a congenital infection), or it may be transmitted after birth, as through breastfeeding.

At birth and for a period shortly thereafter, the antibody response of an infant born to an infected mother mirrors the antibody response of the infant's 10 infected mother, reflecting the presence of maternal antibodies in the baby's fluids. With time, however, if the infant is infected, the infant will begin producing its own antibodies, and the pattern of response will begin to differentiate from that of the mother. Eventually, typically by about six months after birth, the antibody response of the infant will either diminish to near 15 background levels (if the infant is not infected), or will appear distinct from that of the mother, indicating possible infection.

The method for detecting *T. cruzi* infection in an infant, particularly an infant born to a mother with a known or suspected *T. cruzi* infection, therefore includes analyzing at least one biological sample obtained from the infant. 20 Preferably the biological sample is a body fluid such as blood, plasma or serum. The sample is obtained at a time after birth by which the infant's antibody response to the antigenic *T. cruzi* polypeptides, if the infant is infected, is detectably different from the mother's antibody response. Preferably the sample is obtained from the infant at about 6 months of age, but the sample can be 25 obtained earlier, for example at about 5 months, 4 months, 3 months or 2 months. Likewise, the sample can be obtained later since after 6 months the baby is expected to be producing its own antibodies at a detectable level. Analysis is preferably performed using the multiplexed assay of the invention.

An infant that exhibits a background level antibody response to *T. cruzi* 30 antigens in the multiplexed assay is unlikely to be infected with *T. cruzi*. However, an antibody response that exceeds background levels indicates possible infection. Optionally, the method therefore also includes administering a therapeutic agent an infant suspected of having a *T. cruzi* infection.

In a preferred embodiment of the method, the infant's antibody response to the *T. cruzi* antigen panel is first analyzed shortly after birth. When a neonate's antibody response is measured shortly after birth (preferably no later than one month after birth, more preferably no later than two months after birth), the neonate's antibody response will parallel that of its mother, due to the presence of maternal antibodies. Optionally, the mother's antibody response to the antigen panel is thus analyzed. The infant's antibody response at the later time point (when its own antibodies have begun to be produced) is compared to the antibody response of the mother, and/or to its own antibody response at a time shortly after birth. Comparison of the antibody response of the later infant sample with the antibody response of the earlier neonate sample and/or with the antibody response of the mother (preferably using a sample obtained from the mother at about the same time as the sample or samples are obtained from the infant, although the sample from the mother can be obtained at any convenient time as it is expected to be fairly stable) is preferred, as it facilitates the determination as to whether the infant's own antibody response is sufficiently different from the mother's to support the diagnosis of *T. cruzi* infection.

It should be understood that in this method, as in all methods involving the use of the multiplexed assay of the invention, the panel of serodiagnostic targets can include any antigenic *T. cruzi* polypeptide described herein, or subsequently discovered using the screening assay of the invention. Preferably, multiple *T. cruzi* polypeptide antigens are selected from those listed in Tables 1, 2 and/or 4 and used to assess the infant's antibody response in a multiplexed assay.

It should also be noted that the method of detecting *T. cruzi* infection in an infant according to the invention can take the form of either a serodiagnostic method, wherein the sample components that interact with an antigenic *T. cruzi* polypeptides are antibodies, or a cellular assay method, and wherein the sample components that interact with the antigenic *T. cruzi* polypeptides are T cells.

30

#### *Blood supply screening*

The diagnostic test of the invention can be used to detect the presence of *T. cruzi* infection in blood and blood products or fractions include whole blood

as well as such as cellular blood components, including red blood cell concentrates, leukocyte concentrates, and platelet concentrates and extracts; liquid blood components such as plasma and serum; and blood proteins such as clotting factors, enzymes, albumin, plasminogen, and immunoglobulins, or mixtures of cellular, protein and/or liquid blood components. Details regarding the make-up of blood, the usefulness of blood transfusions, cell-types found in blood and proteins found in blood are set forth in U.S. Pat. No. 5,232,844. Techniques regarding blood plasma fractionation are generally well known to those of ordinary skill in the art and an excellent survey of blood fractionation also appears in Kirk-Othmer's Encyclopedia of Chemical Technology, Third Edition, Interscience Publishers, Volume 4.

A sample is contacted with a multicomponent panel of the invention, and a positive or negative response is detected as described above for clinical use of the assay in patients suspected of having *T. cruzi* infection. Advantageously, the diagnostic test is readily automated, for example using microchip technology, for the processing of large numbers of samples.

#### *Prophylactic and therapeutic immunization*

In another aspect, the present invention is directed to both prophylactic and therapeutic immunization against *T. cruzi* infection and the chronic disease state, known as Chagas disease, that often eventually follows initial *T. cruzi* infection. Antigenic *T. cruzi* polypeptides described herein, or identified using a screening method described herein, may be immunogenic. That is, they may elicit a humoral (B cell) response and/or a cell-mediated immune response (i.e., a "T cell" response) in the subject. A cell-mediated response can involve the mobilization helper T cells, cytotoxic T-lymphocytes (CTLs), or both. Preferably, an immunogenic polypeptide elicits one or more of an antibody-mediated response, a CD4<sup>+</sup> Th1-mediated response (Th1: type 1 helper T cell), and a CD8<sup>+</sup> T cell response. Therapeutic administration of the polynucleotide or polypeptide vaccine to infected subjects is expected to be effective to delay or prevent the progression of the *T. cruzi* infection to a chronic disease state, and also to arrest or cure the chronic disease state that follows *T. cruzi* infection. Prophylactic administration of the polynucleotide or polypeptide vaccine to

uninfected subjects is expected to be effective to reduce either or both if the morbidity and mortality associated with infection by *T. cruzi*. Further, if an uninfected, vaccinated subject is subsequently infected with *T. cruzi*, the vaccine is effective to prevent progression of the initial infection to a chronic  
5 disease state. As discussed in more detail hereinbelow, the vaccine can contain or encode a single immunogenic polypeptide or multiple immunogenic polypeptides. Methods for identifying nucleotide sequences encoding such polypeptides from a *T. cruzi* genomic library using, for example, expression library immunization (ELI) or DNA microarray analysis are described below.

10

#### *Advantages of a genetic vaccine*

The choice of polynucleotide delivery as an immunization technique offers several advantages over other vaccine or antigen delivery systems.  
Vaccines containing genetic material are favored over traditional vaccines  
15 because of the ease of construction and production of the vectors, the potential for modification of the sequences by site-directed mutagenesis to enhance the antigenic potency of the individual epitopes or to abolish epitopes that may trigger unwanted response, in the case of DNA vaccines, the stability of DNA, the lack of the dangers associated with live and attenuated vaccines, their ability  
20 to induce both humoral and cell mediated immunity and, in particular, CD8<sup>+</sup> T cell responses, and the persistence of the immune responses. Successful induction of humoral and/or cellular immune responses to plasmid-encoded antigens using various routes of gene delivery have been shown to provide partial or complete protection against numerous infectious agents including  
25 influenza virus, bovine herpes virus I, human hepatitis B virus, human immunodeficiency virus-1, as well as parasitic protozoans like *Plasmodium* and *Leishmania* (Donnelly et al., Ann. Rev. Immunol. 15:617-648, 1997). Representative papers describing the use of DNA vaccines in humans and primates include Endresz et al. (Vaccine 17:50-58, 1999), McCluskie et al.  
30 (Mol. Med. 5:287-300, 1999), Wang et al. (Infect. Immun. 66:4193-202, 1998), Le Borgne et al. (Virology 240:304-315, 1998), Tacket et al. (Vaccine 17:2826-9, 1999), Jones et al. (Vaccine 17:3065-71, 1999) and Wang et al. (Science

282(5388):476-80, 1998). The ability to enhance the immune response by the co-delivery of genes encoding cytokines is also well-established.

*Polynucleotide vaccine*

5       The polynucleotide vaccine of the invention includes at least one, preferably at least two, nucleotide coding regions, each coding region encoding an immunogenic polypeptide component from *T. cruzi* as identified herein and/or using the screening method described herein. When it contains two or more nucleotide coding regions, the polynucleotide vaccine is referred to herein  
10      as a "multicomponent" polynucleotide vaccine. It is desirable to minimize the number of different immunogenic polypeptides encoded by the nucleotide coding regions in the polynucleotide vaccine; however, it is nonetheless contemplated that a polynucleotide vaccine that generates the highest level of protection will encode 10 or more immunogenic polypeptides.

15      The polynucleotide vaccine can contain DNA, RNA, a modified nucleic acid, or any combination thereof. Preferably, the vaccine comprises one or more cloning or expression vectors; more preferably, the vaccine comprises a plurality of expression vectors each capable of autonomous expression of a nucleotide coding region in a mammalian cell to produce at least one  
20      immunogenic polypeptide or cytokine, as further described below. An expression vector preferably includes a eukaryotic promoter sequence, more preferably the nucleotide sequence of a strong eukaryotic promoter, operably linked to one or more coding regions. A promoter is a DNA fragment that acts as a regulatory signal and binds RNA polymerase in a cell to initiate  
25      transcription of a downstream (3' direction) coding sequence; transcription is the formation of an RNA chain in accordance with the genetic information contained in the DNA. A promoter is "operably linked" to a nucleic acid sequence if it does, or can be used to, control or regulate transcription of that nucleic acid sequence. The invention is not limited by the use of any particular  
30      eukaryotic promoter, and a wide variety are known; preferably, however, the expression vector contains a CMV or RSV promoter. The promoter can be, but need not be, heterologous with respect to the host cell. The promoter used is preferably a constitutive promoter.

A vector useful in the present invention can be circular or linear, single-stranded or double stranded and can be a plasmid, cosmid, or episome but is preferably a plasmid. In a preferred embodiment, each nucleotide coding region (whether it encodes an immunogenic polypeptide or a cytokine) is on a separate 5 vector; however, it is to be understood that one or more coding regions can be present on a single vector, and these coding regions can be under the control of a single or multiple promoters.

There are numerous plasmids known to those of ordinary skill in the art useful for the production of polynucleotide vaccines. Preferred embodiments of 10 the polynucleotide vaccine of the invention employ constructs using the plasmids VR1012 (Vical Inc., San Diego CA), pCMVI.UBF3/2 (S. Johnston, University of Texas) or pcDNA3.1 (InVitrogen Corporation, Carlsbad, CA) as the vector. Plasmids VR1012 and pCMVI.UBF3/2 are particularly preferred. In addition, the vector construct can contain immunostimulatory sequences 15 (ISS), such as unmethylated dCpG motifs, that stimulate the animal's immune system. Other possible additions to the polynucleotide vaccine constructs include nucleotide sequences encoding cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-12 (IL-12) and co-stimulatory molecules such B7-1, B7-2, CD40. The cytokines can be used in 20 various combinations to fine-tune the response of the animal's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to control or eliminate the *T. cruzi* infection.

The polynucleotide vaccine can also encode a fusion product containing the antigenic polypeptide and a molecule, such as CTLA-4, that directs the 25 fusion product to antigen-presenting cells inside the host. Plasmid DNA can also be delivered using attenuated bacteria as delivery system, a method that is suitable for DNA vaccines that are administered orally. Bacteria are transformed with an independently replicating plasmid, which becomes released into the host cell cytoplasm following the death of the attenuated bacterium in 30 the host cell.

An alternative approach to delivering the polynucleotide to an animal involves the use of a viral or bacterial vector. Examples of suitable viral vectors include adenovirus, polio virus, pox viruses such as vaccinia, canary pox, and

fowl pox, herpes viruses, including catfish herpes virus, adenovirus-associated vector, and retroviruses. Exemplary bacterial vectors include attenuated forms of *Salmonella*, *Shigella*, *Edwardsiella ictaluri*, *Yersinia ruckerii*, and *Listeria monocytogenes*. Preferably, the polynucleotide is a vector, such as a plasmid, 5 that is capable of autologous expression of the nucleotide sequence encoding the immunogenic polypeptide.

Preferably, the polynucleotide vaccine further includes at least one nucleotide coding region encoding a cytokine. Preferred cytokines include interleukin-12 (IL-12), granulocyte-macrophage colony-stimulating factor (GM-10 CSF), interleukin-6 (IL-6), interleukin-18 (IL-18),  $\gamma$ -interferon,  $\alpha, \beta$ -interferons, and chemokines. Especially preferred cytokines include IL-12 and GM-CSF.

Plasmids and other delivery systems are made using techniques well-known in the art of molecular biology. The invention should be understood as including methods of making and using the polynucleotide vaccine.

15

#### *Polypeptide vaccine*

The polypeptide vaccine of the invention includes at least one, preferably at least two, immunogenic polypeptides from *T. cruzi* as described herein and/or as identified using the screening method described herein. As 20 with the polynucleotide vaccine, it is desirable to minimize the number of different immunogenic polypeptides supplied in the vaccine; however, it is nonetheless contemplated that a polypeptide vaccine that generates the highest level of protection will contain 10 or more immunogenic polypeptides.

Because a CD8<sup>+</sup> T cell response cannot normally be directly triggered 25 by the administration of a conventional protein subunit vaccine, the immunogenic polypeptides contained in the polypeptide vaccine preferably include one or more membrane transporting sequences (MTS) fused to their N-terminus or C-terminus or both. A membrane transporting sequence allows for transport of the immunogenic polypeptide across a lipid bilayer, allowing it to 30 be delivered to the inside of a mammalian cell. In a particularly preferred embodiment, the immunogenic polypeptides are shocked with urea, as described further in Example VIII, prior to administration as a vaccine. From there, portions of the polypeptide can be degraded in the proteasome, and the

resulting peptides can be displayed as class I MHC-peptide complexes on the cell surface. In this way, a polypeptide vaccine can stimulate a CD8+ T cell immune response. In another preferred embodiment, the immunogenic polypeptides are attached to nanoparticles and administered to a subject (e.g., 5 Plebanski et al., J. Immunol. 2004, 173:3148; Plebanski et al., Vaccine, 2004, 23:258). A polypeptide vaccine of the invention is optionally adjuvanted using any convenient and effective adjuvant, as known to one of skill in the art.

The invention should be understood as including methods of making and using the polypeptide vaccine.

10

#### *Pharmaceutical compositions*

The polynucleotide and polypeptide vaccines of the invention are readily formulated as pharmaceutical compositions for veterinary or human use. The pharmaceutical composition optionally includes excipients or diluents that are 15 pharmaceutically acceptable as carriers and compatible with the genetic material. The term “pharmaceutically acceptable carrier” refers to a carrier(s) that is “acceptable” in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like 20 and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, salts, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition. Methods of making and using such pharmaceutical compositions are also included in the invention.

25

#### *Administration of the polynucleotide vaccine*

The polynucleotide vaccine of the invention can be administered to the mammal using any convenient method, such as intramuscular injection, topical or transdermal application to the mammal’s skin, or use of a gene gun, wherein 30 particles coated with the polynucleotide vaccine are shot into the mammal’s skin. The amount of polynucleotide administered to the mammal is affected by the nature, size and disease state of the mammal as well as the delivery method; for example, typically less DNA is required for gene gun administration than for

intramuscular injection. Further, if a polynucleotide encoding a cytokine is co-delivered with nucleotide coding regions encoding the immunogenic polypeptide from *T. cruzi*, the amount of polynucleotide encoding the immunogenic polypeptide from *T. cruzi* in the vaccine is optionally reduced.

5       Hundreds of publications have now reported the efficacy of DNA vaccines in small and large animal models of infectious diseases, cancer and autoimmune diseases (Donnelly et al., Rev. Immunol. 15:617, 1997). Vaccine dosages for humans can be readily extended from the murine models by one skilled in the art of genetic immunization, and a substantial literature on genetic 10 immunization of humans is now available to the skilled practitioner. For example, Wang et al. (Science 282:476-480, 1998) vaccinated humans with plasmid DNA encoding a malaria protein, and the same group has developed a plan for manufacturing and testing the efficacy of a multigene *Plasmodium falciparum* liver-stage DNA vaccine in humans (Hoffman et al., Immunol. Cell 15 Biol. 75:376, 1997). In general, the polynucleotide vaccine of the invention is administered in dosages that contain the smallest amount of polynucleotide necessary for effective immunization. It is typically administered to human subjects in dosages containing about 20 µg to about 2500 µg plasmid DNA; in some instances 500 µg or more of plasmid DNA may be indicated. Typically 20 the vaccine is administered in two or more injections at time intervals, for example at four week intervals.

*Administration of the polypeptide vaccine.*

Like the polynucleotide vaccine, the polypeptide vaccine can be 25 administered to the mammal using any convenient method, such as intramuscular or intraperitoneal injection, topical administration, oral or intranasal administration, inhalation, perfusion and the like. The amount of polypeptide administered to the mammal is affected by the nature, size and disease state of the mammal, as well as by the delivery method. Intraperitoneal 30 injection of 25 to 50 ug of polypeptide containing a membrane transducing sequence has been shown to result in import of the protein into nearly 100% of murine blood and spleen cells within 20 minutes (Schwarze et al., Science 285:1569-1572, 1999) and the sensitization of cytotoxic T cells (Schutze-

Redelmeier et al., J. Immunol. 157:650-655, 1996). Useful dosages of the polypeptide vaccine for humans can be readily determined by evaluating its activity *in vivo* activity in mice.

5   *Administration of a combination of polynucleotide vaccine and polypeptide vaccine.*

The invention contemplates administration of both a polynucleotide vaccine and a polypeptide vaccine to a mammal in a serial protocol. For example, a plasmid-based DNA vaccine may be administered to a mammal to 10 “prime” the immune system, followed by the one or more administrations of a polypeptide vaccine or a viral vaccine (e.g., vaccinia vector carrying the genes that encode the immunogenic polypeptides and, optionally, cytokines) to further stimulate the mammal’s immune system. The order of administration of the different types of vaccines, and the nature of the vaccines administered in any 15 given dose (e.g., polypeptide vaccine, plasmid vaccine, viral vector vaccine) can be readily determined by one of skill in the art to invoke the most effective immune response in the mammal.

*Screening method for identification of antigenic T. cruzi polypeptides*

20       In another aspect, the invention provides high-throughput method to screen putative *T. cruzi* polypeptides for diagnostic potential. The antigenic polypeptides thus identified can be incorporated into a diagnostic test for *T. cruzi* as described herein.

25       *T. cruzi* polypeptides that are preferred candidates for screening, either individually or as part of a pool, have one or more of the following characteristics or features. The *T. cruzi* polypeptides may be abundant in the trypomastigote and/or amastigote stages of the *T. cruzi* life cycle in mammals, as described in more detail above. Additionally or alternatively, the *T. cruzi* polypeptides may be, or may be likely to be, surface-associated or secreted.  
30       Surface associated-antigenic polypeptides include, for example, *T. cruzi* proteins that are anchored to the plasma membrane by glycosylphosphotidylinositol, or GPIs, and those that have transmembrane domains or are otherwise embedded in the plasma membrane. This property

can be evaluated, for example, by analyzing the polypeptide sequence for the presence of an N-terminal leader sequence which directs the polypeptide to the cell membrane; by analyzing the polypeptide sequence for the presence of a known GPI sequence that facilitates attachment of the polypeptide to the cell surface; and/or by analyzing the polypeptide sequence for the presence of a transmembrane domain. Another preferred feature is that the polypeptide is unique to *T. cruzi* and not expressed in other organisms, including other kinetoplastids. This can be determined by performing BLAST searches of GenBank entries for other organisms and/or comparative genomics with *T. brucei* and *Leishmania major*. This feature enhances the specificity of the diagnostic test.

Another preferred feature is that the *T. cruzi* polypeptide be one that is less likely than others to be highly variant. For example, members of large gene families that appear to undergo rearrangements that create new variants are generally not preferred. However, pools of large gene family members (such as the trans-sialidase family, the Mucin-associated surface protein (MASP) family, and other smaller families of genes can be cloned and tested using degenerate primers. In that case, rather than a bead or a spot in the diagnostic test containing only one gene family member, it may have ten or hundreds, thereby circumventing the problem of recombination and variation in these families, and providing a better representation of the family than a single (possibly variant) protein.

The screening method involves providing two substrates that include a plurality of individually addressable candidate antigens derived from *T. cruzi*, in which the antigens present on both substrates are substantially the same. A substrate, as defined herein, is a surface of unreactive material that can be used to contain the individually addressable candidate antigens in isolation from one another. For example, a multi-welled array system such as a 96 well microplate is a substrate useful in the method of screening for serodiagnostic *T. cruzi* antigens. Individually addressed candidate antigens refers to potentially serodiagnostic *T. cruzi* antigens that have been positioned and/or labeled in such a way that differing antigens can be discretely identified using methods known to those skilled in the art. For example, antigens obtained directly or indirectly

from *T. cruzi*, labeled with a fluorescent label with a different wavelength sensitivity from other fluorescent labels used with other antigens and positioned within a specific well or set of wells on a multi-welled array system, are individually addressed candidate antigens.

5 Candidate antigens immobilized on the first substrate are contacted with a body fluid from an organism known to be positive for *T. cruzi* infection based on a detection method such as a T cell assay, polymerase chain reaction (PCR), hemoculture or xenodiagnostic techniques. The organism is preferably a mammal, more preferably a dog or a human. Preferably, the organism exhibits  
10 negative serology when tested for *T. cruzi* infection utilize conventional serodiagnostic tests that rely on antigens from either whole to semi-purified parasite lysates, for example from epimastigotes, that react with anti-*T. cruzi* IgG antibodies.

Candidate antigens immobilized on the first substrate are contacted with  
15 the second substrate with a body fluid from an organism known or reasonably believed to be unexposed to *T. cruzi* infection. The second substrate serves as a control. The organism does not exhibit a strong positive serological signal indicating infection by *T. cruzi*. Preferably, the organism shows no evidence of *T. cruzi* infection by any other diagnostic test as well. Optionally, the screening  
20 method includes testing of additional substrates using body fluids that are strongly, weakly and/or borderline seropositive using conventional tests for *T. cruzi*, as described in more detail below.

The body fluid may be any fluid found within the body of an organism that is capable of containing components of *T. cruzi* or immune system  
25 components prepared in response to exposure to *T. cruzi*. For example, an immune system component may be an antibody that specifically binds to a *T. cruzi* antigen. Such body fluids include, for example, blood, plasma, serum, urine, saliva, tears, lymphatic fluid, and the like.

The organism itself may be any organism that can be infected by *T. cruzi*, including vector organisms. For example, organisms may include insect vectors of Chagas disease belonging to the Hemiptera order, Reduviidae family, and Triatominae subfamily. The organism can also be a vertebrate reservoir of *T. cruzi* infection. Mammals are most susceptible to infection with *T. cruzi*,

with approximately 150 species known to serve as reservoirs. Birds, amphibians, and reptiles are naturally resistant to infection. In the domestic cycle, frequently infected mammals, besides humans, are dogs, cats, mice, rats, guinea pigs, and rabbits. Pigs, goats, cattle, and horses can be infected by *T. cruzi*, but generally only manifest transitory parasitemia. Humans are a preferred organism due to the importance of diagnosing *T. cruzi* infection in humans.

Antigens that exhibit binding to antibodies present in the bodily fluid contacted with the first substrate but little or no binding to antibodies present in the control bodily fluid contacted with the second substrate are identified as antigenic *T. cruzi* polypeptides for use in the multicomponent diagnostic assay. The binding of an antigen to an antibody can be detected by various means known to those skilled in the art. For example, the association may be detected using flow cytometry, or by enzyme immunoassay (EIA) or enzyme-linked immunoassay (ELISA). Preferably, the association of one or more antibodies with multiple antigens is detected using a multiplex analysis system such as the Bio-Plex multiplex analysis system commercially available from, for example, Bio-Rad<sup>®</sup> Laboratories (Hercules, CA).

The Bio-Plex suspension array system is a biomarker assay system that includes a flow-based 96-well fluorescent microplate assay reader integrated with specialized software, automated validation and calibration protocols, and assay kits. The multiplex analysis system utilizes up to 100 fluorescent color-coded bead sets, each of which can be conjugated with a different specific antigen. The term "multiplexing" refers to the ability to analyze many different antigens essentially simultaneously. To perform a multiplexed assay, sample and reporter antibodies are allowed to react with the conjugated bead mixture in microplate wells. The constituents of each well are drawn up into the flow-based Bio-Plex array reader, which identifies each specific reaction based on bead color and quantitates it. The magnitude of the reaction is measured using fluorescently labeled reporter antibodies specific for each antibody that may associate with the antigen being tested.

The Bio-Plex suspension array system uses a liquid suspension array of about 100 sets of micrometer-sized beads, each internally dyed with different

ratios of two spectrally distinct fluorophores to assign it a unique spectral address. The overall operation of the Bio-Plex array system is illustrated in FIGs 1A through 1C. As shown in FIG 1A, polypeptide antigen 10 is bound to a microsphere bead 20 by, for example, a histidine tag. The polypeptide antigen 5 10 is then contacted with a sample of sera containing an antibody; for example, an anti-*T. cruzi* antibody 30. This antibody, in turn, is contacted with a fluorescently labeled reporter antibody 40 to form a microsphere-antigen-antibody complex 50. As shown in FIG 1B, since the microsphere beads 20 provide a large variety of different colors, and the microsphere beads 20 were 10 earlier attached only to specific polypeptide antigens 10, a number of microsphere-antigen-antibody complexes 50 may be present in a microplate well 60. The complexes 50 are then run through a flow cytometry apparatus 70 that includes a classifying laser 90 and a reporting laser 80. The reporting laser 80 determines the amount of a particular antigen present, based on the amount 15 of fluorescently labeled reporter antibody 40. The classifying laser 90, on the other hand, determines the frequency of fluorescence provided by the microsphere bead 20, and based on this frequency, the identity of the polypeptide antigen 10 can be determined.

In the embodiment used in this invention, the Bio-Plex assay utilizes 20 dyed beads containing nickel to capture the His-tagged *T. cruzi* polypeptides produced in the host bacterial cells. Each spectrally addressed bead captures a different protein. The protein-conjugated beads are allowed to react with a sample, and biomolecules in the sample (typically antibodies) bind to the bound protein antigens as further described in the Examples.

25 It should be understood that the invention is not limited to multiplexing as employed in the Bio-Plex assay; other multiplexed approaches can readily be used. For example, protein arrays can be placed on a matrix, and the response to the individual proteins on the solid-phase array can be assayed.

An important advantage of using a multiplexed method is that a plurality 30 of serodiagnostic antigens may be identified during a single run of the analysis. For example, serological responses to as many as a 100 individual proteins can be screened at one time, and the pattern of responsiveness to all 100, or any subset thereof, can be used to make or assist in making a diagnosis. Tests in

current use employ crude antigen preparations from *T. cruzi* itself (an undefined composition thus not very reproducible), or utilize an individual recombinant protein (i.e., a single target thus not very robust) or a string of three portions of three different proteins combined in one synthetic protein. In the latter case,  
5 there is more than one target, but still the response to only a single entity is measured, thus the assay lacks robustness.

Optionally, the method of the invention can utilize more than two substrates that include a plurality of individually addressed candidate antigens. These additional substrates can be used, for example, to evaluate the body fluids  
10 from organisms classified as providing a very high positive sera response, intermediate positive sera response, very low positive sera response or a borderline positive sera response. When used to supplement data obtained on sera from control (uninfected) organisms and organisms that are or have been infected but show little or no positive serological response, these substrates can  
15 provide additional information on candidate *T. cruzi* antigens that may be used as serodiagnostic antigens. Measuring the pattern of responses to many antigens is also useful for evaluating other aspects of the *T. cruzi* infection such as the stage of the disease, its severity, or the particular strain of *T. cruzi* involved.

20 The method of screening for serodiagnostic *T. cruzi* antigens can also be done at the less specific level of an antigen pool, rather than specific antigens. An antigen pool, as defined herein, is a plurality of antigens in a mixture. Antigen pools may be used either as a means of evaluating a wide number of antigens more rapidly, as a means of evaluating mixtures of antigens for  
25 possible interactions, or simply out of necessity when the identify of specific antigens is not known. The method of screening antigen pools can be done either as an independent analysis method, or it may be an optional preliminary step to the screening of individual *T. cruzi* antigens for potential as serodiagnostic antigens. In either case, the method of analyzing antigen pools  
30 includes providing two substrates that include a plurality of individually addressable antigen pools derived from *T. cruzi*, where the antigen pools present on the two substrates are substantially the same. The first substrate is then contacted with a body fluid from an organism known to be serologically

positive for *T. cruzi* infection, while the second substrate is then contacted with a body fluid from an organism known to be not serologically positive for *T. cruzi* infection. Antigen pools that associate with an antibody present in the body fluid from an organism known to be serologically positive but that are 5 absent or present to a lesser degree in the body fluid of an organism known to be not serologically positive for *T. cruzi* infection are thereby identified as serodiagnostic antigen pools that may be used by themselves or further evaluated to determine that specific antigens involved.

The candidate antigens derived from *T. cruzi* that are screened by the 10 method of the invention can include antigens formed from polypeptides, polysaccharides, polynucleotides, or other substances present in *T. cruzi* that are capable of being specifically bound by antibodies. As polypeptides are known by those skilled in the art to be the most common and diverse antigens, the method of the invention preferably identifies polypeptide antigens. Polypeptide 15 antigens can be obtained directly from *T. cruzi* using biochemical separation technology, particularly protein purification methods. More preferably, the polypeptide antigens are obtained using recombinant DNA technology. Using recombinant DNA technology, nucleotide sequences from *T. cruzi* are inserted into a host organism where they are used to direct product of a polypeptide that 20 may contain one or more antigens that may be detected by the screening method.

The smallest useful peptide sequence contemplated to provide an antigen is generally on the order of about 6 amino acids in length. Thus, this size will generally correspond to the smallest polypeptide antigens that are 25 screened for by the method of the invention. It is proposed that short peptides that incorporate a species-specific amino acid sequence will provide advantages in certain circumstances, for example, in the preparation of vaccines or for use in methods of detecting *T. cruzi*. Exemplary advantages of shorter peptides include the ease of preparation and purification, and the relatively low cost and 30 improved reproducibility of production. However, the size of polypeptide antigens may be significantly larger. Longer polypeptide antigens identified by the method may be on the order of 15 to 50 amino acids in length, or may represent an entire protein, including modified proteins such as fusion proteins.

Preparation of the polypeptide antigens will generally include the use of an expression vector. An expression vector is a cloning vector that contains the necessary regulatory sequences to allow transcription and translation of a cloned gene or genes. An expression vector preferably includes a promoter sequence operably linked to one or more coding regions. A promoter is a DNA fragment that acts as a regulatory signal and binds RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding sequence; transcription is the formation of an RNA chain in accordance with the genetic information contained in the DNA. A promoter is "operably linked" to a nucleic acid sequence if it is does, or can be used to, control or regulate transcription of that nucleic acid sequence. The invention is not limited by the use of any particular promoter. A wide variety of promoters are known by those skilled in the art.

A vector useful in the present invention can be circular or linear, single-stranded or double stranded and can be a plasmid, cosmid, or episome but is preferably a plasmid. In a preferred embodiment, each nucleotide coding region encoding an antigenic polypeptide is on a separate vector; however, it is to be understood that one or more coding regions can be present on a single vector, and these coding regions can be under the control of a single or multiple promoters.

There are numerous expression vectors known to those of ordinary skill in the art useful for the production of polypeptide antigens. A preferred expression vector is an expression vector formed using the Gateway® cloning method. The Gateway® cloning method is a universal cloning technique that allows transfer of DNA fragments between different cloning vectors while maintaining the reading frame, and has effectively replaced the use of restriction endonucleases and ligases. The steps involved in the Gateway cloning method are shown in FIG 2. First, a gene is selected for cloning. For the present invention, this would be a gene selected from *T. cruzi*. The gene is then provided with primers and amplified using PCR technology with the help of an attB tagged primer pair, as readily practiced by those skilled in the art. The PCR fragment then combined with a donor vector (pDONR™) that includes attP sites to provide an entry clone, using the BP reaction. An integration reaction between the attB and the attP sites combines the PCR fragment with

the donor vector. The resulting entry clone contains the gene of interest flanked by attL sites. The LR reaction is then used to combine the entry clone with a destination vector to produce an expression vector. In the LR reaction, a recombination reaction is used to link the entry clone with the destination vector 5 (pDEST<sup>TM</sup>) using the attL and attR sites and a clonase enzyme. The attL sites are already found in the entry clone, while the destination vector includes the attR sites. The LR reaction is carried out to transfer the sequence of interest into one or more destination vectors in simultaneous reactions, making the technology high throughput. For example, as this method allows multiple genes 10 to be transferred to one or more vectors in one experiment, this method readily allows multiple antigenic polypeptides to be prepared that can then be screened by the method of the invention, potentially revealing a plurality of serodiagnostic antigens.

The *T. cruzi* genes are cloned into expression vectors, as described, 15 which are then expressed in a host cell, such as a bacterial cell, yeast cell, insect cell, protozoan cell, or mammalian cell. A preferred host cell is a bacterial cell, for example an *E. coli* cell. Another preferred cell is a protozoan cell, more preferably a kinetoplastid cell, most preferably a *Crithidia* cell (U.S. Pat. No. 6,368,827; April 9, 2002). Proteins are isolated from the host cell, purified, and 20 analyzed. Preferably, the proteins are purified onto beads that are then used in a Bio-Plex assay, as described. Various serum samples (e.g., negative, mildly positive, strongly positive) can be efficiently screened for reactivity with a large number of gene products to identify those gene products that are associated with *T. cruzi* infection, for example those indicative of the existence of and, 25 optionally, the extent and/or stage of *T. cruzi* infection. Typically, the identified antigens elicit an antibody response *T. cruzi* in a mammal. The genes and gene products thus identified are useful in diagnostic assays for *T. cruzi*.

The Examples that follow provide representative data from a screening 30 of more than 350 *T. cruzi* gene products. Following FIGs 4-6 in the Examples, a procedure including a preliminary screening of antigen pools, followed by screening for specific antigens, is described. Four different substrates were used to provide data for the reactivity of the antigens in sera with various levels of reactivity to *T. cruzi* antigens. One pool, labeled "3K" on FIG 4D, was

selected for further analysis because it showed good reactivity with the positive sera. The "3K" pool was broken down into individual constituent gene products, and four of the gene products (antigens) were identified as potential candidates for diagnostic use. The "top" antigens identified this way can be  
5 combined into a single, robust diagnostic assay for *T. cruzi*; see, for instance, Example 9. Examples of gene products identified in accordance with the invention are also described.

The method of screening is also capable of identifying antigens that do not consistently elicit a strong B cell response. The majority of conventional  
10 and commercially available serological methods for diagnosis and blood screening of *T. cruzi* infection utilize either crude or semi-purified parasite lysates typically from epimastigotes. However the complex nature of molecules in these lysates creates a test that routinely gives false positive diagnosis.  
Research to improve serological diagnosis techniques has focused on the  
15 identification, characterization and cloning of particular *T. cruzi* antigens that elicit a strong B cell response. Experiments have demonstrated that some individuals declared negative by current serological tests in fact respond to parasite lysate by producing IFN-gamma in ELISPOT assays. These individuals therefore have T cells that have been exposed to parasite antigen but  
20 have a poor B cell antibody response to the antigens in the serological tests that use parasite lysate. The present invention is capable of detecting components present in the sera of such individuals, as shown in FIG 6.

It is to be understood that other screening methods are applicable to the identification of antigenic *T. cruzi* polypeptides to be included in the  
25 multicomponent panel for the diagnostic test. For example, U.S. Pat. No. 6,875,584, issued April 5, 2005, describes screening methods that can be used to identify additional antigenic *T. cruzi* polypeptides for use in a diagnostic test and/or as vaccine components. As another example, a nickel-coated substrate, such as a nanoparticle array, can be used to immobilize His-tagged candidate  
30 antigens which can then be contacted with serum or other blood product (in the case of the serodiagnostic test) or MHC-peptide complexes (in the case of the cellular test) to detect evidence of an immune response in the serum.

It is to be understood that any of the diagnostic, therapeutic or laboratory methods described herein can be performed with one or more protein antigens as set forth in Table 1, Table 3 and/or Table 4 herein; or as elsewhere described in the specification.

5 The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

10

## EXAMPLES

The majority of current serological tests for *T. cruzi* infection utilize whole to semi-purified parasite lysates and are often inconclusive or result in false positives. Recent studies have identified individuals who are seronegative for *T. cruzi* infection by standard tests but are positive by PCR (Salomone et. al. 15 Emerg. Infect. Disease, 2003, 9:1558) or have demonstrable cellular immune responses to *T. cruzi*. With respect to the latter, our lab has recently demonstrated that some individuals declared negative by current serological tests in fact have demonstrable T cell responses to parasite lysate as seen in ELISPOT assays. These individuals therefore have T cells which have been 20 exposed to parasite antigen but have a poor B cell antibody response to the mix of antigens in the serological test. It is apparent that the use of lysates is a poor test for *T. cruzi* infection and we expect that screening with multiple recombinant proteins will be able to reduce the number of false positives, and more importantly false negatives.

25 We have therefore developed a high-throughput method to screen large numbers of recombinantly expressed *T. cruzi* proteins for their serodiagnosis potential. Specifically, we combined a set of putative *T. cruzi* genes cloned into the Gateway System™ with the BioPlex LiquiChip bead technology to screen large numbers of recombinantly expressed proteins for their antigenicity using 30 only a small volume of sample (<100µl). So far, we have produced 34 pools of approximately 10 proteins each and screened them for antigenicity. From the preliminary testing, 11 pools were found to bind readily detectable amounts of antibodies in the sera of *T. cruzi*-infected subjects. These pools were then

broken down and each gene was expressed individually and tested. From these 81 genes we have been able to define more than 15 proteins with serodiagnostic potential.

Our method utilizes a blind screening process that has identified several  
5 known antigens as well as previously unidentified antigenic proteins from  
within pools containing multiple non-antigenic proteins. The use of the BioPlex  
technology is not limited to antigen screening but its full potential may be  
realized as a novel method of blood donor screening. The highly antigenic  
proteins we discovered, and expect to continue to discover, with this method  
10 can be used to create a highly sensitive and specific test for *T. cruzi* infection.

#### Example 1: Buffer and Medium Preparation

A variety of buffers were used in the Bio-Plex multiplex analysis. The  
15 buffers were prepared as follows. To prepare 1 liter of PBS/BSA (10 mM  
NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, and 0.1% (w/v) BSA), 8.77 g NaCl (MW 58.44  
g/mol) and 1.4 g NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O (MW 137.99 g/mol) were dissolved in 900 ml  
H<sub>2</sub>O and the pH was adjusted to 7.4 using NaOH. Then, dissolve 1 gram of  
BSA and adjust the volume to 1 liter. Before use, filter the buffer using a 0.45  
20 μM filter. Sodium Azide should be added to 0.5% when storing the PBS/BSA  
buffer for long term. Azide should not be used with Carboxy Beads.

To prepare 1 liter of coupling buffer (50 mM MES), 11.67 g MES (MW  
233.2 g/mol) was dissolved in 900 ml H<sub>2</sub>O and the pH was adjusted to 5.0 using  
NaOH. The volume was then adjusted to 1 liter using additional H<sub>2</sub>O. Before  
25 use, the buffer should be filtered using a 0.45 μM filter.

To prepare 1 liter of activation buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>), 13.80 g  
NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O (MW 137.99 g/mol) was dissolved in 900 ml H<sub>2</sub>O and the pH  
was adjusted to 6.3 using NaOH. The volume was then adjusted to 1 liter using  
additional H<sub>2</sub>O. Before use, the buffer should be filtered using a 0.45 μM filter.

30 To prepare Buffer Z, 8 M urea, 20 mM Hepes, and 100 mM NaCl are  
combined and dissolved in deionized water to form a solution. The pH of the  
solution is adjusted to 8.0, and the solution is filtered through a 0.45 μm filter  
and stored at room temperature. Imidazole (the side chain molecule in

histidine) is added to Buffer Z at varying concentrations to either prevent the cobalt resin from binding non-specifically to something other than the histidine tag, or to out-competing the binding of the histidine tag and thus causing the protein to elute off the resin.

5 To prepare LB (*Luria-Bertani*) Medium, 10 g tryptone, 5 g yeast extract, and 10 g. NaCl were dissolved in 1 L deionized water and autoclaved for 25 minutes. For plates, 15 grams of agarose were also dissolved into the water prior to autoclaving.

10

#### Example 2: Production of Protein Pools or Individual Proteins

To provide a large set of *T. cruzi* proteins, over 350 proteins in pools of approximately 10 proteins each were prepared. The proteins were prepared using the Gateway® universal cloning technique developed by Invitrogen™.

15 The procedure can be carried out by cloning a pool of several genes together, which results in a pool of proteins, or by cloning an individual gene, resulting in the preparation of an individual protein. For preparation of an individual protein, a gene that codes for a desired *T. cruzi* protein is first selected for cloning. This gene is amplified from *T. cruzi* genomic DNA using gene specific

20 primers flanked by lambda phage recombination sites, attB1 (5') and attB2 (3') and polymerase chain reaction. Gel purification of the att-flanked PCR produced was carried out by separating the PCR reaction product on a 1% agarose gel using electrophoresis. The particular gene is identified by comparison with a DNA standard containing bands of known size. The band of

25 the gene of interest is cut out of the gel and purified using Sigma-Aldrich's GenElute Minus EtBr Spin Columns (Catalog No. 5-6501).

The Gateway® BP reaction is then used to insert the att-flanked *T. cruzi* gene fragment with a pDONR™201 vector (Catalog No. 11798-014, Invitrogen Corp., Carlsbad, CA). The BP reaction is conducted by adding the 5 µl of gel-purified attB-flanked PCR product (40-100 fmoles), 1 µl of the pDONR™201 vector (supercoiled, 150 ng/µl), and 2 µl 5x BP Clonase Reaction Buffer (Catalog No. 11789-013) to obtain a final volume of 8 µl. The BP Clonase™

enzyme mix (Catalog No. 11789-013, Invitrogen Corp., Carlsbad, CA) is mixed gently, and then 2 µl of the enzyme mix was added to the BP reaction mixture and mixed well. The reaction was then incubated at (room temperature) 25° C overnight. Next, 1 µl of Proteinase K solution (Catalog No. 11789-013, Invitrogen Corp., Carlsbad, CA 2 µg/µl) was added, and the mixture was allowed to incubate for 10 minutes at 37° C. Five microliters of the BP reaction are transformed by heat shock into chemical competent DH5 $\alpha$  cells and grown up overnight at 37°C shaking at 280 RPM in 5 mL of LB with 50 mg/L kanamycin to select for pDONR201-transformed cells. The plasmid is then purified from the culture using a QIAprep Spin Miniprep Kit (Catalog No. 27106, Qiagen Inc., Valencia ,CA).

For the next step of protein production, the Gateway LR® recombination reaction was used to insert the gene of interest in pDONR201 into a destination vector to provide the final expression clone. The destination vector in this case is a modified version of Invitrogen's pRSET (Catalog No. V351-20), called pDEST-PTD4. First, the pDEST-PTD4 was linearized by restriction digest of a novel site (PvuII) within the cell death cassette. The linearized plasmid was purified using QIAquick Gel Extraction Kit (Catalog No. 28207, Qiagen Inc., Valencia ,CA). The LR reaction between the gene of interest in the pDONR™201 vector and the desired pDEST-PTD4 expression vector was then set up. First, 300 ng of the pDONR entry clone (prepared above), 300 ng of linearized pDEST-PTD4 (Invitrogen Corp., Carlsbad, CA), and 2 µl LR Clonase Reaction Buffer (Catalog No. 11791-019, Invitrogen Corp., Carlsbad, CA), 2 µl LR Clonase Enzyme Mix, and deionized water are combined to obtain a final volume of 10 µl and mixed thoroughly by flicking the tube. The reaction was then incubated overnight at 25° C. Next, 2 µl proteinase K solution (2 µg/µl) was added and the mix was allowed to incubate for 10 minutes at 37° C. DH5 $\alpha$  cells were then transformed by heat shock with 6 µl of LR reaction products, and plated onto LB agar plates containing 150 mg/L ampicillin and incubated overnight at 37° C to select for ampicillin-resistant expression clones.

Next, all of the colonies were scraped clean with a clean sterile spatula, and used to inoculate a tube of 5 mL LB containing 150 mg/L ampicillin, and

grown overnight at 37° C, 280 RPM. The pDEST-PTD4 containing the gene of interest is purified from the culture using a QIAprep Spin Miniprep Kit (Catalog No. 27106, Qiagen Inc., Valencia ,CA). The miniprep preparation should contain copies of each gene of the pool from the desired pDEST vector. Three 5 microliters of purified pDEST-PTD4 containing the gene of interest was then transformed into BL21(DE3)pLysS chemical competent cells. The culture was then directly inoculated into 10 ml LB/ ampicillin (Amp)/chloramphenicol (CAM) (100 mg/L)/(34 mg/L) and grown overnight, shaking at 37° C at 280 RPM.

10 On the fifth day, a 10 ml starter culture was inoculated into 500 ml LB/Amp/CAM and grown to an OD600 of 0.4. Protein expression was then induced with 0.3 mM concentration of IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside), using 150  $\mu$ l of 1M IPTG in 500 ml culture. The cells were spun down at 5,000 rpm for 8 minutes and 10 mL Buffer Z (8M urea, 20 15 mM Hepes, 100 mM NaCl) containing 15 mM imidazole was added. The cells were then sonicated three times for 25 seconds at an amplitude of 40. The samples were spun down at 13,000 rpm for 10 minutes and the supernatant is combined with 1 ml settled BD TALON™ Metal Affinity Resin (BD Biosciences Clontech, Catalog No 635502) and rocked overnight at 4° C.

20 The resin/cell lysate slurry is then placed into an empty 0.8x4cm chromatography column and the resin bed is allowed to settle. The liquid was allowed to run through and the resin bed was washed with 10 bed volumes (10 mL) of Buffer Z containing 15 mM imidazole. Once the 10 mL wash has run through, the His-tag protein was eluted with 3 bed volumes (3 mL) of Buffer Z 25 containing 250 mM imidazole. The resulting sample contained the purified protein of interest. The sample was then desalted into Buffer Z (without imidazole) using a PD-10 desalting column (Amersham Biosciences, Catalog No.17-0851-01). The resulting imidazole-free sample is quantified and diluted to a concentration of 10  $\mu$ g/mL which is ready to be used to bind to Bio-Plex 30 beads for testing.

### Example 3: Preparation of Bio-Plex Beads

LiquiChip™ Ni-NTA beads (Qiagen Inc., Valencia, CA) were used to bind His-tagged purified proteins in the Bio-Plex assay, but had to be prepared before use. First, the protein samples were desalted into Buffer Z that does not contain Imidazole using Amersham PD-10 desalting columns (Amersham Biosciences Corp, Piscataway, NJ). The protein was then quantified using a BCA assay and diluted to a concentration of 10 µg/ml with Buffer Z. The LiquiChip™ Ni-NTA Bead stock was then vortexed for 30 seconds at full speed. Next, 50 µl of bead suspension was pipetted out and placed into a 1.5 ml microcentrifuge tube. His-tagged protein dilution (50 µl) was then added to the 50 µl LiquiChip™ Bead suspension. The beads were then incubated at 4° C in the dark from at least 4 hours to overnight. Buffer (900 µl PBS/BSA (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 0.1% BSA pH 7.4)) was then added to the protein-coupled LiquiChip™ Bead suspension, adding 0.5% azide as a preservative.

### Example 4: Preparation of Positive Controls

Positive and negative controls were used in the Bio-Plex analysis of *T. cruzi* antigens. The positive control consists of proteins from a *T. cruzi* lysate coupled to LiquiChip™ Carboxy Beads. The beads thus contain a mix of *T. cruzi* proteins bound to their surface, and function as a general antigen mix. The LiquiChip™ Carboxy Beads bind to the proteins in a random manner, forming covalent bonds to amine groups in lysine side chains. The first step in the preparation of positive controls was the activation of Carboxy Beads using EDC/NHS. First, approximately 10 mg each of EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (Fluka catalog No. 03449)) and NHS (N-hydroxysulfosuccinimide (Fluka catalog No. 56485)) were weighed into two microcentrifuge tubes. The LiquiChip™ CarboxyBead suspension (1 ml) was then centrifuged for 5 minutes at 10,000 rpm in a microcentrifuge. The supernatant was removed with a 200 µl pipette and discarded. The beads were then washed twice by adding 80 µl of activation buffer and centrifuged for 5

minutes at 10,000 rpm. The supernatant was then carefully removed. Activation buffer (80 µl) was then added to the bead pellet at the bottom of the tube. The pellet should not be resuspended. The pellet in activation buffer was then vortexed for at least 2 minutes. De-ionized water was then added to the 5 weighed EDC and NHS aliquots to provide solutions with a concentration of 50 mg/ml. NHS solution (10 µl) and EDC solution (10 µl) were then added to the bead suspension, which was then incubated for 20 minutes in the dark. Finally, the beads were centrifuged for 5 minutes at 10,000 rpm, after which the supernatant was removed and discarded.

10       The activated beads were then coupled to the *T. cruzi* lysate. First, the *T. cruzi* pellet was freeze/thawed about 5 times. Insoluble particles were removed by centrifugation. The protein stock was then diluted with coupling buffer to a concentration of 100 µg/ml and a volume of 500 µl. Any foreign protein, azide, glycine, Tris, or other reagent containing primary amine groups 15 present in the protein preparation should be removed by dialysis or gel filtration. Coupling buffer (500 µl) was then added to the beads, which were then resuspended by vortexing. The beads were then washed twice by adding 500 µl of coupling buffer, centrifuging for 5 minutes at 10,000 rpm, removing the supernatant, and then repeating the process. Diluted protein solution (500 20 µl), prepared earlier, was then added. Next, the tube containing the activated beads and the protein solution was gently agitated on a shaker for 2 hours in the dark at room temperature. The beads were then washed twice with PBS/BSA buffer. The beads were then resuspended in 500 µl PBS/BSA, and 0.5% azide was added as a preservative. The bead number was then adjusted to provide the 25 desired concentration per microliter.

#### Example 5: Bio-Plex Analysis of Proteins

At the start of the analysis, a dilution series of the serum to be tested was 30 prepared on a Millipore 96 well filtration plate. The BioPlex Bead/Protein preparation, prepared according to Example 3, was then added to the wells on a Millipore 96 well filtration plate. When preparing beads according to the normal protocol, 10 µl of bead suspension is sufficient to make a useful data

point. However when testing beads in which multiple proteins are bound to an individual bead, it may be necessary to combine the beads into a single tube and distribute them to wells so that enough of each bead is present in a given well to give an accurate data point. Controls are preferably included for each sample 5 (sera/protein) being analyzed. For example, the Bio-Plex analysis for *T. cruzi* antigens included a bead coated with ovalbumin (OVA) as a negative control and with *T. cruzi* lysate as a positive control.

To prepare for the Bio-Plex analysis, 30 µl of PBS/BSA buffer and 10 µl of an individual bead suspension (or a predetermined volume containing 10 multiple beads each with different proteins bound) were added to the Millipore 96 well filtration plate. The filtration plate was then placed on the vacuum manifold and the sample liquid was pulled through the plate. Next, 50 µl of PBS/BSA and 50 µl of serum dilution were added. The beads were then incubated for 1 hour at room temperature while being shaken on a plate shaker. 15 Each well was then washed four times with 200 µl PBS/BSA to remove any unbound IgG antibodies from the well. PBS/BSA buffer (90 µl) was then added to each well and beads that had settled to the bottom of the filtration plate well due to washing were resuspended. An aliquot (10 µl) of the secondary reporter molecule was then added. This provided a 1:30 dilution (0.5 mg/ml) of 20 antibody. A higher dilution may be used, but a 1:30 dilution makes sure that secondary antibody is not limited by residual unbound IgG. The solution was then incubated for 1 hour at room temperature while being shaken.

The assay solution was then drawn into the Bio-Plex array reader, which illuminates and reads the sample. When a red diode "classification" laser (635 25 nm) in the Bio-Plex array reader illuminates a dyed bead, the bead's fluorescent signature identifies it as a member of one of the 100 possible sets. Bio-Plex Manager software correlates each bead set to the assay reagent that has been coupled to it. In this way the Bio-Plex system can distinguish between the different assays combined within a single microplate well. A green "reporter" 30 laser (532 nm) in the array reader simultaneously excites a fluorescent reporter tag (phycoerythrin, or PE) bound to the detection antibody used in the assay. The amount of green fluorescence is proportional to the amount of analyte captured in the immunoassay. Extrapolating to a standard curve allowed

quantitation of the analyte in each sample: The results for specific proteins are described in Example 8, and shown in FIGs 4-6.

5

#### Example 6: Bio-Plex Assay of VV-Ovalbumin Sera

Ovalbumin (OVA) chosen as the protein antigen to develop the BioPlex method. Mice were infected with Vaccinia virus (VV) containing the OVA gene in order to raise serum antibodies to the protein. Sera was collected at 7 days post infection, followed by a boost and an additional sera collection 7 more days later. OVA protein was expressed in *E. coli* and purified using a His-tag and bound to BioPlex beads via a Ni-NTA residue and adsorbed to an ELISA plate for analysis. The sera was diluted and tested using the BioPlex Assay described in Example 5. The results are shown in FIG 3A. The results obtained were very comparable to those obtained using ELISA, as described in Example 7.

15

#### Example 7: ELISA Assay of VV-Ovalbumin Sera

20

A comparison assay on the ovalbumin of mice infected with Vaccinia virus was run using the ELISA (Enzyme-linked Immunosorbent Assay) method. First, a 96-well polystyrene Immunolon microtiter plate (Dynex Technologies, Chantilly, Va.) was coated with 100 µl of 10 µg/ml ovalbumin (OVA) in PBS overnight at 4° C or 2 hours at 37° C. The wells were then washed three times with PBS-T (PBS-Tween 20 buffer) and then blocked with 1% BSA for 2 hours. Serum dilutions were then added to each well and the wells were incubated for 2 hours at room temperature or overnight at 4° C. After incubation, the wells were washed five times with PBST. Biotinylated secondary mouse antibody (1:100 dilution) was then added and the wells were allowed to set for 1 hour at room temperature. The wells were then washed again for five times with PBST. Horseradish peroxidase-conjugated streptavidin was then added for 30 minutes at room temperature at a 1:100 dilution. The wells were then washed again five times with PBST. Finally, a developing reagent (2,2'-azido-di-[3-

ethylbenzthiazoline sulfonate], ABTS) was added. The results of the ELISA assay of ovalbumin sera are shown in FIG 3B.

Example 8: Bio-Plex Assay Results for Pooled and Specific Proteins

5       Using the method of protein production described in Example 2, over  
350 proteins in pools of approximately 10 proteins each were prepared. Each of  
the pools were screened for antigenicity using the Bio-Plex technology, as  
described in Example 5. From the preliminary testing, 11 pools were found to  
bind readily detectable amounts of antibodies in the sera of *T. cruzi*-infected  
10 subjects. These pools were then broken down and each gene was expressed  
individually and tested. From the over 80 genes expressed, 15 proteins have  
been confirmed as having serodiagnostic potential.

A Hemagen® Diagnostics Chagas Disease Test Kit (Hemagen  
Diagnostics, Inc., Columbia, MD) was used to evaluate and confirm the  
15 presence of anti-*T. cruzi* antibodies in sera from areas of active transmission in  
Argentina. Sera from non-endemic uninfected in-house sera served as the  
negative controls. Sera from 4 individuals from areas of active transmission, all  
of which have tested seronegative using standard assays but 1 of which tests  
positive for T cell reactivity to *T. cruzi*, were used for the very low positive  
20 control. Sera from 5 individuals that were borderline positive/negative using  
standard serological assays were used to make up the borderline positive control  
and sera from 7 individuals that were consistently seropositive using standard  
serological assays make up the strong positive control.

Genes of interest were first cloned into the Gateway holding vectors  
25 (pDONR™ vector) and archived as single vectors or are placed into pools.  
Pools of genes in pDONR™ vectors can be moved simultaneously into either  
DNA vaccination vectors or protein expression vectors without the loss of  
individual genes in the pool. The resulting pools were expressed in *E. coli*  
strain BL21(DE3)pLysS cells, minimizing the possible toxic effects of  
30 individual genes. The protein pools are purified and tested using the Bio-Plex  
bead technology for antigenicity. The results from analysis of the protein pools  
using the Bio-Plex analysis method are shown in FIGs 4A-2D. The headings in  
the figures indicate the type of sera being tested, based on the four categories

(negative, very low positive, borderline positive, and strong positive) resulting from the evaluation using the Hemagen® Test Kit. As indicated by the arrow, FIG 4D demonstrates a pool that shows high fluorescence, and hence contains a high level of protein that binds to *T. cruzi*-specific antibodies.

5       Once a pool of proteins was identified using the Bio-Plex screening method as having possible antigenic properties, the individual genes in the pool were examined and tested to find which ones provided reactive antigens. The genes were first moved individually from the pDONR holding vector into an expression vector, followed by expression, purification and testing. Those  
10 proteins that exhibit binding to antibodies in infected individuals were then retested for confirmation and identified. The results of screening the pools for individual proteins is shown in FIGs 5A-5D. The arrow in FIG 5D shows a particular protein that reacted strongly with anti-*T. cruzi* antibodies present in strong positive sera.

15       From the proteins that were screened, many that showed antigenic activity were proteins that had been previously characterized as *T. cruzi* antigens. This provides a level of proof to the capacity of this technique to discover single antigens in pools. Selected ribosomal proteins, ubiquitin, calcium binding proteins, and paraflagellar rod proteins have all been described  
20 previously as being possible targets for serological diagnosis of *T. cruzi* infection. A list of the individual proteins identified as *T. cruzi* antigens using the Bio-Plex screening method are shown below in Table 1. The "Gene ID numbers" represent gene numbers assigned by annotators of the *T. cruzi* genome and are accessed via the *T. cruzi* genome database on the worldwide  
25 web at "TcruziDB.org."

**Table 1**

Assay Protein ID	Protein	T. Cruzi database accession numbers	Gene ID numbers
1a-1	Tc beta-tubulin	Tc00.1047053506563.40	6998.t00004
1a-5	Tc alpha tubulin	Tc00.1047053411235.9	11788.t00001
1c-3	60S ribosomal protein L2, putative	Tc00.1047053508299.60	5568.t00006
2b-3	hypothetical protein, conserved	Tc00.1047053506529.460	6986.t00046
2c-1	cytochrome C oxidase subunit IV, putative	Tc00.1047053506529.360	6986.t00036
2c-9	hypothetical protein	Tc00.1047053506529.610	6986.t00061
2i-1	hypothetical protein, conserved	Tc00.1047053510887.50	6003.t00005
3d-3	iron superoxide dismutase, putative	Tc00.1047053509775.40	5781.t00004
3d-4	trans-splicing factor, putative	Tc00.1047053503583.40	4650.t00004
3j-1	60S ribosomal protein L28, putative	Tc00.1047053506297.270	6890.t00027
3k-1	glycosomal phosphoenolpyruvate carboxykinase, putative (Phosphoenolpyruvate Carboxykinase (Pepck))	Tc00.1047053508441.20	7730.t00002
3k-2	ubiquitin-fusion protein, putative (polyubiquitin / ribosomal protein CEP52)	none	7355.t00001
3k-3	60S acidic ribosomal subunit protein, putative (Calmodulin-ubiquitin associated protein CUB2.8)	Tc00.1047053508355.250	7695.t00025
3k-5	ef-hand protein 5, putative	Tc00.1047053506391.30	6925.t00003
4a-3	paraflagellar rod protein 3	Tc00.1047053509617.20	8152.t00002
B1	axoneme central apparatus protein, putative	Tc00.1047053510955.40	8553.t00004
B2	serine carboxypeptidase (CBP1), putative	Tc00.1047053509695.220	8171.t00022
B5	aminopeptidase, putative	Tc00.1047053511289.30	8647.t00003
B7	elongation factor-1 gamma, putative	Tc00.1047053510163.20	8322.t00002
B8	hypothetical protein, conserved	Tc00.1047053506531.20	6987.t00002
D3	hypothetical protein, conserved	Tc00.1047053506489.30	6967.t00003

Research to improve serological diagnosis techniques has focused on the identification, characterization and cloning of particular *T. cruzi* antigens that elicit a strong B cell response. The use of *T. cruzi* specific antigens in a serological test gives a high level of specificity to a serological test, eliminating the problems that arise due to cross-reactivity to a parasite lysate. However using only a single antigen may not be sensitive enough to detect all individuals that are infected, and thus the use of multiple antigens is preferred. Recent

evidence demonstrates that some individuals declared negative by current serological tests in fact respond to parasite lysate by producing IFN- $\gamma$  in ELISPOT assays. These individuals therefore have T cells that have been exposed to parasite antigen, but have a poor B cell antibody response to the 5 antigens in the serological tests that use parasite lysate. The ability to evaluate the T cell reactivity of individual proteins to sera from various subjects using the Bio-Plex analysis is shown in FIG 6A – 6D.

Example 9: High throughput selection of effective serodiagnostics for *T. cruzi* 10 infection and multiplex diagnostic for Chagas disease

As noted above, diagnosis of *T. cruzi* infection by direct pathogen detection is complicated by the low parasite burden in subjects persistently infected with this agent of human Chagas disease. In this study, we sought to 15 improve upon current diagnostics for *T. cruzi* infection by screening for diagnostic candidates that displayed the ability to detect infection in subjects that went undetected or gave discordant results using other conventional serologic tests. We screened more than 400 recombinant proteins of *T. cruzi*, including randomly selected and those known to be highly expressed in the 20 parasite stages present in mammalian hosts, for the ability to detect anti-parasite antibodies in the sera of subjects with confirmed or suspected *T. cruzi* infection. A set of 16 protein groups were then incorporated into a multiplex bead array format which detected 100% of more than 100 confirmed positive sera and also documented consistent, strong and broad responses in samples undetected or 25 discordant using conventional serologic tests. Each serum had a distinct but highly stable reaction pattern. The end result was thus the identification of a panel of recombinant proteins that more reliably detects *T. cruzi* infection than do a combination of existing conventional tests. Additionally, we show that a multiplex assay utilizing this diagnostic panel has utility in monitoring drug 30 treatment efficacy in chronic Chagas disease. These results substantially extend the variety and quality of diagnostic targets for Chagas disease and offer a useful tool for determining treatment success or failure.

## Methods

*Parasites, gene selection and cloning.* *T. cruzi* epimastigotes of the Sylvio, CL Brenner, Brazil, CL, Tulahuen, M83, M91 and Chapulin isolates were maintained in logarithmic phase growth (Kirchhoff et al., 1984, J Immunol 133: 2731-2735) and used as a source of genomic DNA. Primer sets incorporating lambda phage recombination sites flanking the 18-21 base gene-specific sequence (excluding both start and stop codons), were designed for each gene of interest, the genes cloned by PCR from the pooled DNA of the 8 *T. cruzi* isolates, and Gateway adapted gene product inserted into the pDONR-201 plasmid (Invitrogen, Carlsbad, CA). To speed the cloning process while improving our chances of cloning full-length non-mutated genes, at least 10 clones positive for the appropriate sized insert were pooled for each gene. *T. cruzi* lysate from Brazil strain amastigote and trypomastigotes was prepared as previously described (Laucella et al., 2004, J Infectious Diseases 189:909-918).

*pDEST-PTD construction.* The pDEST-PTD protein expression vector was created from pTAT-HA (Nagahara et al., 1998, Nat Med 4: 1449-1452) by replacing the BamHI-flanked TAT sequence with a BamHI-flanked PTD-4 encoding sequence (Ho et al., 2001, Cancer Res 61: 474-477), followed by Gateway (Invitrogen)- adaptation of the plasmid utilizing the NcoI and XhoI cloning sites. The Gateway cloning cassette was PCR amplified from pDEST-YFP (gift from Dr. Boris Striepen, University of Georgia, GA).

*Protein production and purification.* Genes in pDONR plasmids were transferred to pDEST-PTD4 via a Gateway LR reaction and the proteins expressed in BL21(DE3)pLysS cells were extracted by sonication in 8M urea, 20mM HEPES, 100mM NaCl, pH 8.0 containing 15mM imidazole. The lysate was then applied to TALON Metal Affinity Resin (BD Biosciences Clonetech, Palo Alto, CA) and bound protein was eluted with 250mM imidazole. Imidazole was removed on PD-10 desalting columns (GE Healthcare, Piscataway, NJ) and protein concentration was estimated using a modified Bradford assay. Proteins were diluted to 10 $\mu$ g/mL (in 8M urea) and stored in 1mL aliquots at -20C until use.

*Human sera.* Sera were obtained from individuals living in areas of Santiago del Estero, Argentina endemic for *Trypanosoma cruzi* and were

analyzed using conventional serologic tests (e.g. immunofluorescence assay (IFI), hemagglutination (HAI), and ELISA) performed at the Diagnostic Department of the Instituto Nacional de Parasitología "Dr. Mario Fatala Chabén" and in our laboratory by a commercial ELISA serodiagnostic kit 5 (Hemagen Diagnostics, Columbia, MD). The latter assay was carried out as per the manufacturer's instructions with a positive response defined as 10% above the cutoff (0.250+mean of negative control absorbencies). Three serum pools were created: a "sero-negative" pool consists of 4 sera negative on all assays; a "borderline positive" pool made up of 5 sera with a response at or just above the 10 equivocal zone of the Hemagen test (between cutoff and below cutoff + 10%); a "strong positive" pool containing 7 sera that gave unequivocally positive responses on all tests. True negative controls were obtained from volunteer donors who were not from endemic areas. Sera used for subsequent analysis of individual proteins were obtained from *T. cruzi*-infected adult volunteers aged 15 29 to 61 recruited through the Chagas Disease Section of the Cardiology Department, Hospital Interzonal General de Agudos "Eva Perón", Buenos Aires, Argentina and infection status was determined serologically as described above. In some cases, subjects treated by a 30 day course of benznidazole as previously described (Viotti et al., 2006, Ann Intern Med 144: 724-734) donated 20 serum samples prior to treatment and at regular intervals following treatment. The protocols were approved by the IRBs of the University of Georgia and the Hospital Interzonal General de Agudos "Eva Perón" and signed informed consent was obtained from all individuals prior to inclusion in the study.

*Multiplex assay.* Recombinant proteins were attached to Liquichip™ 25 Ni-NTA beads (Qiagen) or Beadlyte Nickel Beads (Upstate Biotechnology) by overnight incubation at 4°C in the dark. The sets of distinct addressable beads, each with a different protein attached, were pooled in equal volumes along with positive and negative control beads, consisting respectively of Liquichip™ Carboxy Beads (Qiagen) coupled to *T. cruzi* lysate and Liquichip™ Ni-NTA 30 beads coated with recombinant HIS-tagged green fluorescent protein (GFP). Sera at 1:500 dilutions were added and the multiplex assays conducted using standard procedures (Waterboer et al., 2006, J Immunol Methods 309: 200-204). Antibody binding to individual beads was detected with goat anti-human

IgG conjugated to phycoerythrin (Jackson ImmunoResearch, West Grove, PA) and quantified on a BioPlex Suspension Array System (BioRad).

*Statistical analysis.* Serum samples were assayed in duplicate and the weighted mean fluorescence intensity (MFI) was calculated for a minimum of 5 30 beads per determination. The ratio of the specific MFI for each antigen to the MFI of the negative control (GFP- or OVA-coupled) protein was then calculated for each serum and antigen in the assay. Values above the mean plus 4 standard deviations of a minimum of sixteen true negative sera run in the same assay, and individually determined for each antigen, were considered 10 positive.

## Results

As part of a vaccine discovery effort, nearly 1500 genes from *T. cruzi* have been cloned into Gateway entry vector plasmids that allow them to be 15 easily moved into a range of other plasmids. Genes were selected for cloning using a variety of criteria, initially including known expression in *T. cruzi* lifecycle stages that are present throughout infection in mammals (e.g. trypomastigotes and amastigotes), high likelihood of being surface expressed or secreted and expected presence in the genome at low copy number. With the 20 completion of the *T. cruzi* genome sequencing project (El-Sayed et al., 2005, Science 309: 409-415) and whole organism proteome analysis (Atwood et al., 2005, Science 309: 473-476) the additional criterion of being relatively high in abundance in the proteomes of trypomastigotes and amastigotes was added as a basis for selection. Recombinant proteins produced in *E. coli* had N-terminal 25 tags carrying the 6XHis-, PTD (Ho et al., 2001, Cancer Res 61: 474-477) and HA- tags for purification, protein translocation, and identification, respectively were captured by Ni-coupled Luminex beads for use in a multiplex bead array assay.

*Selection of the diagnostic panel.* The initial selection screen (Figure 7) 30 used approximately 420 proteins produced in pools of 8-10 proteins each. Production of pooled proteins was accomplished by moving sets of genes in batch into the PTD-4 expression plasmid and was confirmed by SDS-PAGE analysis (Figure 8). In addition to the individual or pooled recombinant *T. cruzi*

proteins, each screening experiment included negative control recombinant protein (ovalbumin or GFP) expressed from the PTD-4 plasmid as well as a lysate of trypomastigotes and amastigotes of *T. cruzi* that had been chemically coupled to BioPlex beads.

5 To screen the pooled proteins we also took a pooling approach by assembling sera from subjects with and without documented infection with *T. cruzi*. Screening of 51 protein pools revealed 21 pools that were reactive with one or more of the serum pools 1-3 (Figure 7). Reactive pools were then broken down into their individual constituent proteins; a total of 140 proteins were  
10 successfully expressed and individually rescreened with the serum pools, ultimately resulting in the selection of 55 proteins with serodiagnostic potential (Figure 7 and Table 2). An additional 22 proteins that were either identified as high-abundance proteins using proteome analysis (Atwood et al., 2005, Science 309: 473-476) and/or as being unique to *T. cruzi* (and thus not encoded in the *T. brucei* or *Leishmania major* genomes) were then screened using the pooled sera,  
15 and 4 of these 22 were found to be reactive with one or more serum pools. Of the resulting 59 candidate proteins recognized by antibodies in the serum of *T. cruzi*-infected subjects, a substantial number were subsequently excluded from further testing either because they exhibited significant reactivity with sera from  
20 the true negative pool, or because they interfered with other beads in the multibead assays, perhaps because of protein-protein interactions. Preference was also given to *T. cruzi* proteins that detected antibodies in sera from the “borderline” pools. Ultimately 39 proteins (in bold and italics in Table 2) were selected for extensive further testing with a wider array of individual subject  
25 sera.

Table 2: The 59 candidate diagnostic proteins screened independently with individual (non-pooled) sera.

Gene Id	Gene name(s)	Notes	% reactive with 121 known positive sera
Tc00.1047053506391.10_and_Tc00.1047053509233.180	<u>calmodulin and ATPase beta subunit</u>	high abundance	32.23%
Tc00.1047053507029.30	<u>heat shock 70 kDa protein, mitochondrial precursor, putative</u>	high abundance	52.89%
Tc00.1047053510955.40	<u>axoneme central apparatus protein, putative</u>		42.15%
Tc00.1047053511215.119	<u>69 kDa paraflagellar rod protein, putative</u>		23.97%
Tc00.1047053511271.10	<u>dispersed gene family 1 fragment 4</u>	unique to <i>T. cruzi</i>	5.08%
Tc00.1047053506529.610	<u>hypothetical protein</u>		17.27%
Tc00.1047053506391.30	<u>EF-hand protein 5</u>		2.48%
Tc00.1047053506635.130	<u>hypothetical protein, conserved</u>	high abundance	68.60%
Tc00.1047053511265.10	<u>dispersed gene family 1 fragment 5</u>	unique to <i>T. cruzi</i>	8.62%
Tc00.1047053511289.30	<u>aminopeptidase, putative</u>		11.57%
Tc00.1047053506195.110	<u>malate dehydrogenase, putative</u>	high abundance	24.79%
Tc00.1047053508461.140	<u>poly(A)-binding protein</u>	high abundance	34.17%
Tc00.1047053508441.20	<u>glycosomal phosphoenolpyruvate carboxykinase, putative</u>	high abundance	59.29%
Tc00.1047053508355.250	<u>60S acidic ribosomal subunit protein, putative</u>	high abundance	75.21%
Tc00.1047053511633.79	<u>microtubule-associated protein homolog</u>	high abundance	74.38%
Tc00.10470535010433.20_and_Tc00.1047053504277.11_and_Tc00.1047053504157.130	<u>TolT proteins</u>	unique to <i>T. cruzi</i>	74.38%
Tc00.1047053411235.9	<u>alpha tubulin</u>		
Tc00.1047053510877.30	<u>hypothetical protein, conserved</u>		
Tc00.1047053509695.220	<u>serine carboxypeptidase (CBP1), putative</u>		
Tc00.1047053510887.50	<u>hypothetical protein, conserved</u>		
Tc00.1047053509144.40	<u>hypothetical protein, conserved</u>		
Tc00.1047053506247.220	<u>histidine ammonia-lyase</u>		
Tc00.1047053509995.10	<u>60S ribosomal protein L4, putative</u>		
Tc00.1047053504163.50	<u>fructose-bisphosphate aldolase, glycosomal, putative</u>		
Tc00.1047053507089.270	<u>dihydrolipoil dehydrogenase, putative</u>		
Tc00.1047053511019.90	<u>iron superoxide dismutase, putative</u>		
Tc00.1047053509017.20	<u>hypothetical protein, conserved</u>		
Tc00.1047053506529.360	<u>cytochrome C oxidase subunit IV, putative</u>		
Tc00.1047053510187.50	<u>tyrosine aminotransferase, putative</u>		
Tc00.1047053505989.110	<u>hypothetical protein, conserved</u>		
Tc00.1047053508209.140	<u>protein disulfide isomerase, putative</u>		
Tc00.1047053506531.20	<u>hypothetical protein, conserved</u>		
Tc00.1047053504153.280	<u>hypothetical protein, conserved</u>		
Tc00.1047053509233.180	<u>ATPase beta subunit, putative</u>		
Tc00.1047053506563.40	<u>beta tubulin</u>		
Tc00.1047053506459.290	<u>elongation factor-1 gamma, putative</u>		
Tc00.1047053508707.200	<u>nucleoside diphosphate kinase, putative</u>		
Tc00.1047053506529.460	<u>hypothetical protein, conserved</u>		
Tc00.1047053506297.270	<u>60S ribosomal protein L28, putative</u>		
Tc00.1047053511527.34	<u>60S ribosomal protein L2, putative</u>		
Tc00.1047053507483.4	<u>polyubiquitin, putative</u>		
Tc00.1047053509053.70	<u>p22 protein precursor, putative</u>		
Tc00.1047053506585.40	<u>glucose-regulated protein 78, putative</u>		
Tc00.1047053511185	<u>dispersed gene family 1 fragment 8</u>		
Tc00.1047053511589.130	<u>14-3-3 protein, putative</u>		
Tc00.1047053511167.90	<u>14-3-3 protein, putative</u>		
Tc00.1047053507241.30	<u>arginine kinase, putative</u>		
Tc00.1047053510579.70	<u>nascent polypeptide associated complex subunit, putative</u>		
Tc00.1047053506925.300	<u>cyclophilin a</u>		
Tc00.1047053509775.40	<u>iron superoxide dismutase, putative</u>		
Tc00.1047053503583.40	<u>trans-splicing factor, putative</u>		
Tc00.1047053510099.120	<u>d-isomer specific 2-hydroxyacid dehydrogenase-protein, putative</u>		
Tc00.1047053507093.300	<u>hypothetical protein, conserved</u>		
Tc00.1047053508479.340	<u>succinyl-CoA synthetase alpha subunit, putative</u>		
Tc00.1047053509815.120	<u>dispersed gene family 1 fragment 9</u>		
Tc00.1047053511727.270	<u>RNA-binding protein, putative</u>		
Tc00.1047053503781.80	<u>universal minicircle sequence binding protein (UMSBP), putative</u>		
Tc00.1047053506201.39	<u>translation elongation factor 1-beta, putative</u>		
Tc00.1047053506816.50	<u>hypothetical protein</u>		

5 Note: Tc00 numbers indicate closest homologue(s) present in the *T. cruzi* CL Brener sequence database (TcruziDb.org) based upon sequencing of the genes (for top 16) or predicted based upon primer sequences used in cloning. Because some primers for PCR cloning were designed prior to the release of the *T. cruzi* CL Brener sequence and the cloning involved the pooling of multiple clone derived from the PCR of a mixture of *T. cruzi* strains (see Material and Methods), some proteins were derived from mixtures of genes (e.g. numbers 1 and 16) and/or had a percent sequence identity <100% relative to the CL Brener strain (range 94.7 to 100%). In some cases (e.g. # 5 and 9) genes > 2 kb in length were cloned in ~2 kb fragments in order to facilitate cloning and protein production. Items listed in bold type were selected for screening using >100 individual sera. Items underlined were selected to be part of the final 16 set bead array for screening of discordant sera or sera from subjects post-treatment with benznidazole.

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- Although the Luminex bead array technology theoretically accommodates up to 100 distinct, addressable beads in a single well – and thus the ability to assay up to 100 individual proteins - at the time of this work only 17 distinct beads were available with the ability to capture his-tagged proteins.
- 5 Thus our goal in the second part of the screen was to identify a set of the 16 best *T. cruzi* proteins (allowing a bead for a control non-*T. cruzi* protein). The 39 candidate diagnostic proteins were tested in sets of 8-15, with each protein on a separate bead and with a negative control bead (HIS-tagged ovalbumin (OVA)) and a positive control bead (*T. cruzi* lysate) included in each assay sample.
- 10 Between 38 and 48 individual sera from endemic subjects were used to test each protein. These sera are grouped as “uniformly positive” (reactive on all conventional serological tests), “inconclusive” (positive on at least one, but not all, conventional serologic tests), and “negative by conventional tests”, and “known negative” (from residents of North America). Figure 9 shows a
- 15 representative set of 29 proteins tested with 54 individual sera and indicates the range of reactivities of both sera and proteins. In addition to providing the basis on which to select the top proteins, this analysis also revealed that among the 30 sera that were inconclusive or negative on conventional tests, nearly half (14 of 30) had substantial reactivity to 3 or more recombinant *T. cruzi* proteins but not
- 20 with the control OVA protein.

Following repeated screening, 16 proteins were selected to be part of the diagnostic panel (underlined in Table 2). DNA sequencing and mass spectrometric analysis confirmed the identity of each gene and protein and determined that one of the preparations contained two distinct proteins

25 (Calmodulin and an ATPase) and a second contained a mixture of related TolT proteins. This protein set was then used to screen a larger set of sera, most from chronically infected subjects living in Buenos Aires, and the percentage of these proteins reactive with 121 sera from well-characterized subjects was determined (Table 2). A serum was determined to be positive for any particular test

30 antigen if the average luminescence (MFI) was >4 standard deviations above that of a set of true negative sera run in the same assay. Across all experiments, for the 19 true negative sera assayed multiple times (142 sample runs tested on 16 protein preparations for a total of 2272 determinations), none had S.D. >4

and only 17 of the 2272 determinations were >3 S.D. above the average negative serum values (and 9 of these 17 were from one serum sample reactive with the same antigen in multiple tests). Thus this was a highly stringent cutoff. Sera from all 121 of the confirmed chronically infected subjects reacted with at least 1 of the 16 recombinant protein preparations at the >4 S.D. cutoff and all but 7 reacted with >1 protein. As shown in Table 2, 6 of the 16 of the antigens each detected >50% of the sera and 3 antigens approached a 75% detection rate. Of the 121 sera tested, 118 would have been detected as positive using only 4 of the antigens and 100% would be detected using as few as 7 antigens.

Borderline samples. We then used our 16 bead multiplex test to attempt to resolve questionable infection status in subjects due to discordant results on conventional tests (Table 3). In this analysis, a cutoff for reactivity for each protein in the panel was set at the MFI plus 4 SD above the mean of a set of 16 negative sera. For comparison, the result of multiplex analysis of a pool of strongly positive sera assayed on different days is also shown. The strong positive serum pool showed excellent cross-assay consistency with 11 of the 16 protein preparations positive on each of 8 assays and consistent negative reactivity with 3 of 16. Antibodies to the remaining 2 proteins were also detected but at a lower level that sometimes fell below the strict cutoff of 4 S.D. above the mean. The sera classified as “conventional seronegative with no other evidence of infection” broke into 2 groups based upon the results of the multiplex test. Eight of the 16 failed to react with any of the 16 protein panel (although several reacted with the *T. cruzi* lysate) while the remaining 8 reacted with from 2-4 proteins. A similar nearly 50/50 split was observed in the group of 12 conventional seronegatives who were born in an endemic region, and in 5 individuals who had cardiopathologies consistent with Chagas disease. Lastly, testing in the multiplex assay of sera classified as “positive discordant” (based upon reactivity on 2 of the 3 conventional serologic tests but negative on the 3<sup>rd</sup> test) confirmed the positive diagnosis in all 7 cases with reactivity evident on 2-6 recombinant proteins by each serum. Without a clear gold standard diagnostic it is not possible certify on a case-by-case basis that the multiplex assay more accurately detects infection than does conventional serology - particularly in cases where there is reactivity to only 1 or 2 proteins and near the

>4 S.D. cutoff. And while the birth place and presence of heart disease may support a positive diagnostic test, these criteria do not appear to distinguish between those likely to have reactivity with one or more recombinant proteins in the selected panel and those who do not react. However it is clear that

5 conventional serological tests fail to detect a substantial number of individuals, many with antibodies to multiple *T. cruzi* antigens. It is noteworthy that screening of sera with a parasite lysate also routinely fails to detect sera that exhibit reactivity to multiple recombinant *T. cruzi* proteins. The set of 4 most frequently recognized proteins detected all 7 of the discordant positive samples

10 as well as 13 of the 15 discordant negative or negative samples that reacted with at least 1 protein. Expanding the panel to the 7 proteins that detected all of the seropositive samples (see above) allowed us to detect all of these 15 questionable “negative” samples.

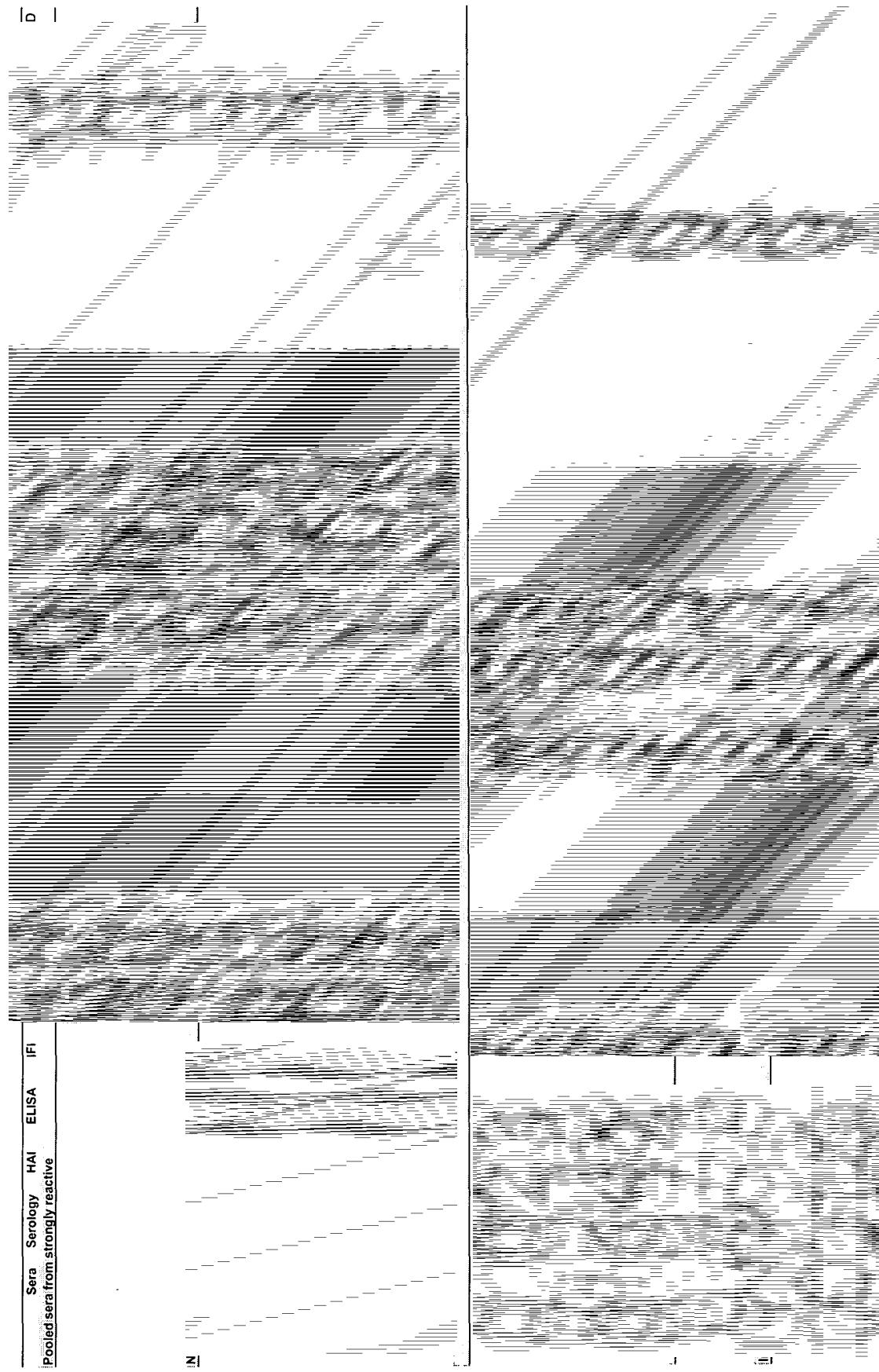
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Table 3: Reactivity of negative, borderline or discordant sera in the 16 protein multiplex assay.



Sera judged cumulatively as “seronegative” based upon conventional serology were grouped into negative but “no other evidence” of exposure (16 sera), those “born in an endemic area” (12), those with evidence of “heart disease” consistent with Chagas disease(5) and compared to (top) pools of strongly positive sera (high reactivity in all serological tests) and to (bottom) sera from subjects who were negative on at least one of the three conventional serologic tests (discordant positive). Reactivity in the conventional serological tests (HAI, ELISA and IFI) and the summary consensus of these tests (neg = below cut-off for all three tests; discord = positive on one of the three tests; discord + = positive on 2 of the 3 tests), as well as reactivity to the 16 recombinant protein sets and the *T. cruzi* lysate are shown. Cutoffs for a positive ELISA is an O.D.> 0.200 and for IFA and HAI is a dilution >1/32 (a reaction at 1/16 is considered “reactive but negative” and < 1/16 non-reactive (nr)). The metric for reactivity of each serum for each protein is expressed as the number of standard deviations that the ratio of the MFI for *T. cruzi* protein to the MFI for GFP was above the average ratios of sixteen true negative sera run in the same assay. Values >4 S.D. above this “background” reactivity are considered reactive and are highlighted. The total number of reactive recombinant proteins for each serum is indicated in the right-most column. nd = not determined (insufficient numbers of beads detected in this sample)

*Monitoring treatment efficacy.* There is a pressing need for a means to assess treatment efficacy in Chagas disease so we next used the multiplex assay to monitor changes in serology over time in subjects treated with benznidazole (BZ). Representative data from a set of 16 non-endemic normals (Figure 10A) demonstrates the background level of detection of responses in uninfected individuals, displayed as the MFI for each protein. To establish the stability of serological responses over time in the absence of treatment, serial serum samples were obtained from chronically infected, seropositive subjects, all without clinical disease; a representative set of 6 subjects screened at 4 times points for up to 21 months is shown in Figure 10B. Each subject exhibits a distinct pattern of serological responses and both the pattern and the potency of those responses are remarkably stable over time. In contrast, a representative set of 4 (from a total of 38) subjects followed for up to 36 months after treatment with BZ shows that some subjects exhibit a post-treatment decrease in the strength of responses to most *T. cruzi* antigens tested (Figure 11A). In many cases this fall is evident by 2 months post-treatment (e.g. PP001, PP115, PP164) and is followed by a transient increase at 6 months. Interestingly, this early drop in antibody levels following treatment is also sometimes evident, although less consistently so, with conventional serological tests, particularly with indirect hemagglutination (Figure 11A). Subject PP117 has borderline positive

serology in both the multiplex and the conventional serologic assays, and is representative of a case in which documenting changes following treatment would be difficult. Figure 11A). Figure 11B presents 2 other patterns of responses following treatment. PP044 shows essentially no change in the 5 pattern or potency of antibody responses up to 24 months post-treatment. Subject PP024 is similar in that responses to the several prominently detected proteins are relatively stable over time. However the MFI reading for numerous other antigens falls consistently over the 24 month monitoring period. Thus, although it might take more time and additional assays to determine treatment 10 efficacy in these two subjects, a preliminary assessment would be that treatment failed in the case of PP044 but was successful for subject PP024.

## Discussion

The poor quality of diagnostics for *T. cruzi* infection is a major 15 impediment to coping with a disease that affects as many as 20 million people. Without quality diagnostics, the statistic of the disease burden is at best a guess, the ability to conclusively identify who should be treated, or should be allowed to donate blood or tissues is greatly compromised and the effectiveness of interventions to limit transmission or drugs to treat those infected is impossible 20 to determine with any certainty.

In the early stages of *T. cruzi* infection, parasites can often be detected in blood. However, as immunity develops, even amplification techniques such as xenodiagnosis, hemaculture, and PCR, despite being repeated multiple times, routinely fail to detect infection (Castro et al., 2002, Parasitol Res 88: 894-900; 25 Picka et al., 2007, Braz J Infect Dis 11: 226-233; Salomone et al., 2000, Am J Cardiol 85: 1274-1276; Duarte et al., 2006, Rev Soc Bras Med Trop 39: 385-387). Consequently, determination of infection status is largely dependent on the consensus results of multiple tests with different formats (e.g. ELISA, indirect fluorescent antibody, indirect hemagglutination, complement fixation). 30 However the unreliability of these tests is well documented (Pirard et al., 2005, Transfusion 45: 554-561; Salomone et al., 2003, Emerg Infect Dis 9: 1558-1562; Avila et al., 1993, J Clin Microbiol 31: 2421-2426; Castro et al., 2002, Parasitol Res 88: 894-900; Caballero et al., 2007, Clin Vaccine Immunol.

14:1045-1049; Silveira-Lacerda et al., 2004, Vox Sang 87: 204-207; Wincker et  
al., 1994, Am J Trop Med Hyg 51: 771-777; Gutierrez et al., 2004, Parasitology  
129: 439-444; Marcon et al., 2002, Diagn Microbiol Infect Dis 43: 39-43; Picka  
et al., 2007, Braz J Infect Dis 11: 226-233; Zarate-Blades et al., 2007, Diagn  
5 Microbiol Infect Dis 57: 229-232). Many of these tests, including one recently  
licensed by the United States Food and Drug Administration for use as a blood  
screening test in the U.S. (Tobler et al., 2007, Transfusion 47: 90-96), use crude  
or semi-purified parasite preparations derived from parasite stages present in the  
insect vector but not in infected humans. Recently a number of recombinant  
10 parasite proteins or peptides have also come into limited use for diagnosis (da  
Silveira et al., 2001, Trends Parasitol 17: 286-291; Chang et al., 2006,  
Transfusion 46: 1737-1744; Kirchhoff et al., 1984, J Immunol 133: 2731-2735;  
Laucella et al., 2004, J Infectious Diseases 189:909-918).

A subject whose serum is consistently positive on multiple of the  
15 currently used tests is relatively easily determined to be infected. But the  
infection status of individuals positive on only one test (as in blood bank  
screening) is unclear and detection of parasites in subjects who are negative  
using multiple conventional serologic tests (Salomone et al., 2003, Emerg Infect  
Dis 9: 1558-1562; Gutierrez et al., 2004, Parasitology 129: 439-444; Marcon et  
20 al., 2002, Diagn Microbiol Infect Dis 43: 39-43; Picka, et al., 2007, Braz J  
Infect Dis 11: 226-233; Wincker et al., 1994, FEMS Microbiol Lett 124: 419-  
423) or who are positive by alternative but not widely available serological tests  
(Caballero et al., 2007, Clin Vaccine Immunol. 14:1045-1049; Zarate-Blades et  
al., 2007, Diagn Microbiol Infect Dis 57: 229-232) is not uncommon.  
25 Furthermore, currently available tests are inadequate for monitoring treatment  
efficacy (Sanchez Negrette et al., 2008, Clin Vaccine Immunol 15: 297-302;  
Bahia-Oliveira et al., 2000, J Infect Dis 182: 634-638; Solari et al., 2001, J  
Antimicrob Chemother 48: 515-519) and thus may also give inaccurate  
measurements of the effectiveness of other interventions.

30 With these deficits in mind, we set out to identify parasite proteins that  
would more effectively detect *T. cruzi* infection and provide a tool for  
monitoring changes in infection status over time. Development of a repository  
of nearly 1500 *T. cruzi* genes cloned into Gateway entry vectors provided a

relatively straightforward approach to producing a large number and diversity of *T. cruzi* proteins appropriate for high-throughput screening of diagnostics. Adding the targeted approach of selecting proteins documented for high level expression in trypomastigote and amastigote stages of *T. cruzi* allowed us to

5 also focus on the proteins that would be predicted to elicit the strongest antibody response in infected humans. The Luminex-based multiplex bead array system permitted us to screen many proteins simultaneously with very low requirements for serum. The production of histidine-tagged proteins also made it relatively uncomplicated to attach the recombinant proteins to Luminex

10 beads. This latter point is not trivial as the proteins could be coupled to the assay beads directly from the denaturing urea-based lysis buffer without the requirement of movement to a non-denaturing buffer, wherein many of the proteins precipitated. The strong response detected using proteins prepared in this way suggests either that natively folded proteins are not required for the

15 detection of these antibodies or that re-folding of the proteins attached to the Luminex beads during buffer exchange resulted in the formation of native conformational epitopes.

In addition to its utility for screening of a large number of proteins, the Luminex system also excels as a platform for multiplex analysis of antibodies to

20 a relatively large set of targets. We were restricted in this work by the number of Luminex bead sets manufactured with Ni<sup>+2</sup> and thus sought to identify a maximum of 16 independent *T. cruzi* proteins that gave informative results from a large set of human sera. The ultimate panel selected by the screen included at least one protein previously identified as a potential diagnostic, the

25 mitochondrial HSP-70 (Krautz et al., 1998, Am J Trop Med Hyg 58: 137-143). It is possible that other proteins revealed in our screen have been studied previously. However since the identity of some of these previously assayed proteins is somewhat cryptic (da Silveira et al., 2001, Trends Parasitol 17: 286-291) and few have been associated with annotated genes in the sequenced *T.*

30 *cruzi* genome, this is difficult to determine. Also, over half of the antigens selected in our screen were among the 50 most abundant proteins in the trypomastigote and amastigote proteomes (Atwood et al., 2005, Science 309: 473-476). Two hypothetical proteins and 2 proteins unique to *T. cruzi* among

the sequenced kinetoplastids, including 2 fragments from the very large and multicopy dispersed gene family protein, were among the proteins selected. Proteins that are unique to *T. cruzi* could be particularly useful in a serological screen as they are absent from *Leishmania*, one of the potentially confounding infections in terms of diagnosis of *T. cruzi*. However the dispersed gene family fragments were among the worst performers in the large scale screen – with only 5-9% of all confirmed positive sera having detectable antibodies to these. Similarly, other gene family proteins, including trans-sialidases, mucins and mucin-associated proteins (MASPS) were part of the screen but failed to make even the initial selection cuts in our assays, presumably because only a small fraction of their diversity would be represented in the recombinant proteins screened.

A multiplex approach like the Luminex also provided a more detailed examination of responses than is possible using a single target consisting of either an individual protein or a protein/peptide mixture. Each individual was seen clearly to have a distinct pattern of responses to the protein panel and that this pattern was impressively stable over time (several years). This is both interesting scientifically and serves as further validation of the quality and consistency of the data generated using this multiplex methodology. This heterogeneity of responses to pathogens among individuals appears to be more the norm than the exception, as similar results have been reported for individuals infected or immunized with viral (vaccinia), bacterial (*Francisella tularensis*) and protozoal (*Plasmodium falciparum*) pathogens (Davies et al., 2007, Proteomics 7: 1678-1686; Sundaresh et al., 2006, Bioinformatics 22: 1760-1766; Sundaresh et al., 2007, Bioinformatics 23: i508-518). Thus serodiagnostics in general are likely to need to move toward multiplex assays, as single antigens that are recognized by all individuals infected by any pathogen appear to be rare (Davies et al., 2007, Proteomics 7: 1678-1686).

The ability to simultaneously and independently assess antibody responses to multiple targets was instrumental to our success in addressing the issues of the detection of serological responses in subjects who are negative by conventional serology and the relatively rapid detection of changes in selected responses following drug treatment. The multiplex assay detected 100% of 121

samples consistently positive by conventional serology, and 100% of samples positive on 2 out of 3 conventional tests. In addition, however, we also detected antibodies specific for one or more recombinant proteins in 18 of 33 subjects judged as negative by conventional serology. Other investigators have

5 documented cases of conventional seronegative subjects being seropositive on alternative tests or even parasite positive (Salomone et al., 2003, Emerg Infect Dis 9: 1558-1562; Caballero et al., 2007, Clin Vaccine Immunol. 14:1045-1049; Gutierrez et al., 2004, Parasitology 129: 439-44; Marcon et al., 2002, Diagn Microbiol Infect Dis 43: 39-43; Picka et al., 2007, Braz J Infect Dis 11: 226-

10 233; Zarate-Blades et al., 2007, Diagn Microbiol Infect Dis 57: 229-232; Wincker et al., 1994, FEMS Microbiol Lett 124: 419-423) although these previous reports of “infected seronegatives” have been somewhat anecdotal – presumably because investigators rarely screen for parasites in seronegative subjects. However in some studies parasite-positive conventional seronegatives

15 are very well documented. For example Picka et al. (Braz J Infect Dis 11: 226-233, 2007) reported on one subject who was negative by up to 5 replicates of 4 different conventional serological tests yet was positive by a combined hemaculture-PCR approach. The multiple examples of failed conventional serology to detect infection in combination with the well-documented

20 unreliability of parasitological tests, supports the conclusion that individuals who are seropositive in our multiplex assay are likely to be infected with *T. cruzi*. This conclusion is further supported by on-going studies demonstrating *T. cruzi* –specific T cell responses in subjects who are negative by conventional serology but positive in our multiplex assays. Without more sensitive

25 parasitological tests we cannot conclusively determine if the subjects who are negative by conventional serology but positive in our multiplex assay are infected or possibly “exposed” but not still infected with *T. cruzi*. And without additional extensive validation, we cannot exclude the possibility that other infections or immunological conditions resulted in some of the multiplex

30 positive responses, although standard clinical analysis failed to detect other complicating infections in these subjects. However, especially for subjects who have antibodies to up to 8 different recombinant *T. cruzi* proteins and were born in endemic areas and/or have evidence of heart disease, it is reasonable to

conclude that they are indeed infected with *T. cruzi* despite their negative results with conventional serologic assays. Overall these studies support the already documented conclusion that current serological tests can misdiagnose infection – perhaps to a significant extent.

5 A second issue we addressed using the multiplex serological assay for *T. cruzi* infection was that of efficacy of therapeutic treatment. Because most subjects are negative by parasitological assays prior to treatment (making a negative result after treatment uninformative) and remain positive by conventional serology for extensive periods of time after treatment, assessing  
10 whether treatment actually achieved cure) has been problematic. When combined with other evidence of treatment failures and the adverse effects of the drugs, the absence of a method to detect treatment efficacy has resulted in a very low rate of treatment in chronic Chagas disease. This absence of a reliable and timely test for treatment efficacy is also a major impediment to the  
15 development and testing of new drugs – an area that has been at a virtual standstill for decades.

Herein we show that the multiplex assay using the selected set of recombinant proteins can detect significant changes in antibody levels, in some cases as early as the first post-treatment assay point (2 months post-treatment  
20 completion). These changes are not evident in all cases – an outcome that is not surprising given that treatment failure is common (Viotti et al., 1994, Am Heart J 127: 151-162). Our ability to assess responses to multiple targets on an individual basis appears to be crucial to the success of detection of serologic changes following treatment, as similar changes are not consistently observed  
25 using conventional serologic tests. Previous studies have suggested that various recombinant antigens may provide better assessment of treatment efficacy relative to conventional serology (Sanchez Negrette et al., 2008, Clin Vaccine Immunol 15: 297-302; Sosa Estani et al., 1998, Am J Trop Med Hyg 59: 526-529).

30 In conclusion, we define a set of diagnostic targets and an assay approach that we believe is a significant improvement upon current diagnostic tests for *T. cruzi* infection both for more consistently detecting infection and for assessing the effectiveness of treatment. Additional validation of these targets

and the general methodology will require analysis of a larger set of subjects, a process that is currently on-going. Herein we have also not addressed the question of whether the antigens we identify would be useful throughout the wide endemic range for *T. cruzi*. Heterogeneity among different parasites  
5 strains in distinct regions could present a challenge. However here again this is a concern that a multiplex assay might rather easily address – it seems unlikely that all 16 proteins in our pool, most of which are abundant housekeeping proteins, would vary substantially among parasites in various regions. The problem of infection confirmation by detection of parasites or parasite products  
10 is likely to continue to be a roadblock to full acceptance of the results of this test, or any other, when they conflict with conventional serologic tests – despite the proven inadequacy of these “standard” tests. A downside of the Luminex system for multiplex analysis is the reagent expense as well as the requirement for specialized equipment to “read” the results. However, other multiplex  
15 platforms such as protein microarrays could be more cost conservative and require less infrastructure (Davies et al., 2007, Proteomics 7: 1678-1686; Kartalov et al., 2006, Biotechniques 40: 85-90). Also, our results suggest that the number of proteins in the analysis could be reduced without substantial loss of sensitivity and the possibility exists for additional improvements in  
20 sensitivity by the inclusion of *T. cruzi* proteins previously validated by others, or that could be detected in additional screens like that described herein. At a minimum, these results begin to lay the groundwork for the removal of one of the major impediments to the development and effective implementation of treatments for *T. cruzi* infection.

25

#### Example 10. Preliminary Panel of Serodiagnostic Proteins

In a preliminary study that eventually resulted in the serodiagnostic  
30 proteins described in Example 9, 53 diagnostic proteins was selected (Table 4) from a group of 59 candidate proteins identified through screening with serum pools and individual sera. The preliminary serodiagnostic panel selected for further study consisted of the top 16 proteins in Table 4.

Table 4. Protein antigens

Current panel constituents	Published as antigens?	Common Name	Annotated gene or Tc00 id of closest homolog
1		hypothetical protein	Tc00.1047053508767.10
2		hypothetical protein, conserved	Tc00.1047053506635.130
3	1,3 (ribosomal P protein, TcP0?)	60S acidic ribosomal subunit protein, putative	Tc00.1047053508355.250
4	1-2 (flagellar CaBP, 1F8)	flagellar calcium-binding protein, putative	Tc00.1047053507491.151
5	1.3 (MAP)	microtubule-associated protein, putative	Tc00.1047053511633.79
6	1 (heat shock)	heat shock 70 kDa protein, mitochondrial precursor, putative	Tc00.1047053507029.30
7	1 (parafagellar assoc. prot)	69 kDa parafagellar rod protein, putative	Tc00.1047053511215.119
8		EF-hand protein 5	Tc00.1047053506391.30
9		aminopeptidase, putative	Tc00.1047053511289.30
10		axoneme central apparatus protein, putative	Tc00.1047053510955.40
11		hypothetical protein	Tc00.1047053506529.610
12		glycosomal phosphoenolpyruvate carboxykinase, putative	Tc00.1047053508441.20
13		dispersed gene family protein 1 fragment	Tc00.1047053511271.10
14		malate dehydrogenase, putative	Tc00.1047053506195.110
15		dispersed gene family protein 1 fragment	Tc00.1047053511265.10
16		poly(A)-binding protein	Tc00.1047053508461.140
<b>Understudy - ranked by approximate potential</b>			
17	1 (cytoskeleton assoc?)	beta tubulin	Tc00.1047053506563.40
18	1 (cytoskeleton assoc?)	alpha tubulin	Tc00.1047053411235.9
19	1 (ribosomal prot)	60S ribosomal protein L28, putative	Tc00.1047053506297.270
20	1 (ribosomal prot)	polyubiquitin (pseudogene), putative	Tc00.1047053507483.4
21		iron superoxide dismutase, putative	Tc00.1047053511019.90
22		iron superoxide dismutase, putative	Tc00.1047053509775.40
23		elongation factor-1 gamma, putative	Tc00.1047053506459.290
24		hypothetical protein, to be annotated	Tc00.1047053507515.4
25		dispersed gene family protein 1 fragment	Tc00.1047053509815.120
26		hypothetical protein, conserved	Tc00.1047053504153.280
27		p22 protein precursor, putative	Tc00.1047053509053.70
28		25 kDa translation elongation factor 1-beta, putative	Tc00.1047053506201.39
29		universal minicircle sequence binding protein (UMSBP), putative	Tc00.1047053503781.80
30		hypothetical protein, conserved	Tc00.1047053510877.30
31		d-isomer specific 2-hydroxyacid dehydrogenase-protein, putative	Tc00.1047053510099.120
32		RNA-binding protein, putative	Tc00.1047053511727.270
33		stress-induced protein st1, putative	Tc00.1047053506321.290
34		glutamanyl carboxypeptidase, putative	Tc00.1047053510837.20
35	1 (trans-sialidase)	chunk of conserved hypothetical protein	Tc00.1047053509099.160
36		centrin, putative	Tc00.1047053506559.380
37		possible salivary proline-rich protein rp15	Tc00.1047053506835.110
38		tuzin	Tc00.1047053507485.140
39	1 (trans-sialidase)	chunk of putative trans-sialidase	Tc00.1047053507997.14
40	4 (cruzipain)	cysteine peptidase, putative	Tc00.1047053507603.270
41	1 (heat shock)	Tc 85 kDa antigen with homology to heat shock proteins	Tc00.1047053509643.130
42	1 (heat shock)	Tc hsp 70	Tc00.1047053511211.170
43		serine carboxypeptidase (CBP1), putative	Tc00.1047053509695.220
44		hypothetical protein, conserved	Tc00.1047053510887.50
45		hypothetical protein, conserved	Tc00.1047053509141.40
46		dihydrolipooyl dehydrogenase, putative	Tc00.1047053507089.270
47		hypothetical protein, conserved	Tc00.1047053506529.460
48		trans-splicing factor, putative	Tc00.1047053503583.40
49		GTP-biNDing nuclear protein rtb2, putative	Tc00.1047053503539.30
50		14-3-3 protein, putative	Tc00.1047053511167.90
51		nascent polypeptide associated complex subunit, putative	Tc00.1047053510579.70
52		hypothetical protein, conserved	Tc00.1047053507093.300
53		succinyl-CoA synthetase alpha subunit, putative	Tc00.1047053508479.340

1 da Silveira, et al., TRENDS in Parasitology Vol.17 No.6 June 2001, p.286-291

2 Umezawa, et al., TRANSFUSION Volume 43, Jan. 2003, p. 91-97

3 Umezawa, et al., JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2004, p. 449-452

4 Martinez, et al., INFECTION AND IMMUNITY, Nov. 1991, p. 4275-4277

- 5 Figure 12 shows the pattern of responses of 11 subjects to 11 antigens and 2 controls (ovalbumin as the negative control, and *T. cruzi* lysate as a positive control) at the time of the first bleed (Figure 12A) and 12 months later (Figure 12B). Three aspects are worthy of mention. First, the pattern of responses is unique in each individual; no one appears to respond similarly.
- 10 Second, the pattern of responses is stable over the 12 month period. This is a strong indicator that the assay is indeed reproducibly detecting a persistent

response. This is also important with respect to using the test to monitor changes after therapy. Third, no single recombinant protein (or even combination of 3 or 4 proteins) detects all responders. This result validates the need for a multicomponent test for *T. cruzi* infection.

5

Example 11. Monitoring treatment for Chagas disease

Benzindazole is the primary compound used for treatment of Chagas disease, although there is controversy about its efficacy in treating chronically infected subjects, such as those who have been infected >20 years). The left side of Figure 13 below shows consistent serologic responses at 0 and 12 months in the absence of treatment in 4 subjects. The right side shows 6 treated subjects, treated at time 0 and reassayed at 12 and 24 months post-treatment. Changes in serology are obvious in 3 of the treated subjects at 12 months. The other 3 subjects have little to no change, even at 24 months. It is worth noting that conventional serology conducted on these same sera showed inconsistent or no changes. Furthermore, the percentage of individuals showing serological changes consistent with cure is similar to that reported in other like studies but using much longer follow-ups. E.g., a recent study by Fabbro et al. (Rev Soc Bras Med Trop. 2007 Jan-Feb;40(1):1-10), reported a treatment efficacy rate of 35-63% based upon conventional serology but required an average 16 year follow-up to see this change. Moreover, nearly 100% of those showing changes in serology also showed changes in T cell responses (Figure 14; note decreasing to undetectable responses within 12-25 months). Similar changes were not seen in untreated subjects or in treated subjects who failed to exhibit serological changes. Overall we conclude that this multiplex assay has the capability to relatively rapidly detect treatment success or failure – especially when coupled with assays of T cell reactivity.

30

Example 12. Maternal/neonate diagnostics

The unique pattern of responses in each individual may have utility with respect to monitoring congenital infection, which is currently very difficult. If  
5 the pattern of the serologic response in mother and newborn is similar, then we would suspect that the infant's serologic response is a result of maternally derived antibodies rather than to antibodies produced by the infant. However if the patterns are different, then we would suspect that the child is infected and therefore should be treated.

10 Four separate pairs of mother and newborn were evaluated. Serum antibody titers against individual recombinant *T. cruzi* proteins were determined for both the mother and the infant at a time point relatively soon after birth (within weeks), and again approximately 6 months later. For all pairs, the infant's pattern of response shortly after birth resembles the mother's pattern of  
15 response, indicating the presence of maternal antibodies (Figure 15). At the later time point, two of the infants (Figure 15B, and D) show a pattern of response differs from the mother's, indicating that these infants may be infected and producing their own antibodies. Another infant (Figure 15A) shows a response that is near background levels, suggesting that the infant is likely not  
20 infected. Circles over the individual measurements indicate statistically significant responses.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance,  
25 nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be  
30 understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. An article comprising:
  - a substrate comprising a surface; and
- 5 a plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof, immobilized on said substrate surface; wherein at least one polypeptide is selected from the polypeptides listed in Table 2 or Table 4, with the proviso that the at least one polypeptide is not listed in Table 1.
- 10 2. The article of claim 1 wherein the polypeptides are immobilized on the substrate surface to form a microarray.
- 15 3. The article of claim 1 wherein the substrate comprises at least one nanoparticle, and wherein the polypeptides are immobilized on the surface of the nanoparticle.
- 20 4. A kit for diagnosis of *T. cruzi* infection comprising:
  - an article according to claim 1; and
  - packaging materials and instructions for use.
5. The kit of claim 4 formulated for medical or veterinary use.
6. A method for obtaining information about a known or suspected *T. cruzi* infection in a mammal, or for determining whether a mammal is or has been infected by *T. cruzi*, said method comprising:
  - obtaining a biological sample from the mammal;
  - contacting the biological sample with a plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof; wherein at least one polypeptide is selected from the polypeptides listed in Table 2 or Table 4, with the proviso that the at least one polypeptide is not listed in Table 1; and

evaluating the presence, absence, intensity or pattern of interaction of components of the biological sample with the antigenic *T. cruzi* polypeptides to determine the presence or absence of *T. cruzi* infection, the identity of the infective strain, the length of the infection, the stage of the infection, whether 5 the infection is still present or the mammal has been cured, the vaccination status of the mammal, the success of treatment, or any combination thereof.

7. The method of claim 6 wherein the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof, is 10 immobilized on a substrate surface.

8. The method of claim 6 wherein the mammal is a human or a dog.

9. The method of claim 6 wherein the biological sample comprises a body 15 fluid comprising an antibody.

10. The method of claim 9 wherein the body fluid comprises blood, plasma or serum.

20 11. The method of claim 6 wherein the biological sample comprises a mononuclear blood cell.

12. The method of claim 6 wherein the biological sample comprises a peripheral blood mononuclear cell (PBMC) fraction of blood from the mammal.

25 13. The method of claim 6 wherein the method is a serodiagnostic method, and wherein the biological sample component that interacts with the antigenic *T. cruzi* polypeptide is an antibody from the mammal.

30 14. The method of claim 6 wherein the method is a cellular assay method, and wherein the biological sample component that interacts with the antigenic *T. cruzi* polypeptide is T cell from the mammal.

15. The method of claim 6 wherein the method comprises a multiplexed assay wherein the biological sample is contacted simultaneously with the plurality of antigenic *T. cruzi* polypeptides.
- 5 16. The method of claim 6 wherein the biological sample is obtained from an actual or potential blood donor or transplant donor.
17. The method of claim 6 wherein the biological sample is obtained from a pooled blood product supply intended for use in transfusions or research.
- 10 18. A method for detecting contamination of a blood product supply with *T. cruzi*, the method comprising:
  - selecting a sample from the blood supply;
  - contacting the sample with a plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof; wherein at least one polypeptide is selected from the polypeptides listed in Table 2 or Table 4, with the proviso that the at least one polypeptide is not listed in Table 1; and
  - evaluating the presence, absence, intensity or pattern of interaction of components of the sample with the antigenic *T. cruzi* polypeptides to determine whether the blood supply is contaminated with *T. cruzi*.
- 15 19. The method of claim 18 wherein the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof, is immobilized on a substrate surface.
- 20 20. The method of claim 18 wherein the blood product supply comprises whole blood, a blood product, or a blood fraction.
- 25 30 21. The method of claim 18 wherein the blood product supply comprises a cellular blood component, a liquid blood component, a blood protein, or mixtures thereof.

22. The method of claim 18 wherein the blood product supply comprises a red blood cell concentrate, a leukocyte concentrate, a platelet concentrate, plasma, serum, a clotting factor, an enzymes, albumin, plasminogen, or a immunoglobulin, or mixtures of thereof.

5

23. The method of claim 18 wherein the method is a serodiagnostic method, and wherein the sample component that interacts with the antigenic *T. cruzi* polypeptide is an antibody.

10 24. A method for determining whether an infant has a *T. cruzi* infection, said method comprising:

obtaining a biological sample from an infant born to a mother known to have or suspected of having a *T. cruzi* infection, wherein said biological sample is obtained later than about 3 months after birth of the infant;

15 contacting the infant's biological sample with a plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof; and

evaluating the presence, absence, intensity or pattern of interaction of components of the biological sample with the antigenic *T. cruzi* polypeptides to 20 determine whether the infant exhibits an antibody response that exceeds background levels.

25. The method of claim 24 further comprising:

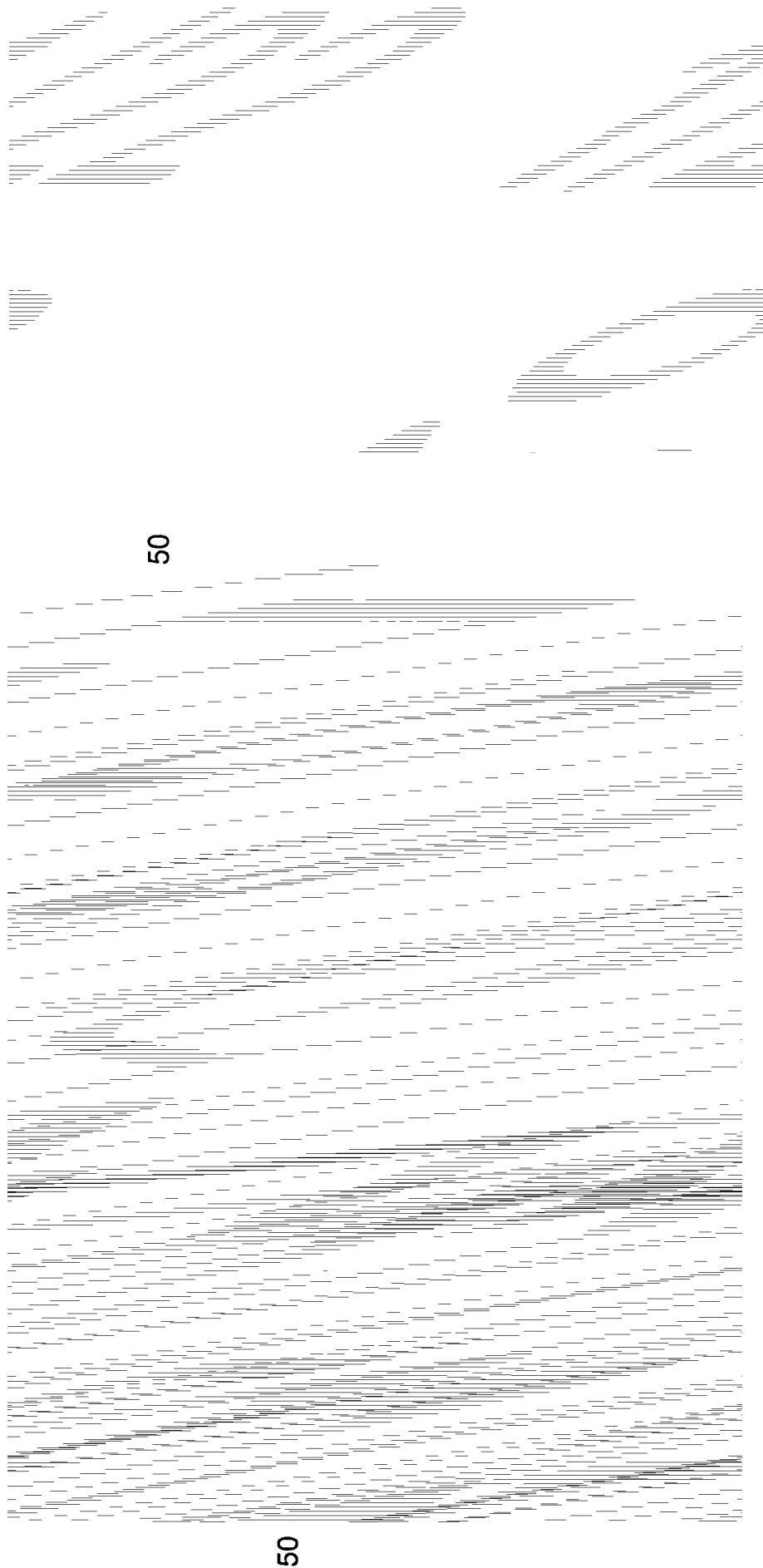
obtaining a biological sample from the infant's mother;

contacting the mother's biological sample with the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof; and

comparing the presence, absence, intensity or pattern of interaction of components of the mother's biological sample with the antigenic *T. cruzi* 30 polypeptides, to the presence, absence, intensity or pattern of interaction of components of the infant's biological sample with the antigenic *T. cruzi* polypeptides, to determine whether the infant's antibody response differs from

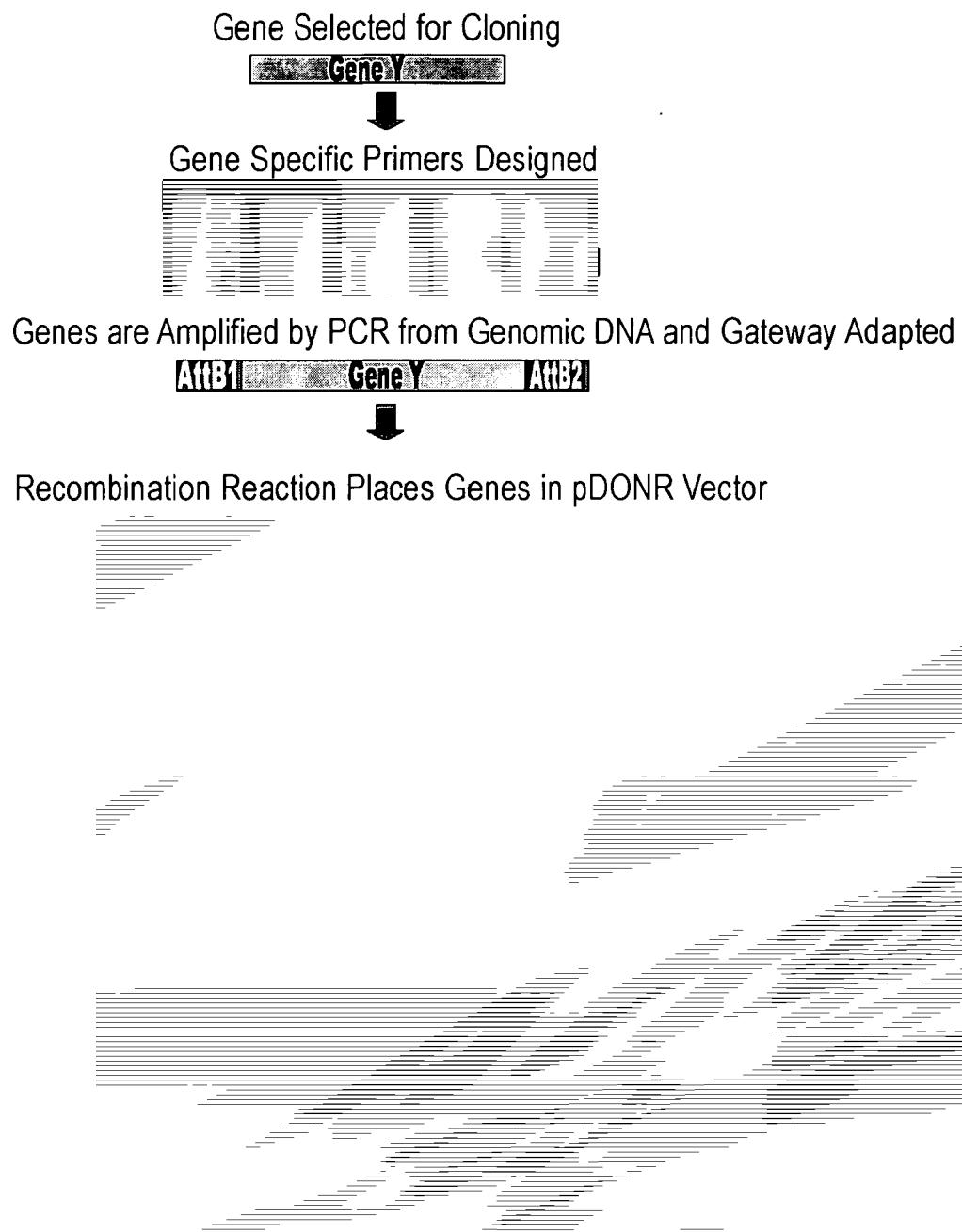
the mother's antibody response, wherein a difference in antibody responses indicates that the infant may have a *T. cruzi* infection.

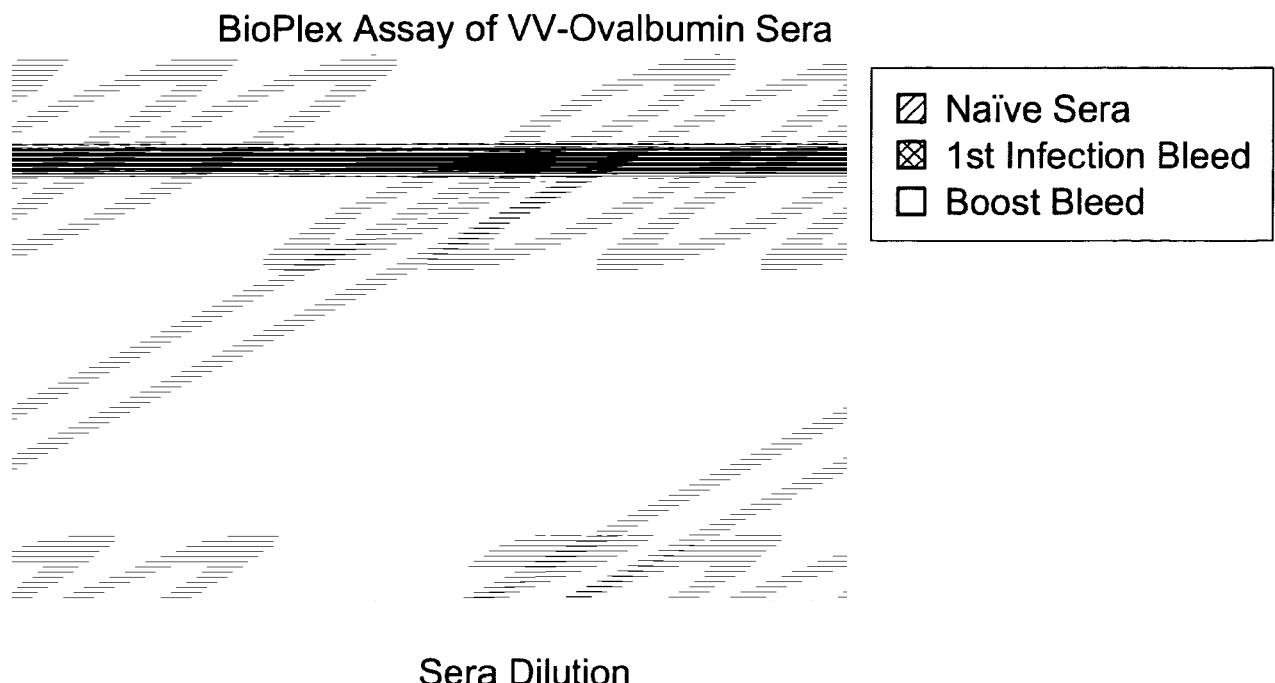
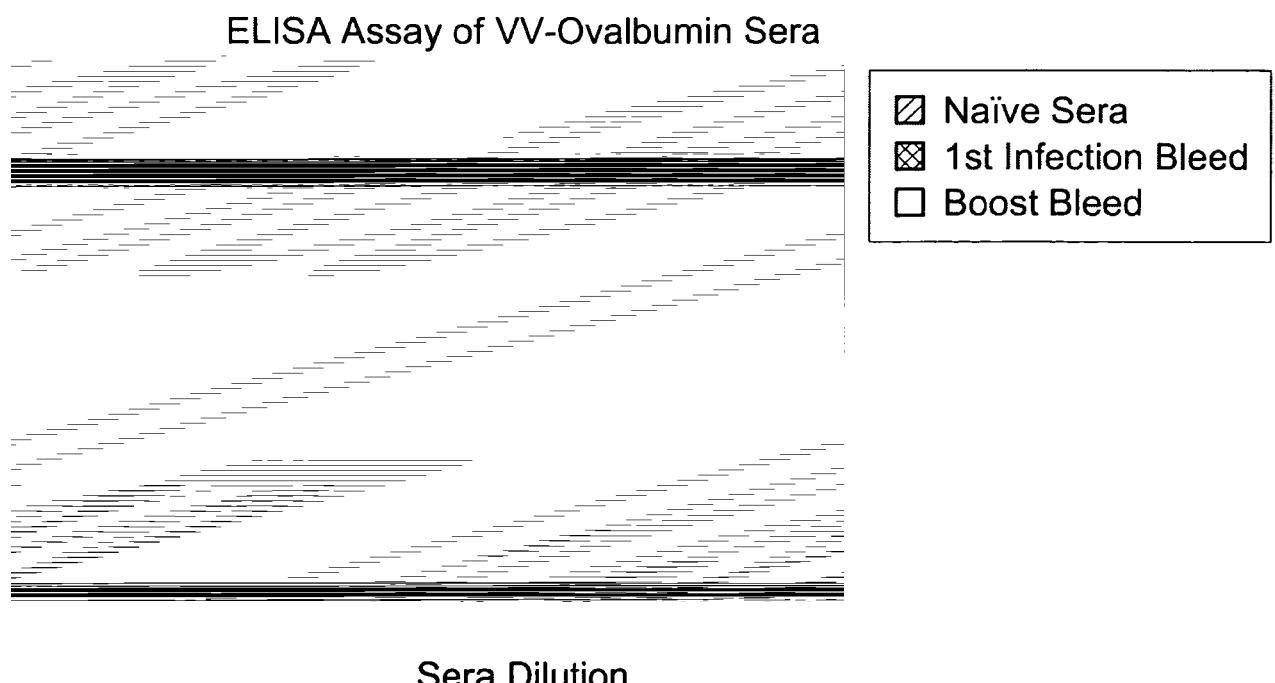
26. The method of claim 24 wherein at least one polypeptide is selected  
5 from the polypeptides listed in Table 1, Table 2 or Table 4
27. The method of claim 24 further comprising:  
obtaining an earlier biological sample from the infant shortly after birth;  
contacting the infant's earlier biological sample with the plurality of  
10 individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or  
subunits thereof; and  
comparing the presence, absence, intensity or pattern of interaction of  
components of the infant's earlier biological sample with the antigenic *T. cruzi*  
polypeptides, to the presence, absence, intensity or pattern of interaction of  
15 components of the infant's later biological sample, or of the mother's biological  
sample, or both, with the antigenic *T. cruzi* polypeptides, to determine whether  
the infant's later antibody response differs from the mother's antibody response,  
wherein a difference in antibody responses indicates that the infant may have a  
*T. cruzi* infection.  
20
28. The method of claim 24 wherein the biological sample comprises a body  
fluid comprising an antibody.
29. The method of claim 24 wherein the body fluid comprises blood, plasma  
25 or serum.
30. The method of claim 25 further comprising administering a therapeutic  
agent to the infant to treat a *T. cruzi* infection.



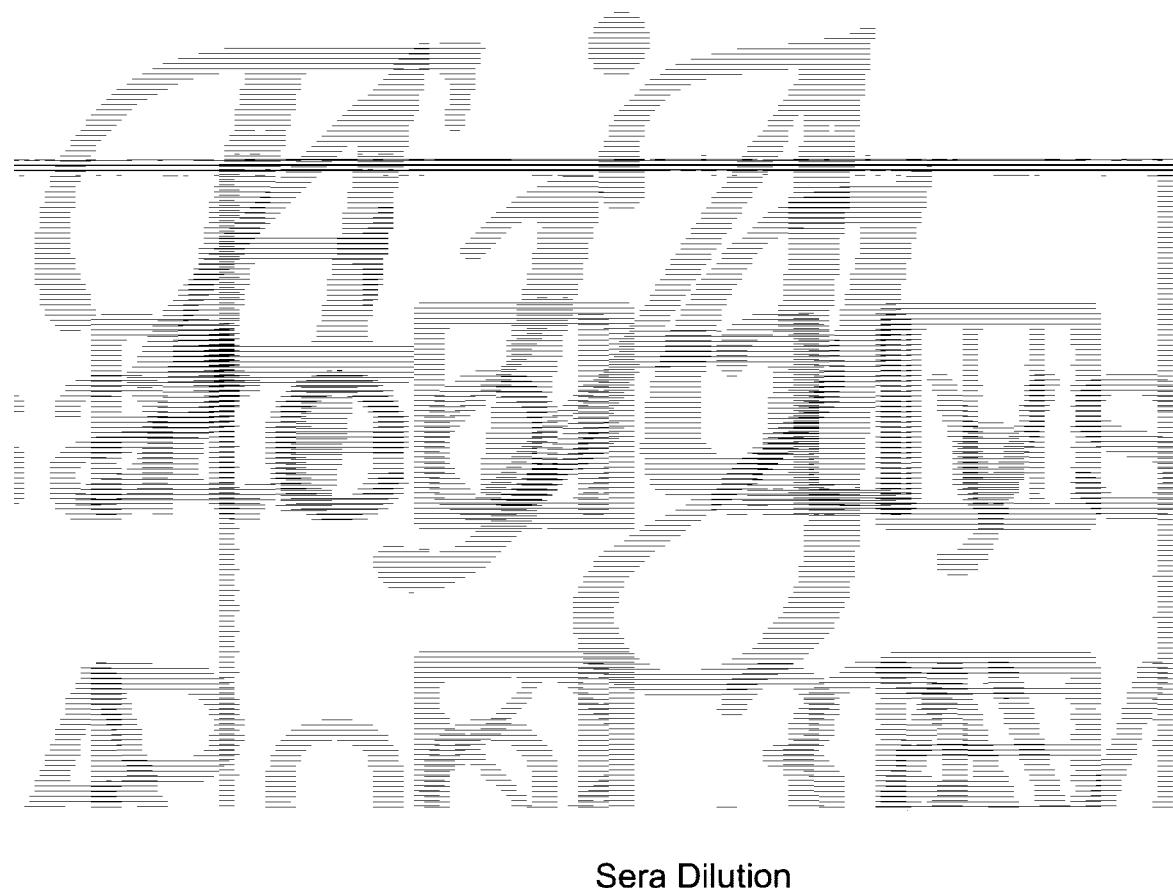
50

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*Fig. 2*

*Fig. 3A**Fig. 3B*

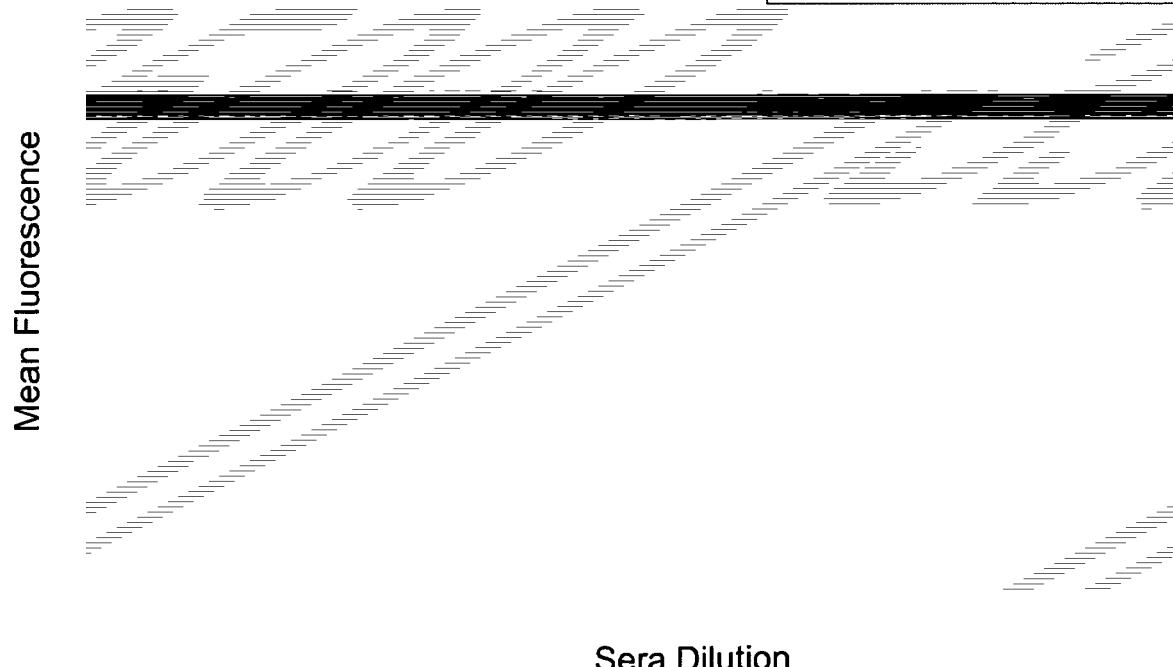
4/22



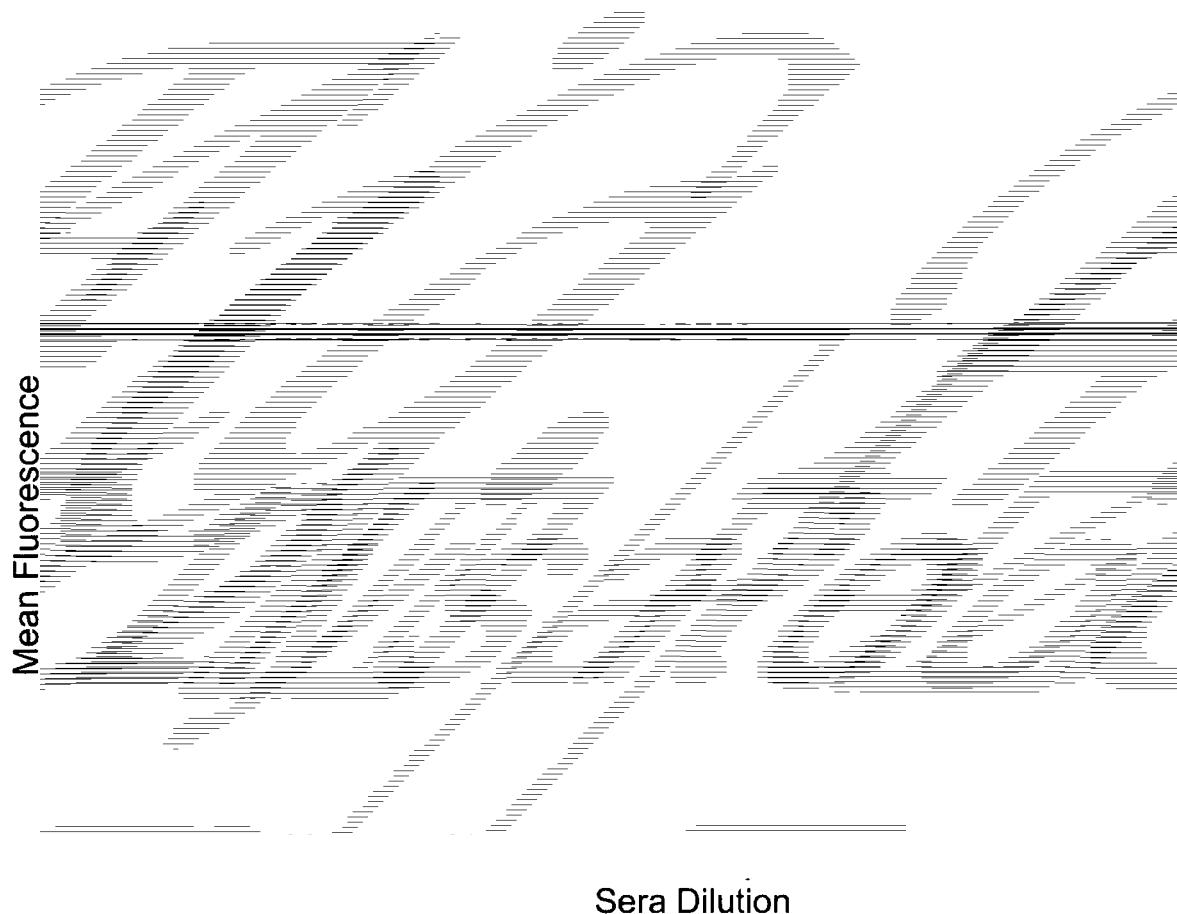
*Fig. 4B*

Very Low Positive Sera

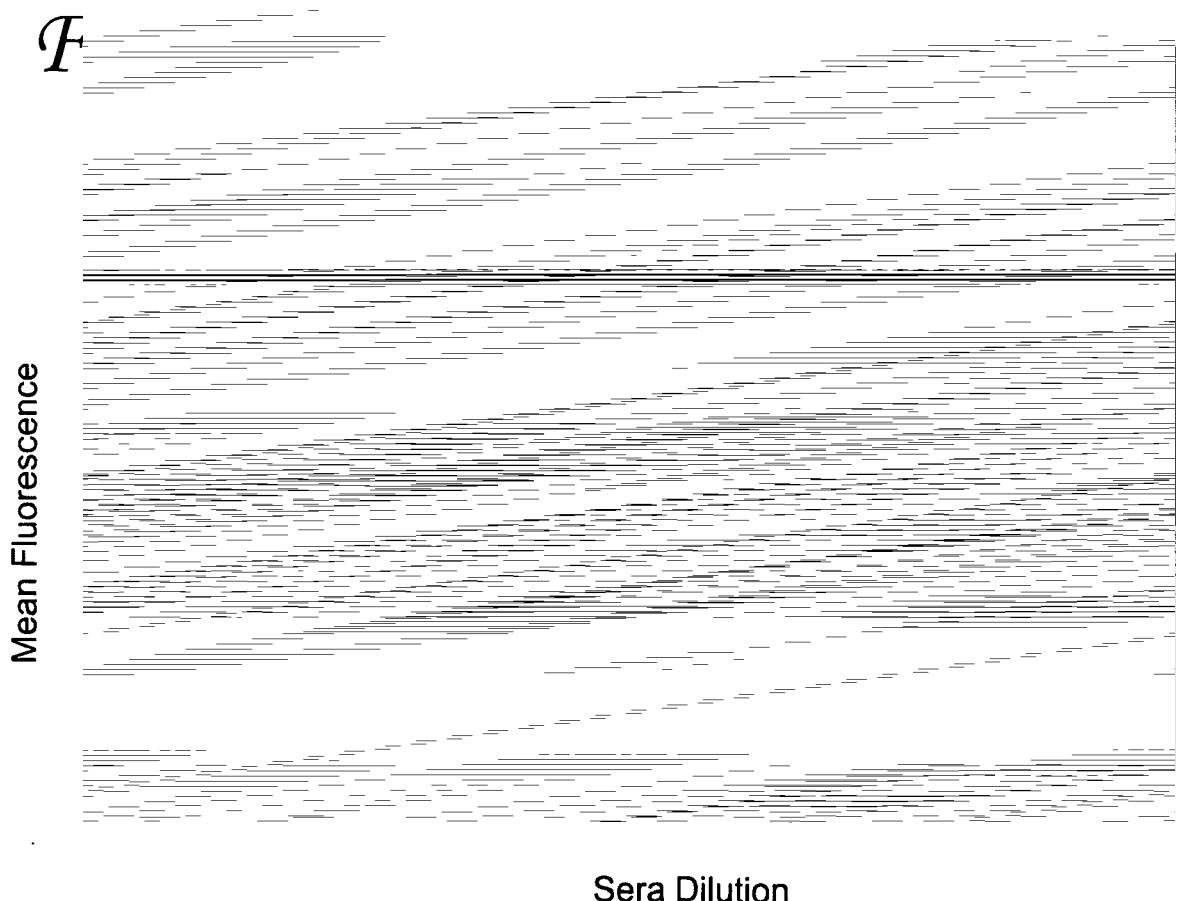
□ Lysate	■ Pool 2I
□ OVA	■ Pool 3A
□ Pool 1C	■ Pool 3K
□ Pool 2A	■ Pool 6



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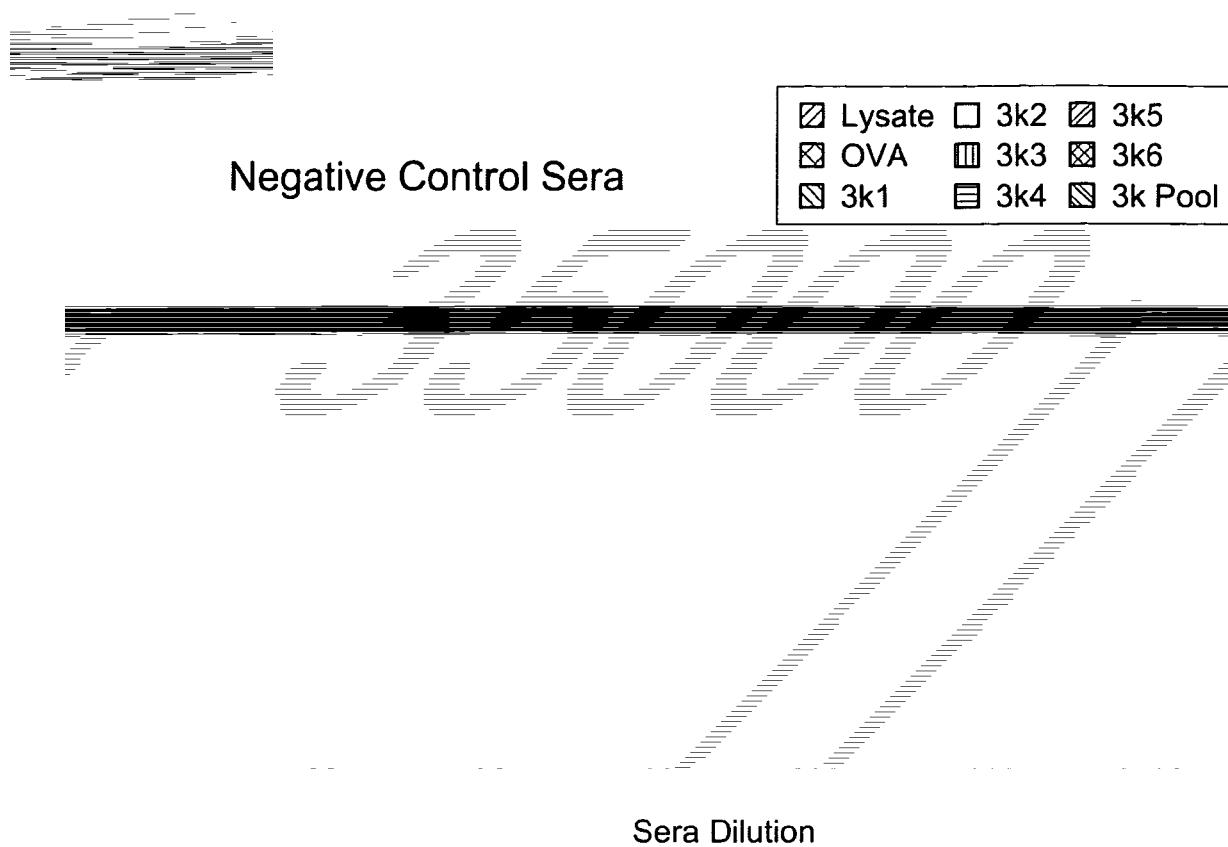


Sera Dilution

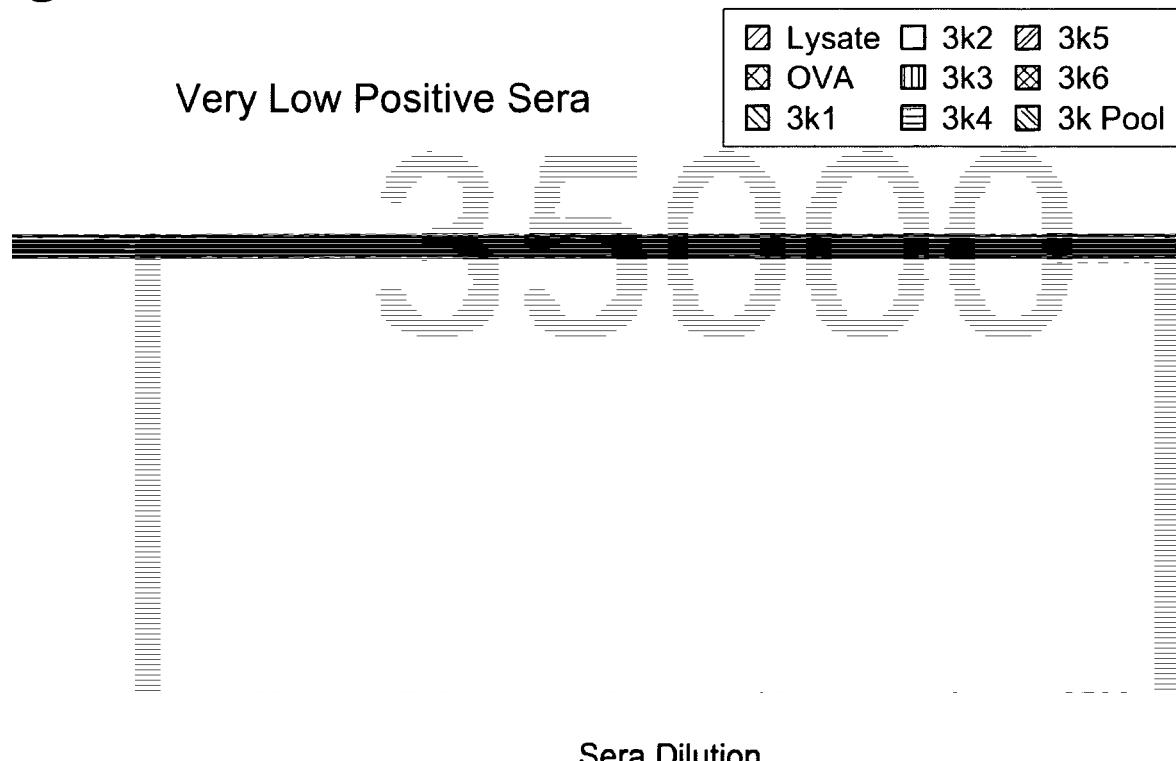


Sera Dilution

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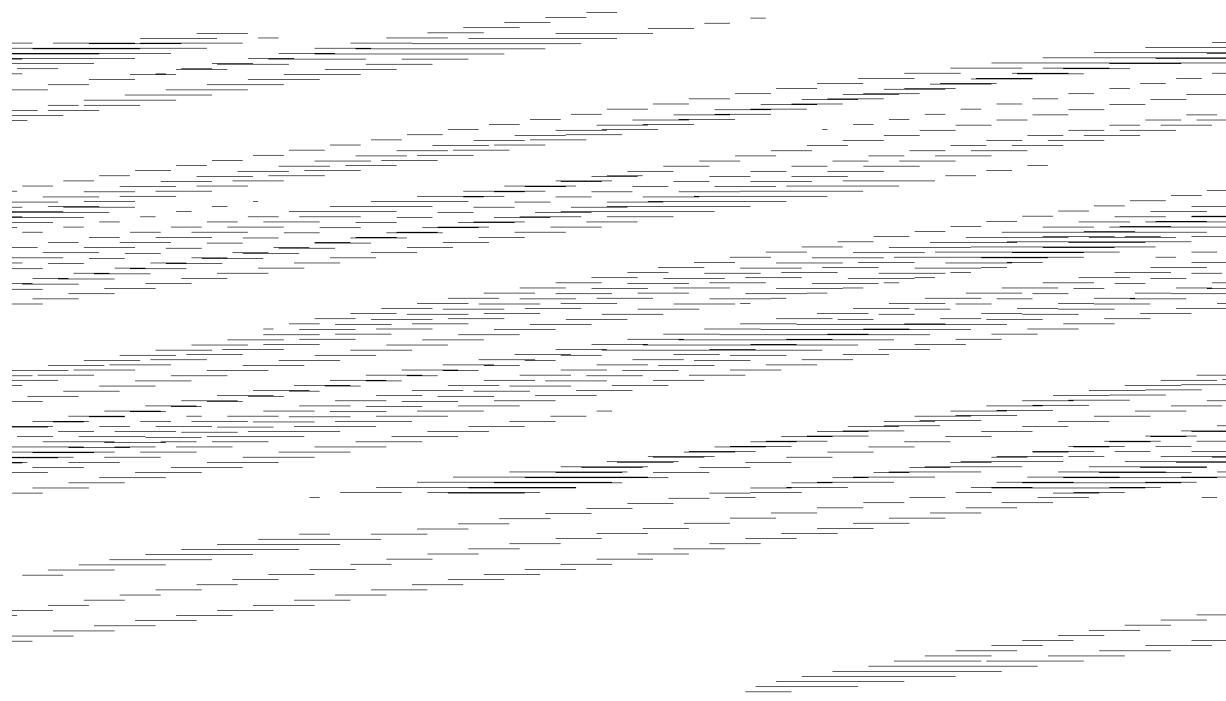
*Fig. 5B*



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*Fig. 5C*

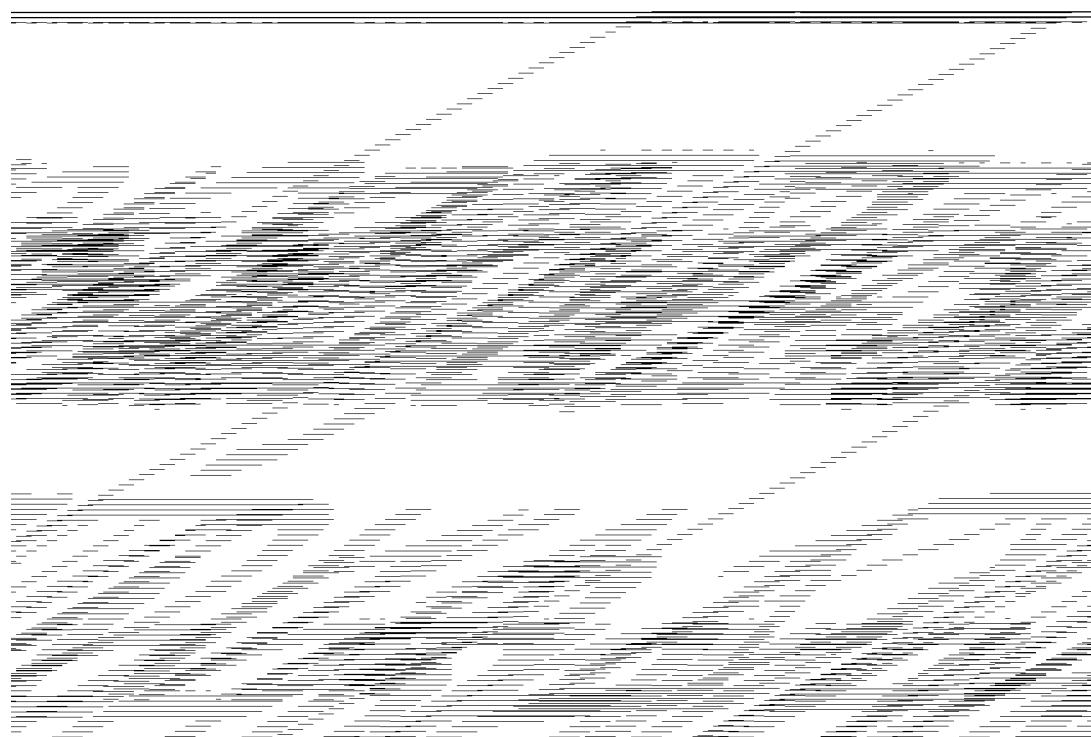
8/22



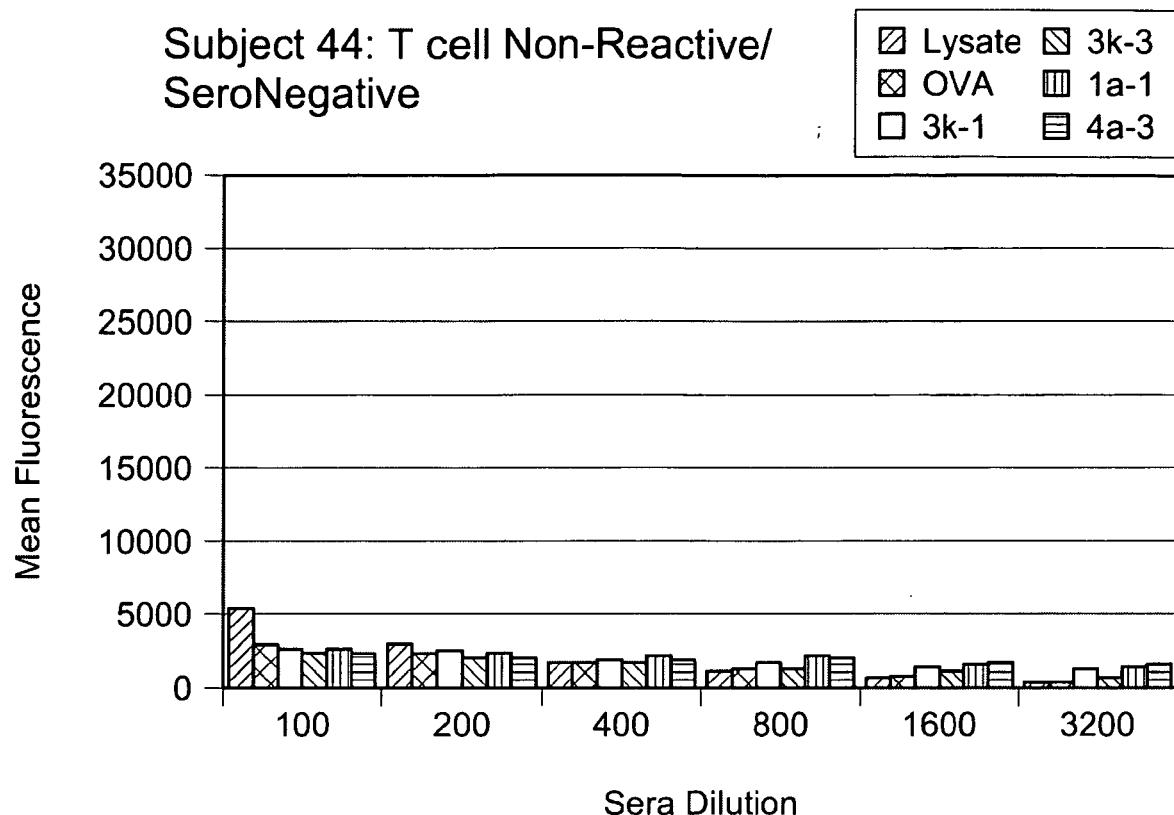
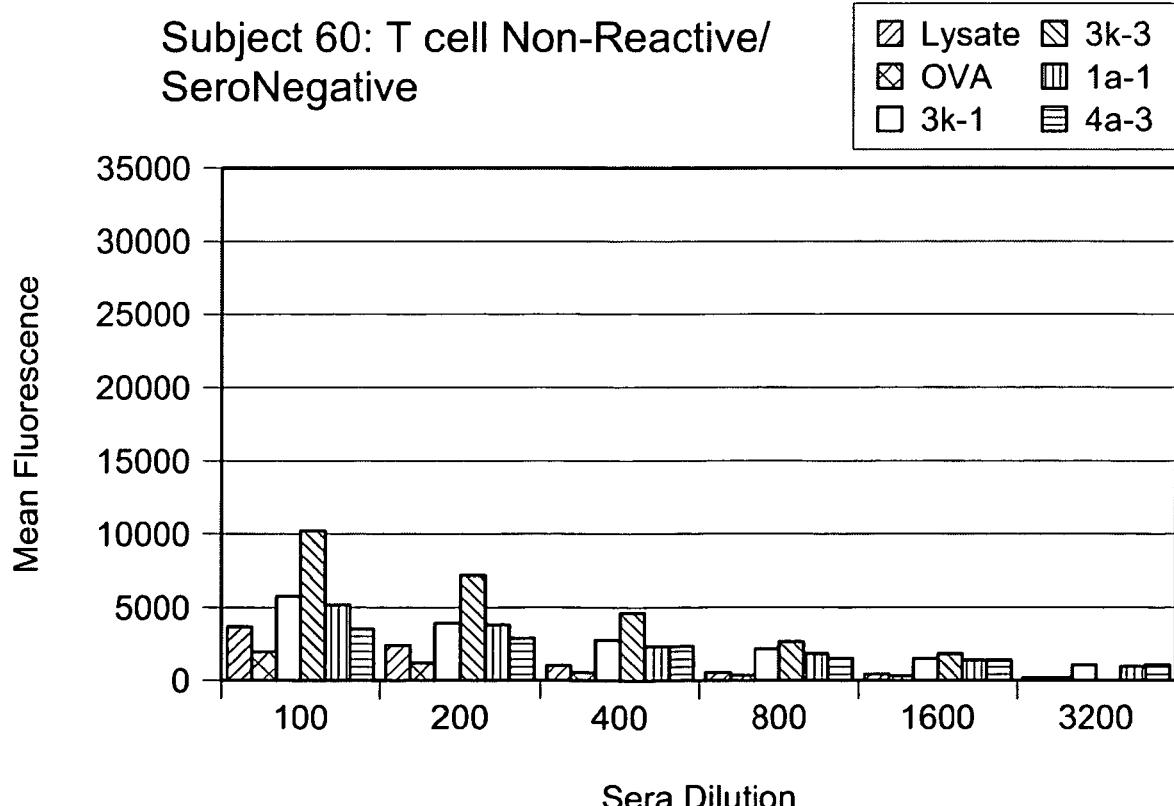
Sera Dilution

Fig. 6B

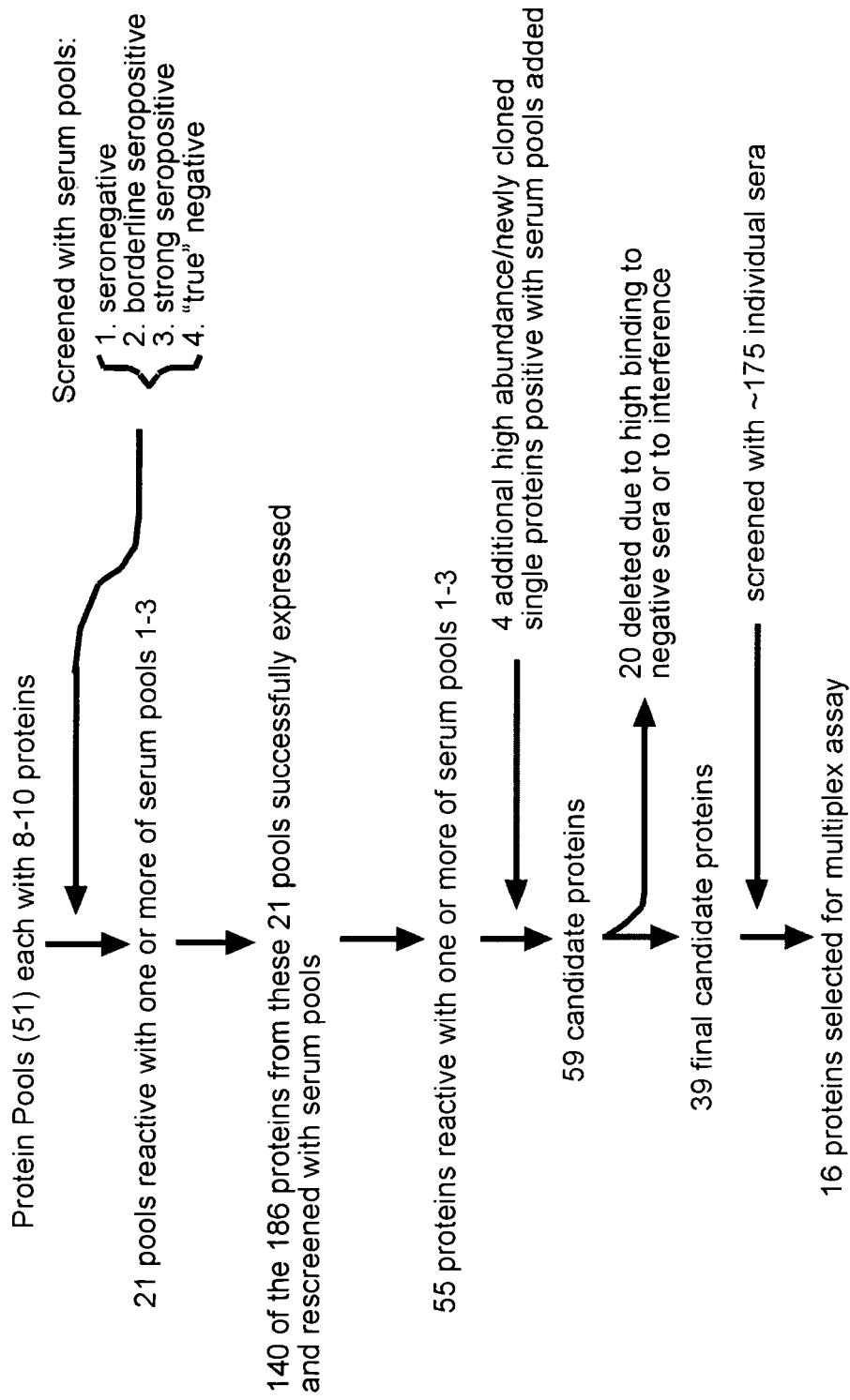
Mean Fluorescence



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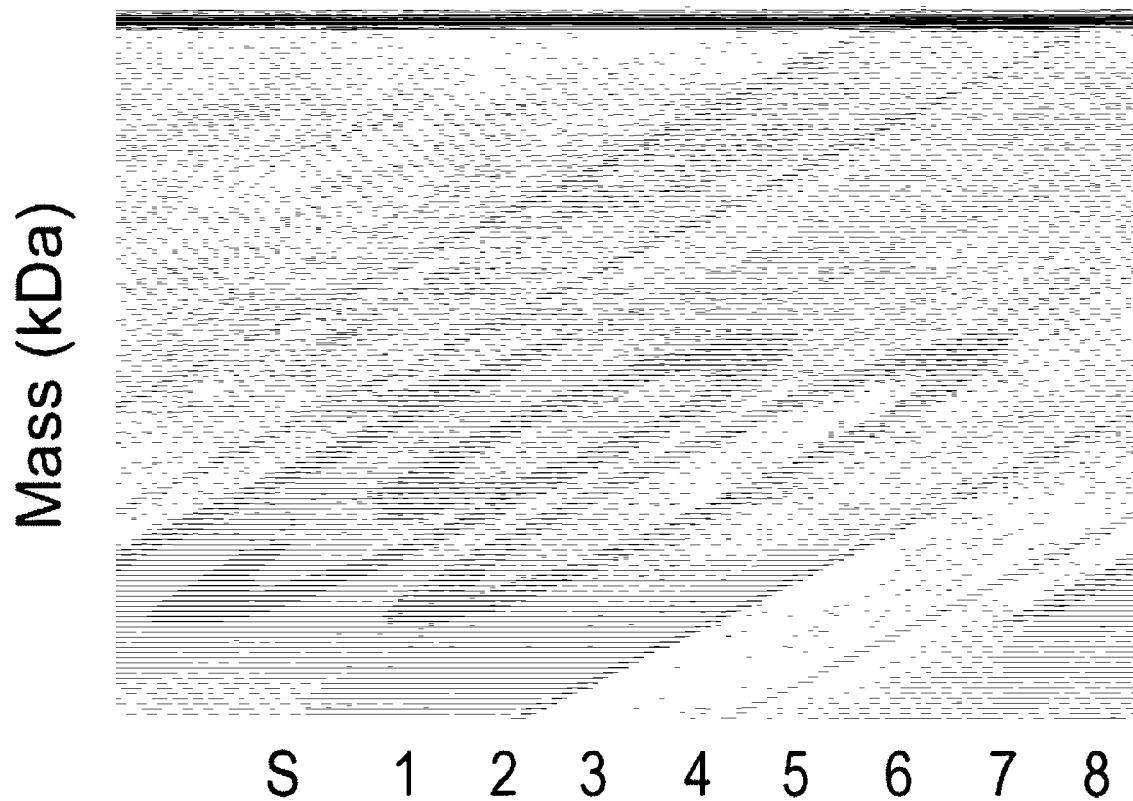
*Fig. 6C**Fig. 6D*

## Selection of Diagnostic Proteins



*Fig. 7*

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*Fig. 8*

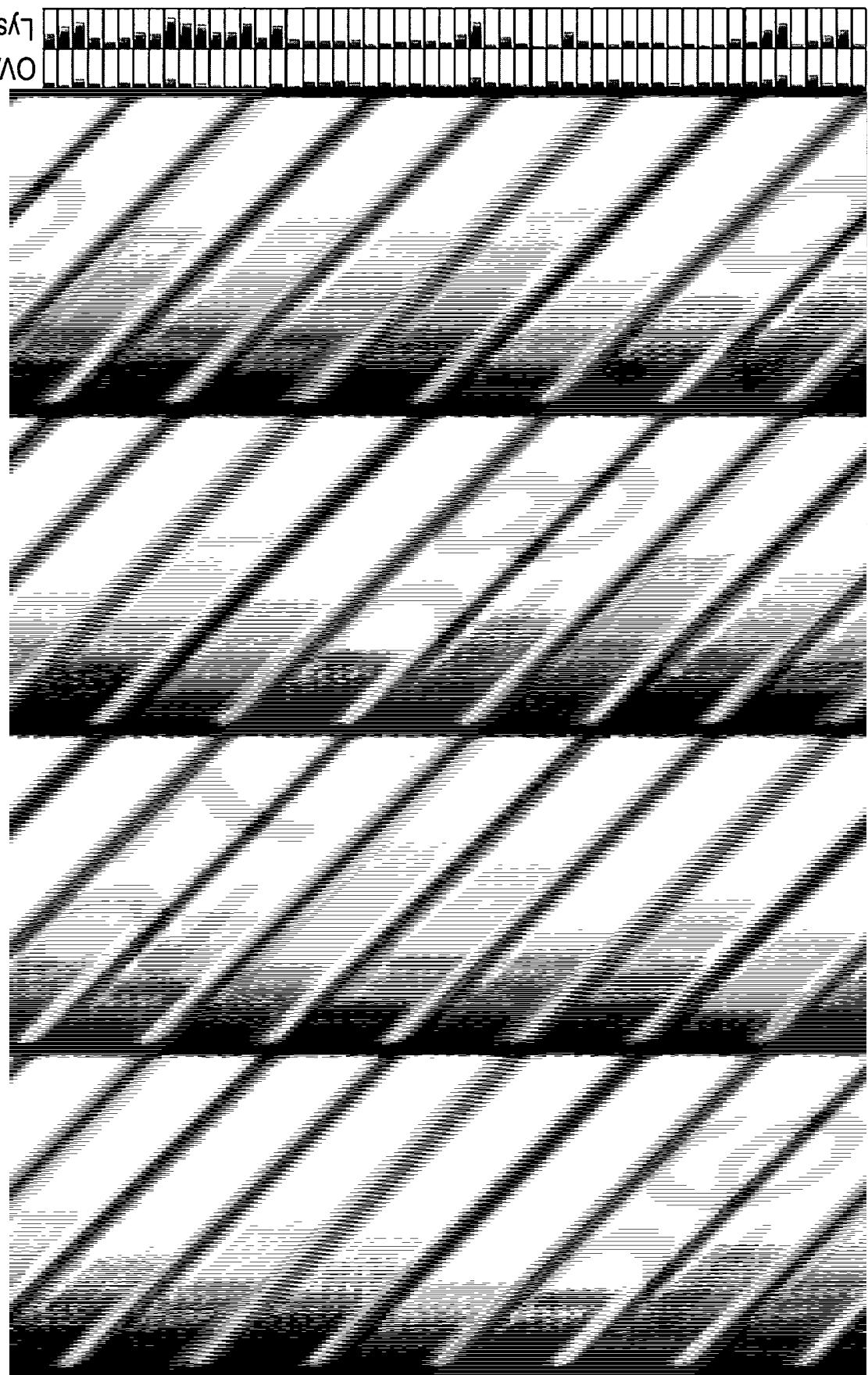
12/22

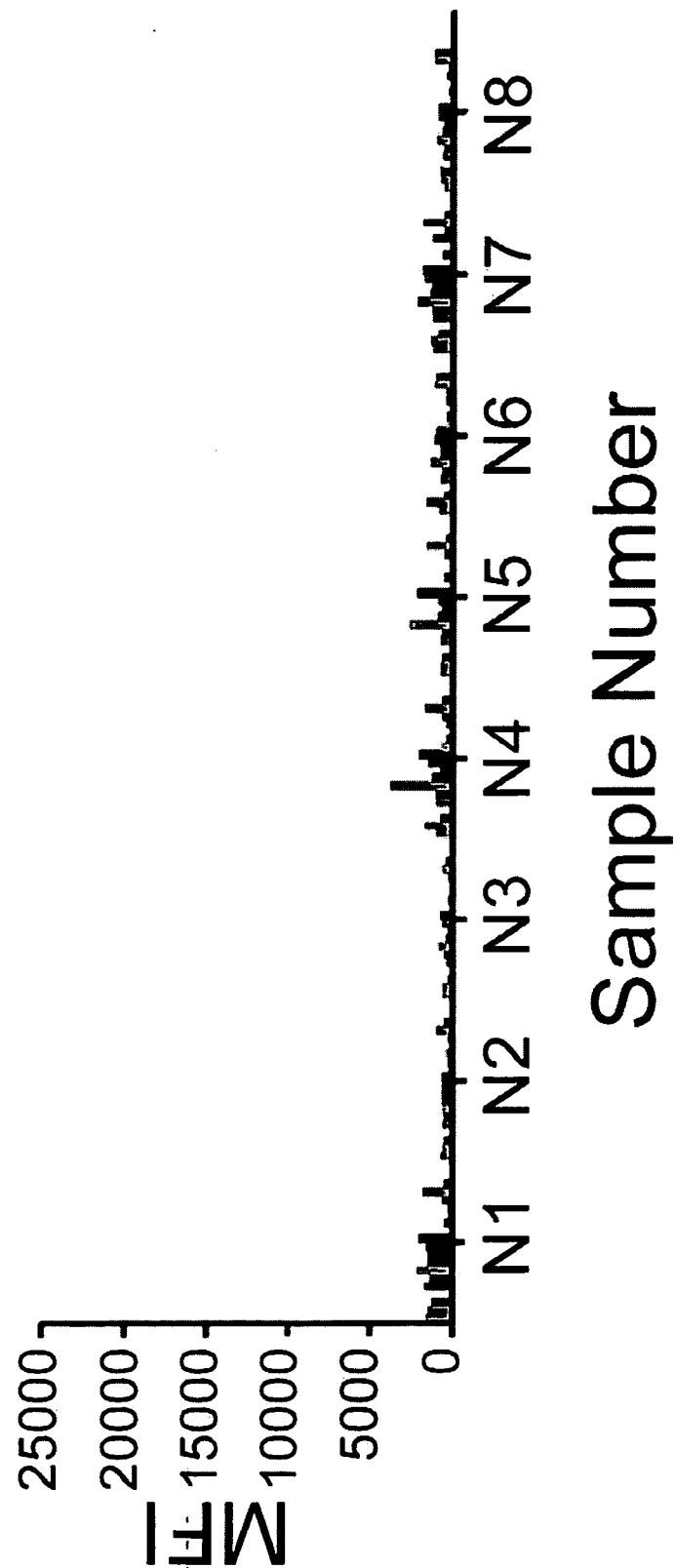
## Recombinant Proteins

Lysate

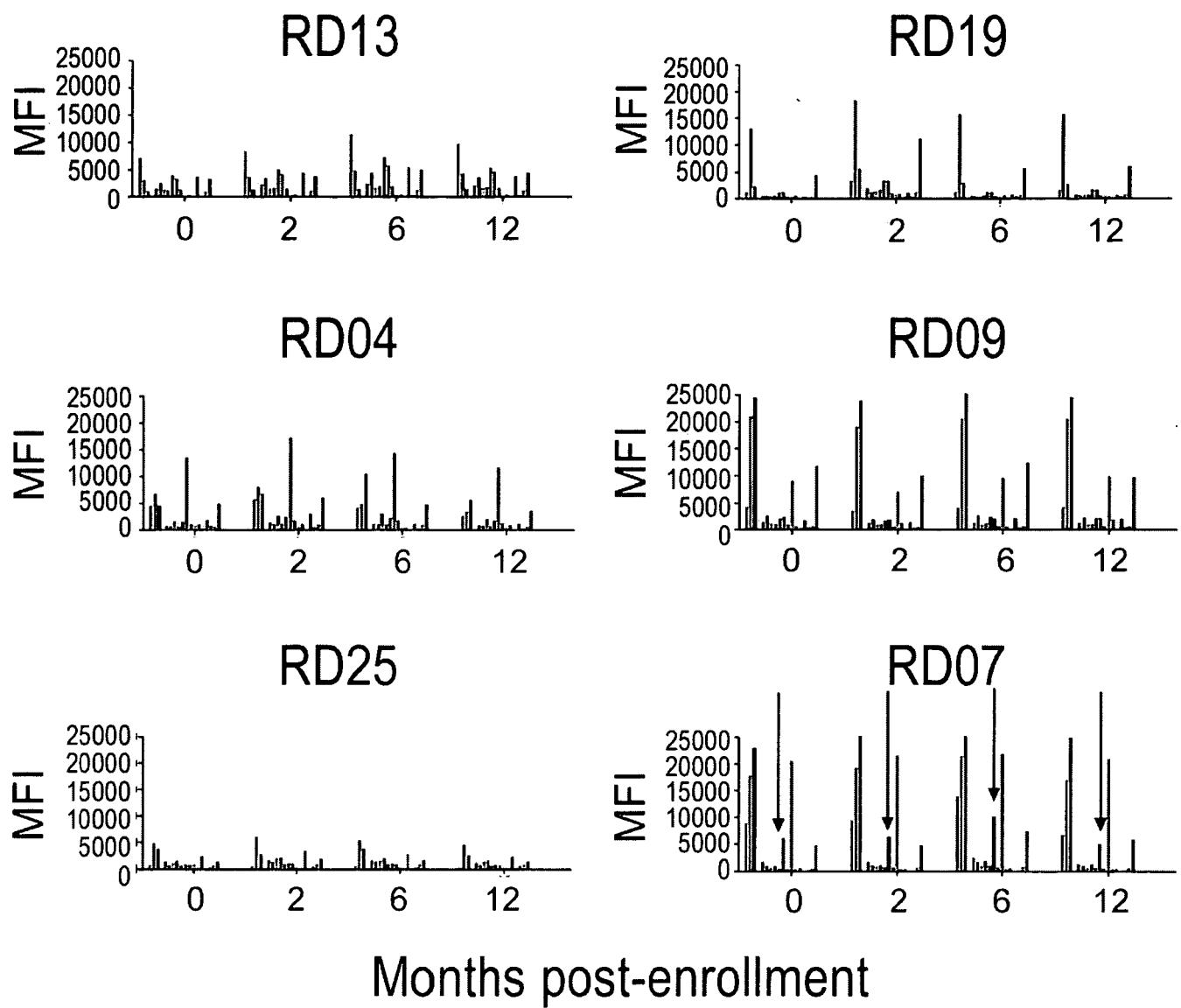
OVA

MFI



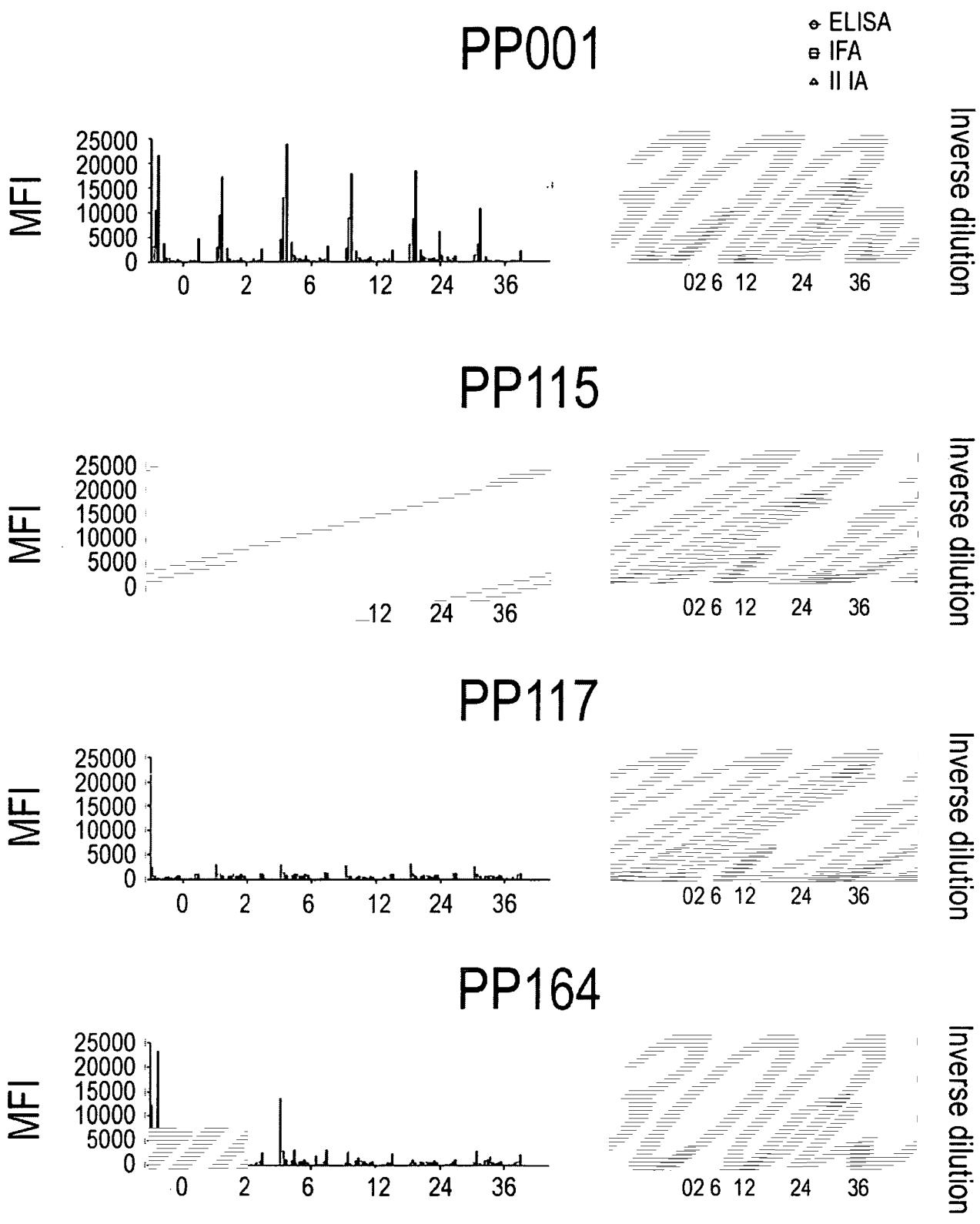
*Fig. 10A*

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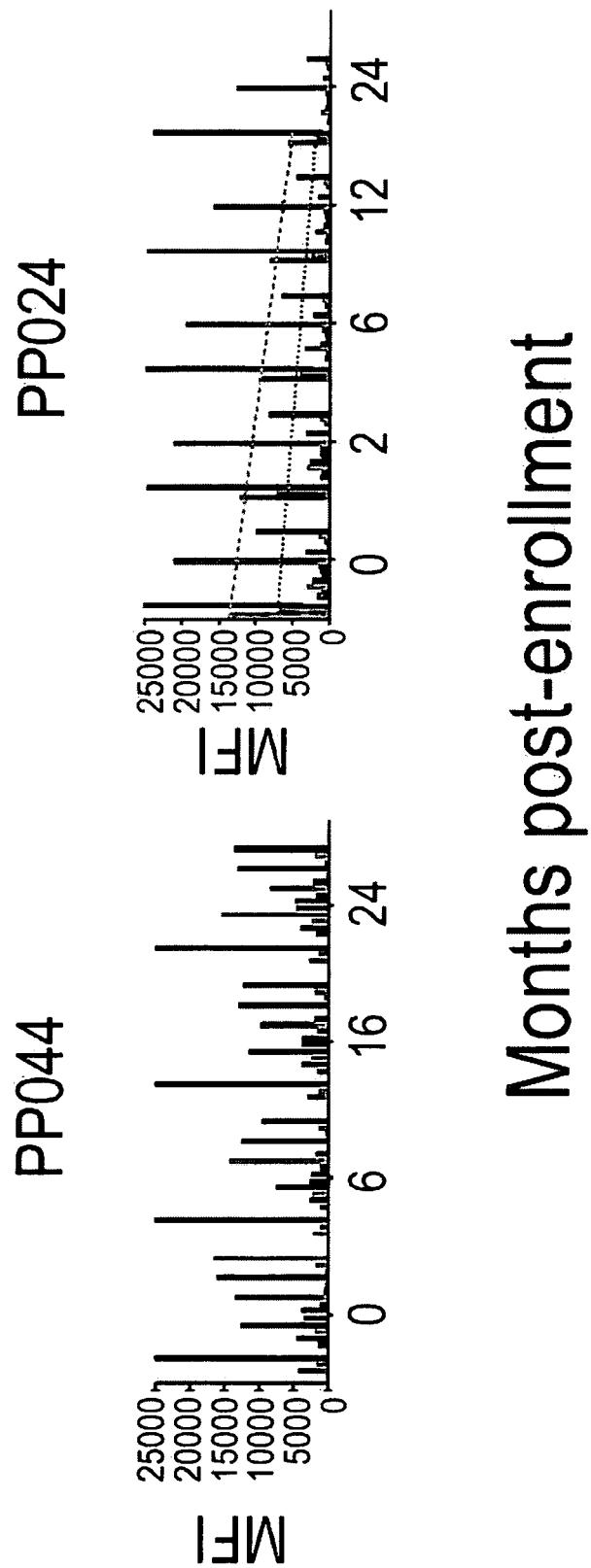
*Fig. 10B*

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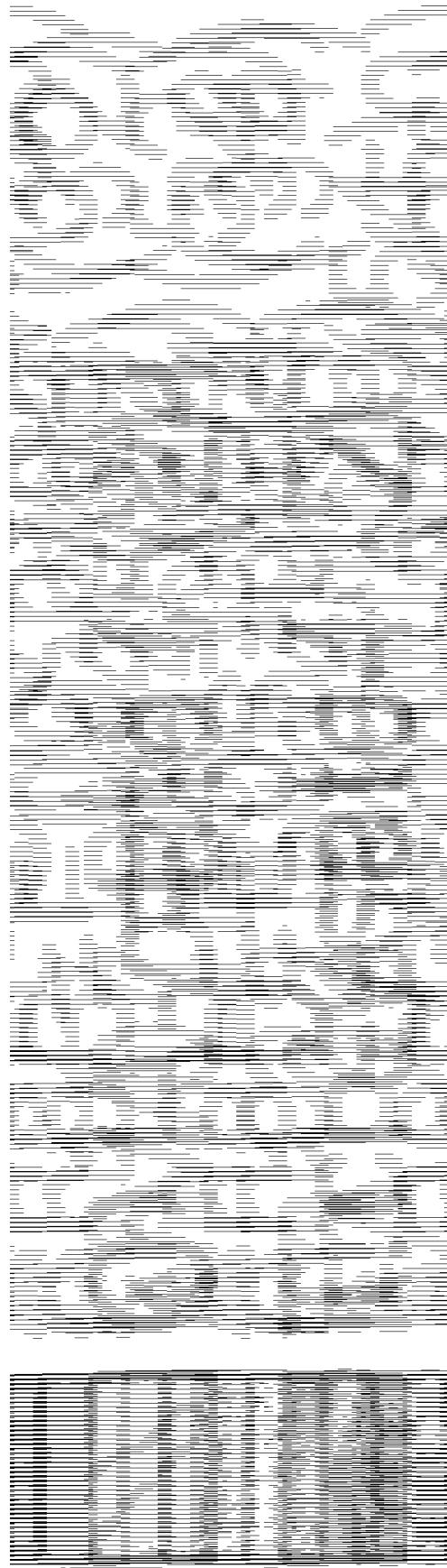
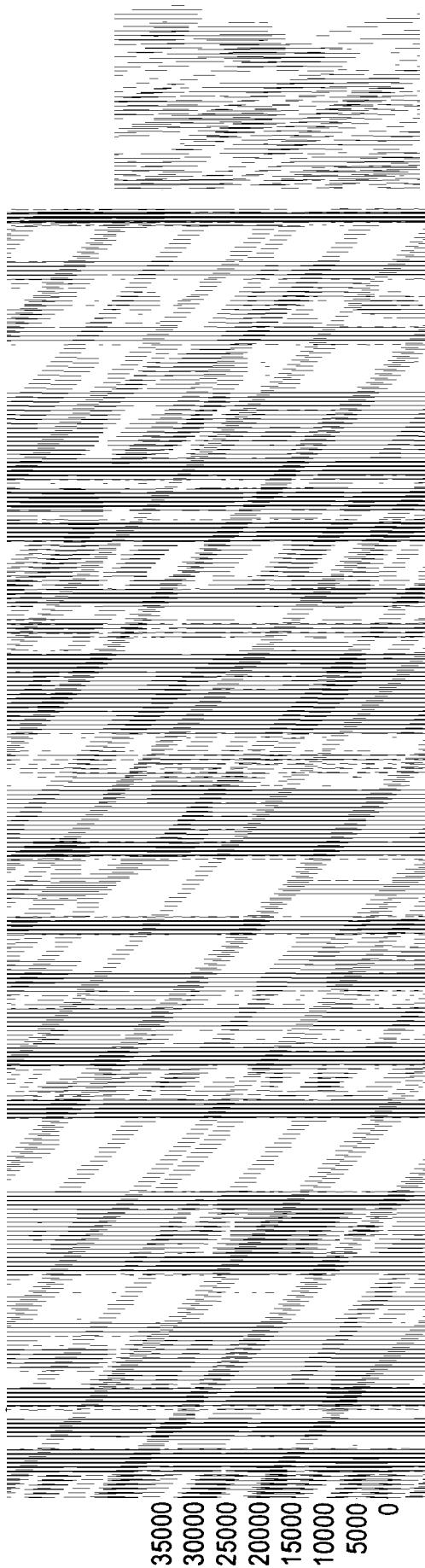


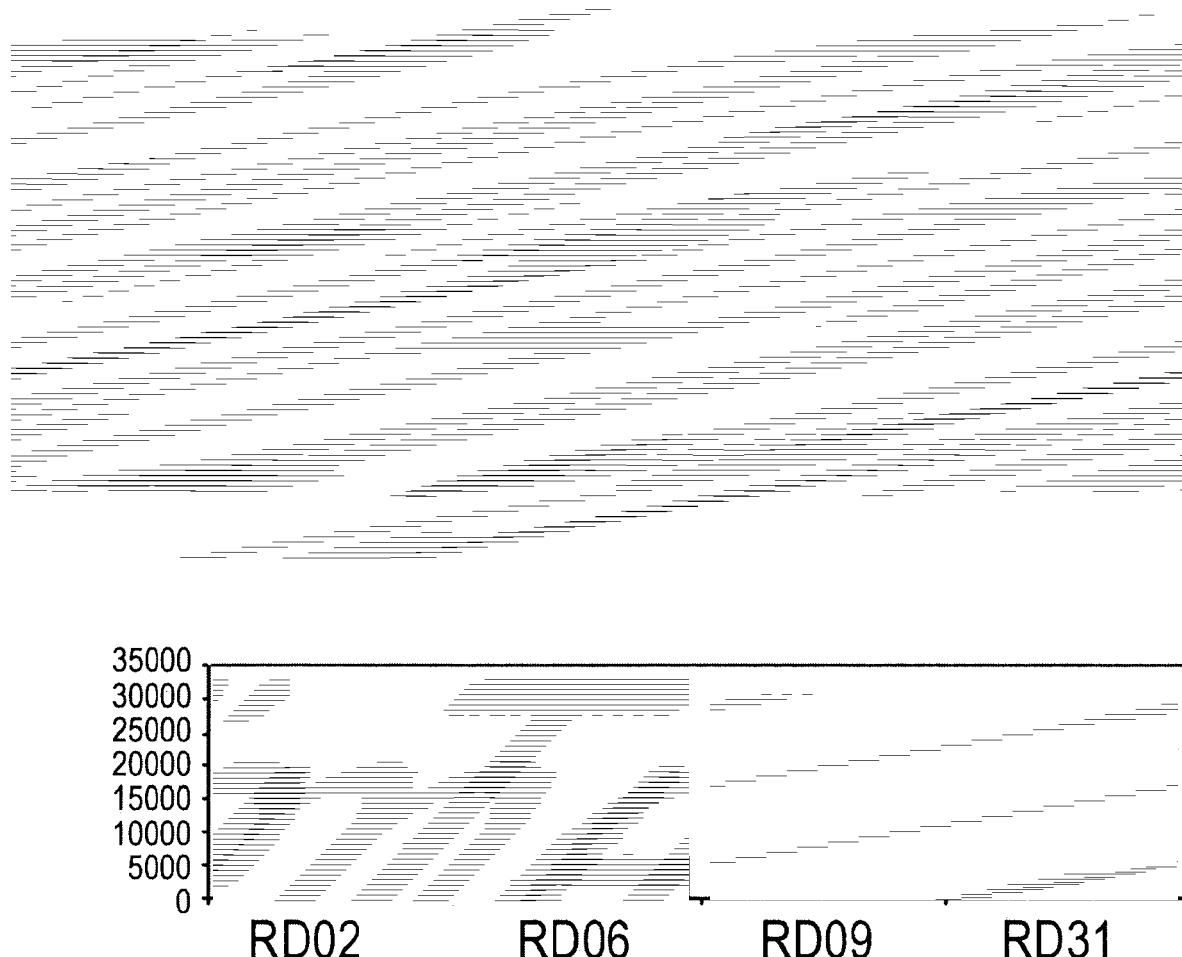
Months post-enrollment

*Fig. 11A*

*Fig. 11B*

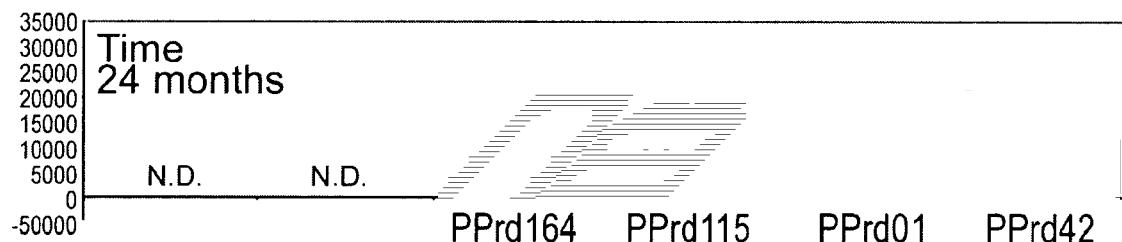
17/22





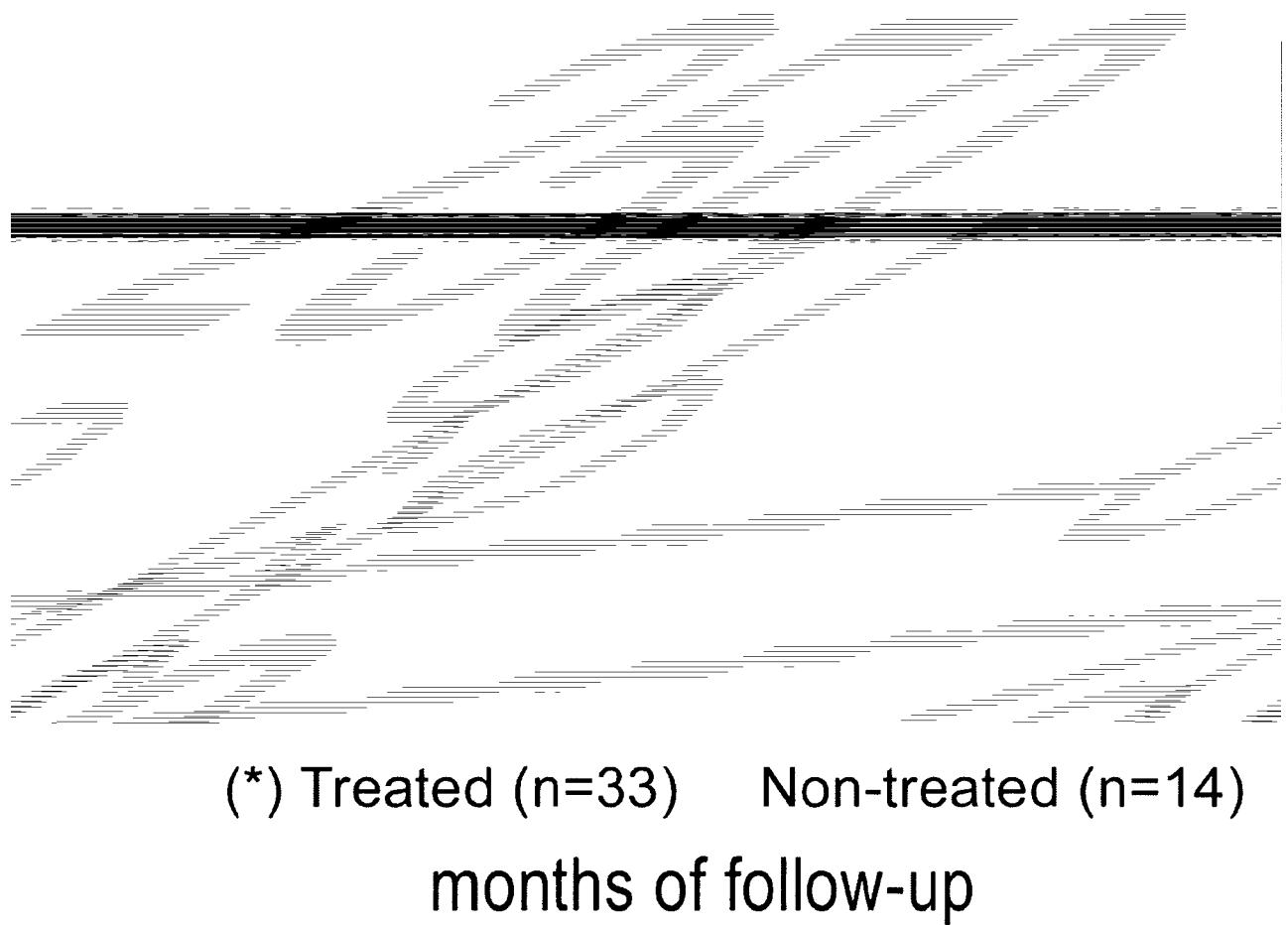
- kn107-ptd4 (24)
- kn109-ptd4 (26)
- kn117-ptd4 (28)
- kn122-ptd4 (30)
- AnoB-E09-ptd4 (32)
- AnoD-B06-ptd4 (34)
- AnoF-F10-ptd4 (36)
- AnoH-E01-ptd4 (38)
- AnoH-G10-ptd4 (50)
- AnoL-E02-ptd4 (52)
- FAB-A04-ptd4 (54)
- OVA-ptd4 (58)
- T.c. lysate (15)

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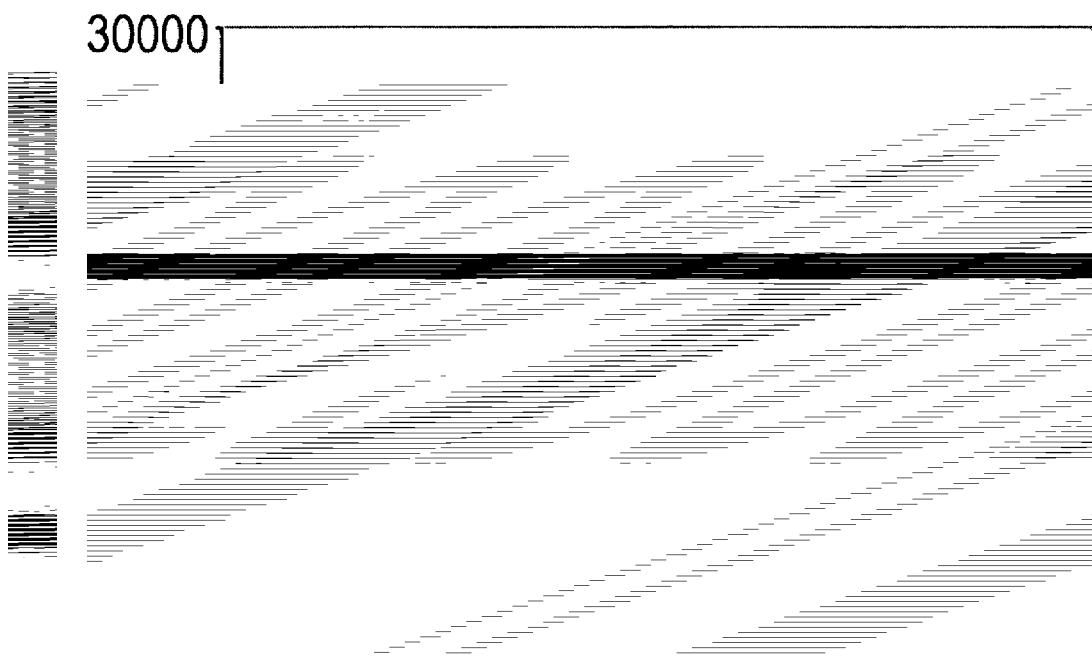
- kn107-ptd4 (24)
- kn109-ptd4 (26)
- kn117-ptd4 (28)
- kn122-ptd4 (30)
- AnoB-E09-ptd4 (32)
- AnoD-B06-ptd4 (34)
- AnoF-F10-ptd4 (36)
- AnoH-E01-ptd4 (38)
- AnoH-G10-ptd4 (50)
- AnoL-E02-ptd4 (52)
- FAB-A04-ptd4 (54)
- OVA-ptd4 (58)
- T.c. lysate (15)

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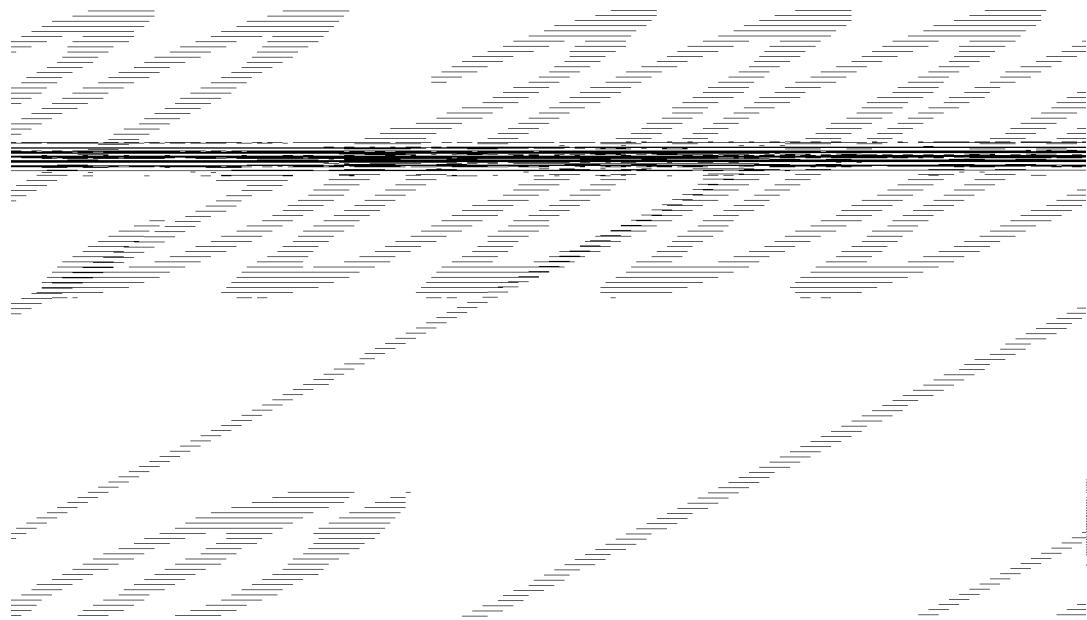
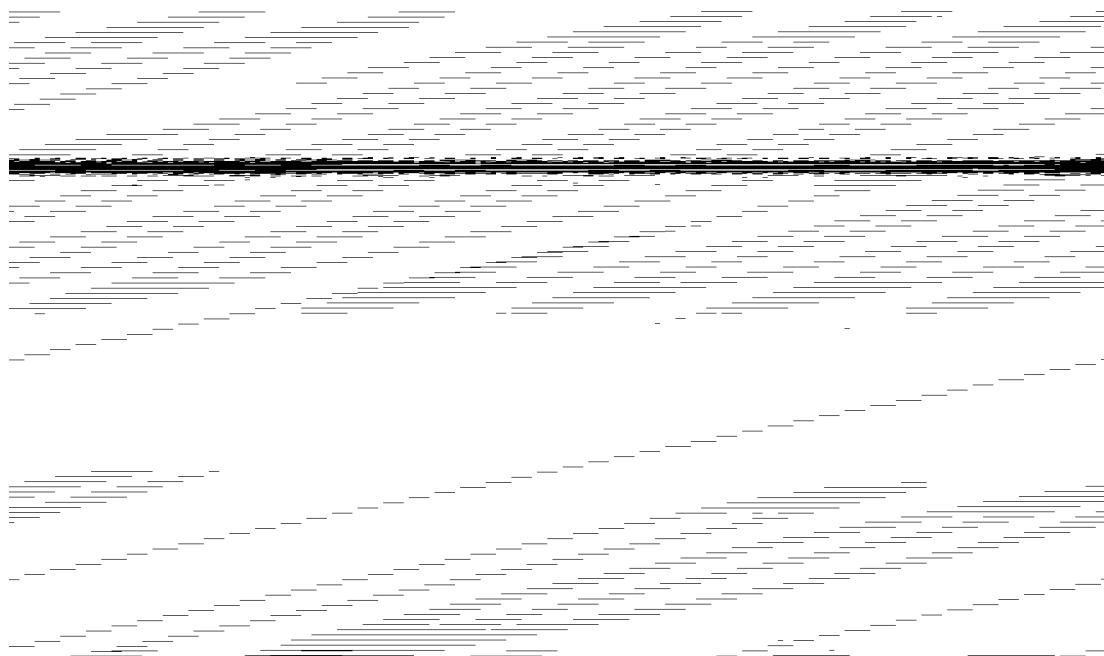


*Fig. 14*

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*Fig. 15A**Fig. 15B*

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*Fig. 15C**Fig. 15D*

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 08/09174

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(8) - A61K 39/00 (2008.04)

USPC - 424/191.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

USPC: 424/191.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
USPC: 424/191.1;435/7.22;436/86

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST (PGPB,USPT,USOC,EPAB,JPAB) Google (Patents, Scholar, and Web)

Search Terms Used: trypanosoma cruzi (polypeptide OR peptide) substrate (human OR dog) therapeutic agent microtubule.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,419,933 B1 (REED et al.) 16 July 2002 (16.07.2002), entire document, especially: Abstract; col. 2, ln 16-29; col. 5, ln19-25; col. 9, ln 59-64	24, 25, 27-29
Y		1-23, 26, 30
Y	US 2006/0228300 A1 (CHANG et al.) 12 October 2006 (12.10.2006), para [0082], [0111], [0160], [0161], [0215], [0418]	1-23, 26, 30
A	US 2005/0158347 A1 (TARLETON et al.) 21 July 2005 (21.07.2005)	1-30
A	US 2004/0241729 A1 (LIEW) 2 December 2004 (02.12.2004)	1-30
A	US 2005/0244505 A1 (HIGBEE et al.) 3 November 2005 (03.11.2005)	1-30

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 October 2008 (19.10.2008)

Date of mailing of the international search report

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PCT OSP: 571-272-7774



# Genome-Wide Screening and Identification of New *Trypanosoma cruzi* Antigens with Potential Application for Chronic Chagas Disease Diagnosis

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## Abstract

The protozoan *Trypanosoma cruzi* is the etiologic agent of Chagas disease, an infection that afflicts approximately 8 million people in Latin America. Diagnosis of chronic Chagas disease is currently based on serological tests because this condition is usually characterized by high anti-*T. cruzi* IgG titers and low parasitemia. The antigens used in these assays may have low specificity due to cross reactivity with antigens from related parasite infections, such as leishmaniasis, and low sensitivity caused by the high polymorphism among *T. cruzi* strains. Therefore, the identification of new *T. cruzi*-specific antigens that are conserved among the various parasite discrete typing units (DTUs) is still required. In the present study, we have explored the hybrid nature of the *T. cruzi* CL Brener strain using a broad genome screening approach to select new *T. cruzi* antigens that are conserved among the different parasite DTUs and that are absent in other trypanosomatid species. Peptide arrays containing the conserved antigens with the highest epitope prediction scores were synthesized, and the reactivity of the peptides were tested by immunoblot using sera from C57BL/6 mice chronically infected with *T. cruzi* strains from the TcI, TcII or TcVI DTU. The two *T. cruzi* proteins that contained the most promising peptides were expressed as recombinant proteins and tested in ELISA experiments with sera from chagasic patients with distinct clinical manifestations: those infected with *T. cruzi* from different DTUs and those with cutaneous or visceral leishmaniasis. These proteins, named rTc\_11623.20 and rTc\_N\_10421.310, exhibited 94.83 and 89.66% sensitivity, 98.2 and 94.6% specificity, respectively, and a pool of these 2 proteins exhibited 96.55% sensitivity and 98.18% specificity. This work led to the identification of two new antigens with great potential application in the diagnosis of chronic Chagas disease.

**Citation:** Reis-Cunha JL, Mendes TAdO, de Almeida Lourdes R, Ribeiro DRdS, Machado-de-Avila RA, et al. (2014) Genome-Wide Screening and Identification of New *Trypanosoma cruzi* Antigens with Potential Application for Chronic Chagas Disease Diagnosis. PLoS ONE 9(9): e106304. doi:10.1371/journal.pone.0106304

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**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

The protozoan *Trypanosoma cruzi* is the etiologic agent of Chagas disease, an infection that afflicts 8 million people in Latin America, causes high morbidity and accounts for 662,000 disability-adjusted life years (DALYs) [1–4]. The parasite is distributed from Southern USA to Southern Argentina, comprising 22 countries, where bloodsucking insects of the Triatominae subfamily are incriminated in the vector-borne transmission route of Chagas disease [2].

Immigration and travel of chagasic patients to non-endemic countries, such as the US [5], Australia and Spain [6], has spread this infection worldwide [7,8], highlighting other important

transmission routes, such as blood transfusion, congenital transmission and organ transplantation [7,8]. These alternative transmission routes have also gained importance in endemic countries where vector-borne transmission has been controlled [2,9].

The progression of Chagas disease begins with an acute phase that lasts 10–90 days and is characterized by high parasitemia but is usually asymptomatic. After approximately three months of infection, the parasitemia is controlled by the host immune response, and the patients reach the chronic phase, which is usually characterized by high anti-*T. cruzi* IgG titers [10]. Chronic Chagas disease is asymptomatic in 70% of the patients but evolves

into cardiac, digestive or mixed clinical forms in 30% of cases [10–12].

Because parasitemia is low during chronic Chagas disease, diagnosed is mainly carried out using serologic assays, such as indirect ELISA, indirect hemagglutination and indirect immunofluorescence [13–15]. The use of whole or fractionated parasite protein extracts as antigens has been replaced by recombinant proteins or peptides, which have higher specificity due to lower cross-reactivity with other infections, such as leishmaniasis [16–18] and *T. rangeli* [15,19]. In addition, the use of recombinant proteins or peptides facilitates the standardization of methods for antigen preparation [15,20].

The *T. cruzi* taxon has been extensively documented as highly polymorphic [21–24], and currently, six different DTUs (discrete typing units), named TcI – TcVI, have been recognized [25]. This high variability among *T. cruzi* strains compromises the sensitivity of serological tests for Chagas disease [15,26]. The TcV and TcVI DTUs are derived from recent recombination events between strains from TcII and TcIII [27,28]. CL Brener, the first strain to have its genome sequenced [29], belongs to the TcVI DTU. Because CL Brener is a hybrid strain, its genome is composed of two haplotypes named esmo-like and non-esmo-like, which are derived from TcII and TcIII, respectively [29]. TcI, TcII, TcV and TcVI are the most common lineages associated with human infections [30,31].

In the present study, we have explored the hybrid nature of the CL Brener strain using a broad genome screening approach to select new *T. cruzi* antigens that are potentially conserved among the different parasite DTUs and that are absent in other trypanosomatid species. This analysis led to the identification of two new candidate antigens for use in the serodiagnosis of chronic Chagas disease.

## Materials and Methods

### Ethics statement

The design and methodology of all experiments involving mice were performed in accordance with the guidelines of COBEA (Brazilian College of Animal Experimentation), strictly followed the Brazilian law for “Procedures for the Scientific Use of Animals” (11.794/2008) and were approved by the animal-care ethics committee of the Federal University of Minas Gerais (protocol number 143/2009).

The study protocol involving human samples was approved by the Ethics Committee of the Federal University of Minas Gerais (UFMG) under protocol number No. 312/06. All subjects provided written informed consent before blood sample was collected.

### Mouse sera

Each experimental group was composed of five 6–8-week old C57BL/6 female mice, which were infected intraperitoneally with *T. cruzi* bloodstream trypomastigotes from Colombiana, Y or CL Brener strains. All parasites were previously genotyped according to Souto *et al.*, 1996 [32], de-Freitas *et al.*, 2006 [28] and Burgos *et al.*, 2007 [33]. Infection was confirmed by the observation of trypomastigote forms in blood collected from the infected mice’s tail seven days after the parasite inoculation. An additional group was infected with *T. rangeli* trypomastigotes from the SC-58 strain, and infection was confirmed by PCR [34]. Six uninfected mice were used as the control group. The chronic phase of infection was confirmed 4 months post-infection by negative parasitemia and the presence of anti-parasite IgG (as tested against *T. cruzi* and *T. rangeli* crude antigens) by ELISA [35]. Blood from

chronically infected mice was then collected by cardiac puncture followed by 30 minutes incubation at room temperature and centrifugation at 4,000 × g for 15 minutes at 4°C to obtain serum, which was stored at -80°C until use.

We performed ELISA using sera from mice chronically infected with *T. cruzi* and the epimastigote raw extract as antigen and determined that the 1:100 dilution would be adequate to discriminate between sera from infected and non-infected mice.

### Human sera

A total of 58 sera samples from chagasic patients were used in this study. Of these samples, 43 were from chagasic patients infected with untyped parasites collected from Rio Grande do Norte and Minas Gerais States, Brazil, 8 were samples from chagasic patients previously characterized to be infected with TcII [36], and 7 were samples from patients known to be infected with TcVI. Of the 43 samples from chagasic patients infected with untyped parasites, 23 are from patients with defined clinical forms of Chagas disease: 9 are from patients with indeterminate forms, 14 with chronic chagasic cardiopathy [37]. Infection was confirmed by testing the reactivity of sera from chagasic patients using Chagatest recombinant ELISA v. 3.0 kit, Chagatest hemagglutination inhibition (HAI), screening A-V kit (Wiener Lab, Rosário, Argentina) using titers of 1:40 as the cutoff value, and indirect immunofluorescence (IIF) using a *T. cruzi* Y strain epimastigotes maintained in culture and fixed with 20% formaldehyde as the antigen all according to the manufacturer’s instructions. Anti-human IgG immunoglobulin labeled with fluorescein isothiocyanate (Sigma Chemical Company, Missouri, USA) was used as the secondary antibody at titers of 1:40 as the cutoff value [38]. The serum samples were considered positive for *T. cruzi* infection when two methods with different principles were reactive, indeterminate when only one method was reactive, and negative when these methods were nonreactive in accordance with the recommendations of the World Health Organization [39]. Sera exhibiting indeterminate results were evaluated using western blot (TESAcruzi, bioMérieux Brazil) to measure anti-*T. cruzi* reactivity [40].

To evaluate the cross-reactivity with leishmaniasis, sera from 5 patients with clinical signs of cutaneous leishmaniasis and confirmed infections by microscopic analysis of biopsy from the cutaneous lesion from the Centro de Referência em Leishmaniose in Januária/MG Brazil were used. In addition, sera from 5 patients with visceral leishmaniasis with reactivity confirmed by serology, molecular analysis and microscopic analysis of bone marrow aspirates from Hospital Universitário Clemente de Faria in Montes Claros/MG Brazil were used.

The sera of 45 healthy Brazilian individuals residing in Belo Horizonte, Minas Gerais were used as a negative control.

### In silico prediction of linear B-cell epitopes

Linear B-cell epitope predictions were performed on all predicted proteins in CL Brener genome release 4.1 [29] using the BepiPred 1.0 program with a cutoff of 1.3 [41] as previously described [42]. To select epitopes that are potentially conserved among *T. cruzi* strains, the predicted proteome from the esmo-like and non-esmo-like haplotypes of the *T. cruzi* CL Brener strain [29] were aligned using the ClustalW program [43]. The predicted epitopes that had identical sequences in the two CL Brener haplotypes were selected, and 15–18 mer peptides with mean score  $\geq 1.3$  were retained. To reduce the chance of cross-reaction with other parasites, the selected peptides were analyzed with BLASTP searches against the predicted *L. braziliensis*, *L. infantum* and *L. major* proteomes, and those that showed greater

than 70% similarity along 70% of the length were discarded. After analysis, 450 peptides with the highest mean BepiPred score that were identical between the two CL Brener haplotypes and absent in the predicted *Leishmania major*, *L. braziliensis* and *L. infantum* proteomes were selected for synthesis.

### Spot synthesis and immunoblotting

Peptide arrays containing 30, 120 or 300 distinct peptides (450 in total) were synthesized as previously described [42] (Table S1). These membranes were used in immunoblotting assays with pools of sera from five C57BL/6 mice chronically infected with Colombiana (TcI), Y (TcII) or CL Brener (TcVI) *T. cruzi* strains or uninfected mice. Briefly, the membranes were blocked for 16 hours with 5% BSA, 4% sucrose in PBS, washed 3 times for 10 minutes with 0.1% Tween-20 in PBS and incubated with one of the pools of sera diluted 1:2,500 in 0.1% Tween in PBS for one hour. Subsequently, the membranes were washed as described above, incubated with horseradish peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich) diluted 1:35,000 in 0.1% Tween in PBS for one hour, washed as described above and visualized by chemiluminescence using ECL Plus western blotting detection system (GE-Healthcare) with 20 minutes exposure on an ImageQuant LAS 4000 digital imaging system (GE-Healthcare). After data acquisition, the membranes were regenerated for use with another pool of sera. The regeneration was performed by washing the membranes 3 times for 10 minutes with dimethylformamide, followed by incubation for 16 hours with an 8 M urea, 10% SDS solution. The membranes were then washed twice for 30 minutes in the 8 M urea, 10% SDS solution, washed once with deionized water, and then washed 3 times in 55% ethanol and 10% acetic acid. Lastly, the membranes were washed for 5 minutes with deionized water. The densitometric value of each spot was calculated using ImageMaster 2D Platinum software (GE-Healthcare). The relative value (RV) for each spot was calculated using the formula  $RV = PDV/NDV$ , where PDV corresponds to the spot densitometric value when a positive pool of sera were used and NDV corresponds to the densitometric value when the negative pool of sera were used. Spots with RV value of 2 or higher were considered reactive.

### Expression and purification of recombinant proteins

The primer sequences used to amplify the entire coding sequence of the Tc00.1047053511623.20 gene and the 5' end of the Tc00.1047053510421.310 gene are listed in Table S2. The PCR reactions were performed using 100 ng of genomic DNA from the *T. cruzi* CL Brener strain and Platinum Taq DNA High Fidelity Polymerase (Invitrogen) according to the manufacturer's instructions. The amplicons were purified from an agarose gel using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions. The purified fragments were cloned into the pET-28a-TEV expression vector (CeBiMe, Campinas/SP) in frame with the N-terminal poly-histidine tag. The constructs were sequenced to confirm that the fragments were cloned in frame, and the recombinant proteins were expressed in the *Escherichia coli* BL-21Star strain by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture for 3 h at 37°C shaking at 180 rpm. Recombinant proteins were purified using affinity chromatography with HisTrap HP 5 mL columns (GE Healthcare Life Sciences) in the AKTA primeplus (GE Healthcare Life Sciences) system. The purified proteins were separated by SDS-PAGE and visualized by western blotting to confirm their identity and purity (data not shown).

### ELISA

ELISA half area plates (Greiner-Bio-One-675061) were coated with 50 μL/well of a solution containing 5 μg/mL recombinant Tc00.1047053511623.20 (*rTc\_11623.20*) protein, 10 μg/mL recombinant protein corresponding to the N-terminal portion of the Tc00.1047053510421.310 protein (*rTc\_N\_10421.310*), or 10 μg/mL protein mixture containing both recombinant proteins at a ratio of 1:1 diluted in ultrapure H<sub>2</sub>O for 18 h at 4°C. Plates were blocked with 200 μL 5% BSA in PBS for 1 hour at 37°C. The human or mouse sera, diluted 1:100 in 2.5% BSA in PBS was added and incubated for 1 hour at 37°C. The plates were washed four times with 200 μL PBS containing 0.05% Tween 20 and incubated for 1 hour at 37°C with 50 μL secondary horseradish peroxidase-conjugated anti-human or anti-mouse IgG antibody (Sigma-Aldrich) diluted 1:5,000 in 2.5% BSA in PBS. Then, the plates were washed four times as previously described and incubated for 15 minutes at 37°C in a dark room with 50 μL revealing solution (0.1 M citric acid, 0.2 M Na<sub>2</sub>PO<sub>4</sub>, 0.05% OPD, 0.1% H<sub>2</sub>O<sub>2</sub>). The reaction was interrupted by adding 50 μL 4N HCl, and the absorbance was measured at 490 nm in an automated Versa Max Microplate Reader. Each sample was assayed in triplicate.

### Statistical analysis

All statistical analyses were performed using Graph Prism 5.0 software. The normal distribution of data was evaluated by the Kolmogorov-Smirnov test, an unpaired *t*-test was used for the comparative analysis between the two data sets, and ANOVA was used to evaluate three or more experimental groups. P values lower than 0.05 were considered statistically significant. The values of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated for the human sera. The cutoff value was determined based on the Receiver Operating Characteristic (ROC) curve to maximize sensibility and specificity.

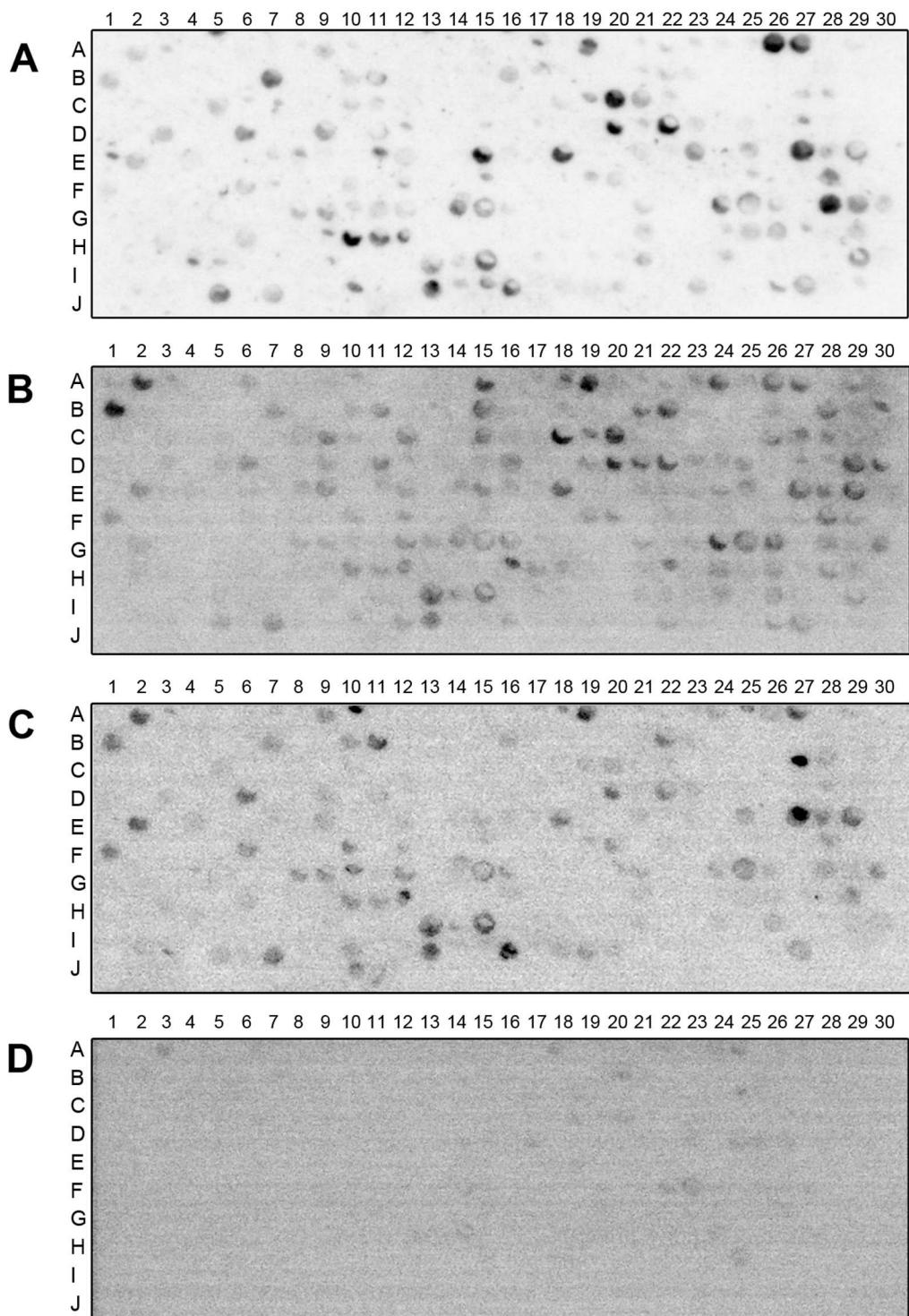
### Results

#### Evaluation of the reactivity of the selected peptides with the sera from C57BL/6 mice infected with different *T. cruzi* strains using immunoscreening of peptide arrays

To correctly diagnose patients with chronic Chagas disease, identifying antigens that are conserved among parasite strains but do not cross-react with sera from individuals infected with related parasites, such as *T. rangeli* and *Leishmania* species, is imperative.

As an attempt to identify epitopes conserved in distinct *T. cruzi* DTUs, we have performed polymorphism analysis and B-cell epitope prediction on all proteins derived from single copy genes represented by allele pairs in the CL Brener genome. We assumed that because CL Brener is a recent hybrid between TcII and TcIII DTUs, alleles pairs that contain conserved epitopes are likely to be conserved in the parental genotypes. To this end, we aligned 3,983 proteins derived from allele pair from esmo-like and non-esmo-like haplotypes, and the conserved sequences were analyzed by epitope prediction using the BepiPred algorithm [41]. A total of 1,488 conserved predicted B cell epitopes were identified. To reduce the chance of cross-reactivity with related parasites, we excluded 402 sequences that had high sequence similarity to the predicted proteome of human-infectious *Leishmania* species. A total of 1,086 peptides ranging from 15–18 amino acids that are potentially conserved among different *T. cruzi* strains but are absent in *Leishmania* sp. were selected.

To determine the reactivity of the sera from mice experimentally infected with *T. cruzi* to these antigens, 450 peptides with



**Figure 1. Immunoblot of a peptide array with sera from C57BL/6 mice chronically infected with different *T. cruzi* strains.** (A) Sera from mice infected with Colombiana (TcI); (B), Y (TcII) or (C), CL Brener (TcVI); or (D) uninfected mice.  
doi:10.1371/journal.pone.0106304.g001

high BepiPred scores were synthesized on cellulose membranes and probed using a pool of sera from C57BL/6 mice chronically infected with the *T. cruzi* strains Colombiana (TcI), Y (TcII) or CL Brener (TcVI) (Figure 1). These strains correspond to the *T. cruzi* DTUs commonly associated with human infections.

The antigen spots that were reactive with pooled serum from uninfected mice were excluded from further analysis. The densitometric value of each spot with each pool of serum was determined, and their relative value (RV) was calculated using the formula  $RV = PDV/NDV$ . Spots with an RV value of 2 or higher

were categorized as reactive. Peptides that were reactive with at least one serum pool from *T. cruzi*-infected mice are listed in the Table S3. Two peptides, named C6-30 and E27-300, had RV higher than 2 for all pools of sera from the mice chronically infected with each one of the *T. cruzi* strains assayed. The genes that encode the proteins containing these peptides are Tc00.1047053510421.310 and Tc00.1047053511623.20, respectively.

These protein sequences were analyzed for linear B cell epitopes and intrinsically unstructured regions. The presence of these unstructured regions suggest that a given protein region is in an unfolded structure and therefore possibly accessible for antibody binding. Hence, the co-occurrence of these two features reinforces the accuracy of the B cell linear epitope prediction. All selected proteins had a high density of predicted B cell epitopes and large predicted unfolded regions that co-localize with these epitopes (Figure 2).

### Expression and purification of recombinant proteins

The full-length Tc00.1047053511623.20 gene was cloned into the pET28a-TEV vector for recombinant expression. Due to large size of the Tc00.1047053510421.310 gene, only its first 1,704 nucleotides which contains the coding sequence of the peptide C6-30 were cloned into the expression vector. Both sequences were cloned in frame in the expression vector as confirmed by sequencing (data not shown). The recombinant proteins with a predicted molecular weight of 47 kDa for Tc00.1047053511623.20 (named rTc\_11623.20) and 66 kDa for the N-terminal portion of the protein Tc00.1047053510421.310 (rTc\_N\_10421.310) were successfully expressed in *E. coli* BL21 cells as insoluble his-tagged proteins and purified by Ni<sup>2+</sup>-affinity chromatography (GE Healthcare). The recombinant protein expression was confirmed by western blot using mouse anti-His antibody (GE Healthcare) (data not shown).

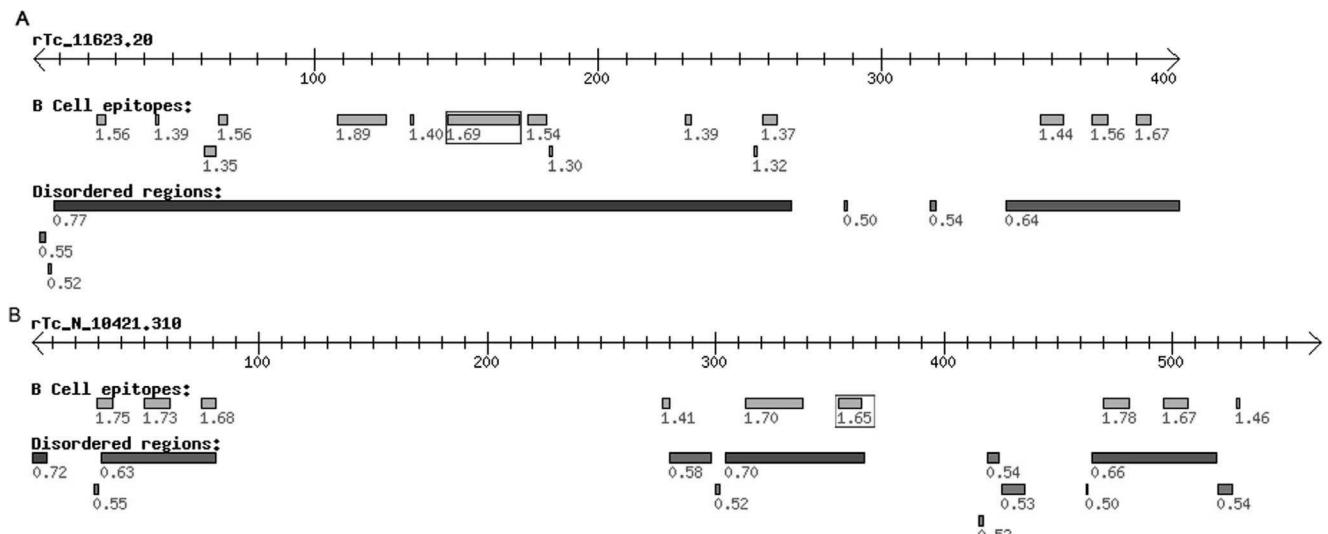
### Reactivity of sera from C57BL/6 mice chronically infected with *T. cruzi* to the purified recombinant proteins

Initially, the recombinant proteins were analyzed by ELISA using individual sera from three groups of C57BL/6 mice: (i) mice chronically infected with Colombiana (TcI), Y (TcII) or CL Brener (TcVI) *T. cruzi* strains, (ii) mice infected with the SC-58 strain of *T. rangeli*, (iii) or uninfected mice. The serum samples used in this assay were the same as the pooled samples used for the immunoblotting screen. Both rTc\_11623.20 and rTc\_N\_10421.310 had high sensitivity and specificity (Figure 3, Figure S1 and Table S4). In concordance with the immunoblotting assays, all sera from mice chronically infected with each one of the three *T. cruzi* strains were reactive with rTc\_11623.20 and rTc\_N\_10421.310 when they were assayed individually and when assayed using an antigen pool containing both recombinant proteins by ELISA. The sera from *T. rangeli*-infected mice or uninfected mice exhibited results below the cutoff for all antigens (Figure 3).

### Performance of rTc\_11623.20 and rTc\_N\_10421.310 for diagnosing Chagas disease by ELISA

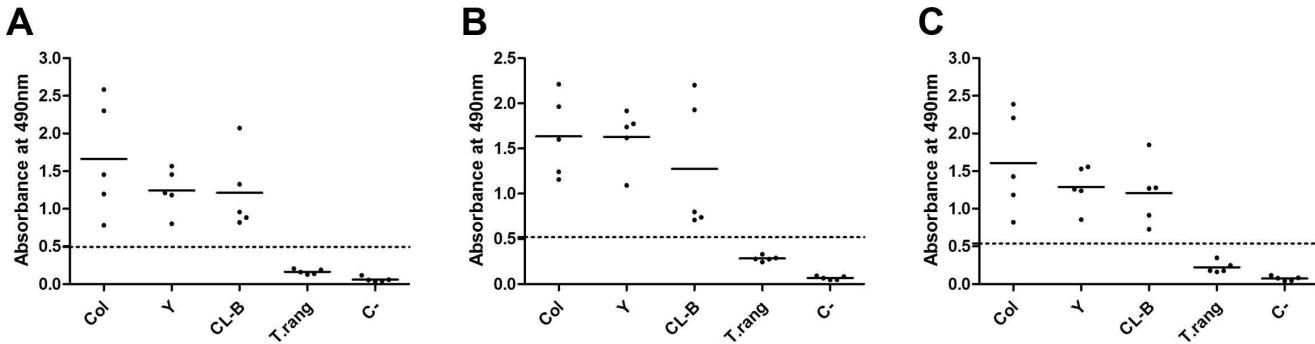
To evaluate the accuracy of the recombinant proteins rTc\_11623.20 and rTc\_N\_10421.310 in the diagnosis of chronic human Chagas disease, a total of 113 human sera, of which 58 samples correspond to chronic chagasic patients and 55 to non-chagasic control patients, were screened. All serum samples were tested by ELISA using both recombinant proteins (Figure 4, Figure S2).

Of the 58 sera from chagasic patients, 55 were reactive with rTc\_11623.20 (Figure 4A), 52 with rTc\_N\_10421.310 (Figure 4B) and 56 with a pool of both recombinant proteins (Figure 4C). All three sera that were below the cutoff for rTc\_11623.20 belong to the non-typed group (Figure 4D). Of the six sera from chagasic patients that were below the cutoff value for rTc\_N\_10421.310, four samples belong to the non-typed group, one to the TcVI group and one to the Tc-Card group (Figure 4E). The two sera



**Figure 2. Predictions of B-cell linear epitopes and intrinsically unstructured/disordered regions in rTc\_11623.20 (A) and rTc\_N\_10421.310 (B).** The complete sequences of the recombinant proteins were submitted to the BepiPred and IUPred algorithms. The dashed arrow corresponds to the complete amino acid sequence of the recombinant proteins. The orange boxes indicate linear B-cell epitopes as predicted by BepiPred, and the gray boxes indicate unfolded regions that were predicted by IUPred. The epitope region that contains the peptide screened in the immunoblotting for each one of the proteins is highlighted by a blue box.

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**Figure 3. Recognition of *rTc\_11623.20* and *rTc\_N\_10421.310* by sera from C57BL/6 mice chronically infected with different *T. cruzi* strains.** Sera from mice chronically infected with CL Brener (CL-B), Colombiana (Col) or Y (*T. cruzi* strain), mice infected with *T. rangeli* (T.rang) or uninfected mice (C-) were screened by ELISA with *rTc\_11623.20* (A), *rTc\_N\_10421.310* (B) or with a pool of these two recombinant proteins (C). The dotted line represents the cutoff value that was obtained based on the ROC curve. The solid line corresponds to the mean values. Col, mice infected with Colombiana; Y, mice infected with the Y strain; CL-B, mice infected with the CL Brener; T. rang, *T. rangeli*-infected mice. C-, uninfected mice. doi:10.1371/journal.pone.0106304.g003

that were below the cutoff for the pool of both recombinant proteins belong to the non-typed group (Figure 4F).

Of the 55 sera from non-chagasic individuals, 54, 52, and 54 samples were below cutoff for *rTc\_11623.20* (Figure 4A), *rTc\_N\_10421.310* (Figure 4B) and the pool of both proteins, respectively (Figure 4C). The only non-chagasic serum sample that was above cutoff value for *rTc\_11623.20* was from a patient with visceral leishmaniasis (Figure 4D). Of the three sera from non-chagasic individuals that had values above cutoff for *rTc\_N\_10421.310*, two were from patients with visceral leishmaniasis and one from a patient with cutaneous leishmaniasis (Figure 4E). The only non-chagasic serum sample that had values above cutoff for the pooled recombinant proteins belonged to the uninfected group (Figure 4F).

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy values were 94.83, 98.18, 98.30, 94.82 and 96.4, respectively for *rTc\_11623.20*; 89.66, 94.55, 95.08, 90.16, and 92.03 respectively for *rTc\_N\_10421.310* and 96.55, 98.18, 98.30, 96.49 and 97.34, respectively for the pooled antigens (Table S4).

## Discussion

The lack of highly accurate methods to diagnose Chagas disease hampers the correct identification and treatment of infected individuals and restricts the evaluation of effectiveness of any initiative aiming at blocking transmission or vaccination in endemic countries [13,15]. Additionally, reports of blood transfusion transmission in the USA, Spain and Canada, countries that receive a high contingency of Latin-American immigrants also highlights the importance of an accurate diagnostic test to identify Chagas disease infection in samples from blood banks of non-endemic countries [5–8,44,45].

Due to the high genetic variability among the *T. cruzi* strains, the sensitivity of many diagnostic tests varies widely with sera from patients infected with parasites from different geographic regions, where different *T. cruzi* DTUs are found [31]. To identify new antigens that are potentially conserved among distinct *T. cruzi* strains, in this study, we applied a genome-wide screening aiming at identifying epitopes that are conserved between esmo-like and non-esmo haplotypes of the CL Brener hybrid strain. Because the esmo-like haplotype is derived from TcII and non-esmo haplotype is derived from the TcIII, antigens that are conserved between

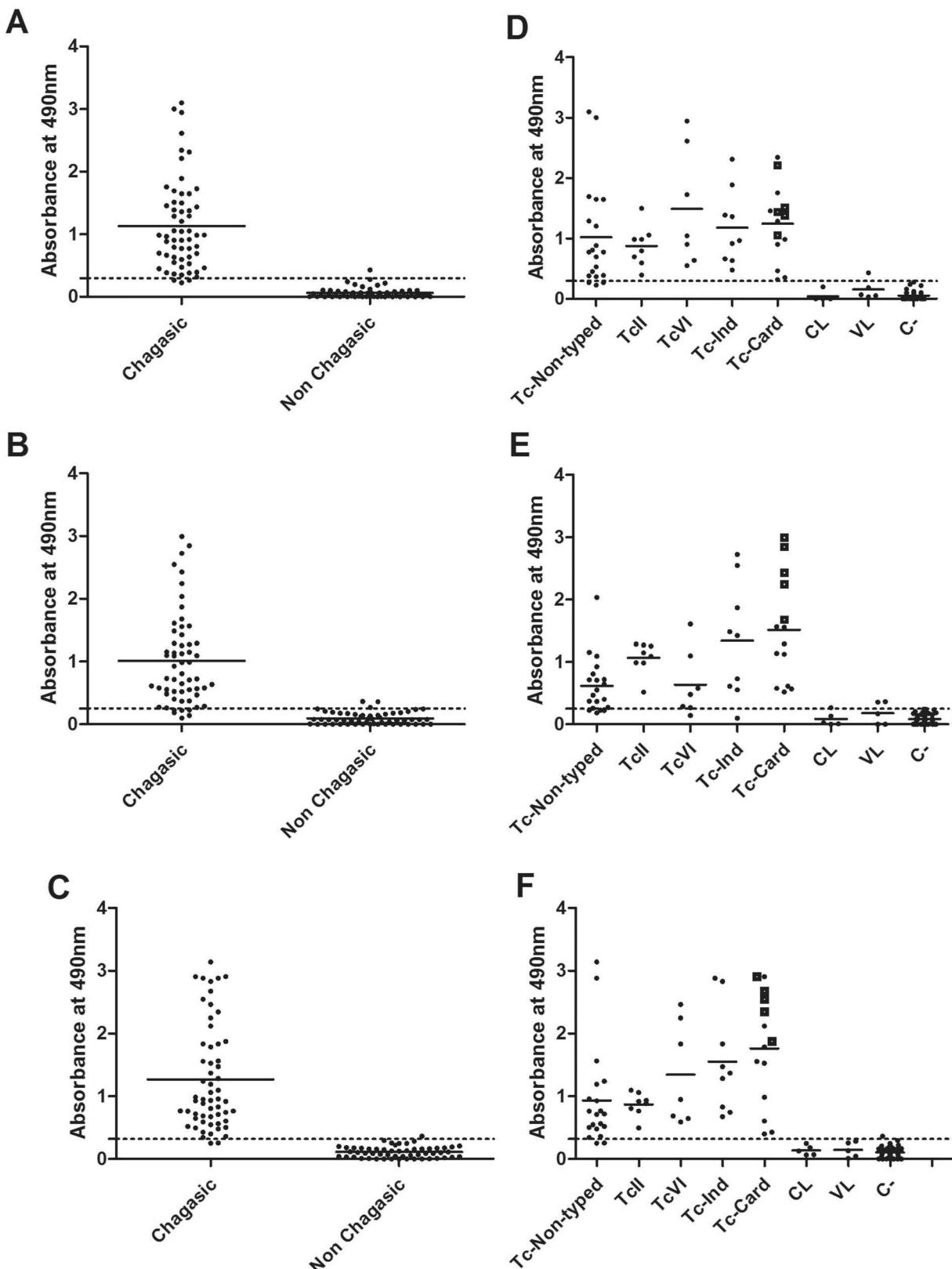
these two haplotypes are likely to also be conserved among other *T. cruzi* DTUs.

The major drawback of the specificity of Chagas disease serologic tests is cross-reaction with sera from patients infected with related parasites, such as *T. rangeli* and the *Leishmania* genus [15,18,46,47]. In an attempt to increase the specificity of the selected epitopes, we excluded sequences that had high similarity to the predicted proteome of sequenced, human-infectious *Leishmania* species. This approach allowed us to identify two new antigens, named *rTc\_11623.20* and *rTc\_N\_10421.310*, designed for Chagas disease diagnosis.

These two antigens were first screened with the sera from C57BL/6 mice chronically infected with *T. cruzi* strains from TcI (Colombiana), TcII (Y) and TcVI (CL Brener) DTUs. The selection of these *T. cruzi* DTUs was due to their broad geographic distribution, importance in human infection in Latin America and their categorization into highly divergent *T. cruzi* DTUs [25,31]. Both proteins, used individually or pooled, were able to discriminate 100% of the sera from mice infected with all the three *T. cruzi* strains from the uninfected mice or from mice infected with *T. rangeli* (Figure 3).

Next, we tested the accuracy of *rTc\_11623.20* and *rTc\_N\_10421.310* as antigens to diagnose human patients with chronic Chagas disease. To this end, sera from patients infected with TcII and TcVI *T. cruzi* DTUs, sera from patients with different clinical forms of Chagas disease and sera from patients infected with non-typed *T. cruzi* were used. This sera panel was used because patients that are infected with different DTUs or present different clinical forms have been reported to exhibit discordant results when evaluated with conventional Chagas serologic tests [14,15,26]. Additionally, cross-reactivity between sera from patients infected with *T. cruzi* and *Leishmania* spp. is well documented [15,17,18]. To determine the specificity of both recombinant proteins, sera from healthy humans and patients with cutaneous or visceral leishmaniasis were also assayed.

The sensitivity and specificity of the *rTc\_11623.20*, *rTc\_N\_10421.310* and pooled recombinant proteins were comparable with the results from commercial tests as the INNO-LIA Chagas assay, which contains a combination of recombinant antigens and exhibited 99.4% sensitivity and 98.1% specificity [47]. The *rTc\_11623.20* and *rTc\_N\_10421.310* antigens also showed slightly better results than the recombinant protein TSSA VI, which exhibits 87% sensitivity and 97.4% specificity [48]. Additionally, *rTc\_11623.20* and *rTc\_N\_10421.310* showed sim-



**Figure 4. Recognition of *rTc\_11623.20* and *rTc\_N\_10421.310* by the sera from chronic chagasic patients or *Leishmania*-infected individuals.** Sera from chronic chagasic patients and sera from patients with cutaneous and visceral leishmaniasis were assayed by ELISA using *rTc\_11623.20* (A, D), *rTc\_N\_10421.310* (B, E) and pooled recombinant proteins (C, F). Tc-non typed, sera from chronic chagasic patients; TcII, sera from patients infected with *T. cruzi* TcII DTU; TcVI, sera from patients infected with *T. cruzi* TcVI DTU, Tc-Ind, sera from chagasic patients in the indeterminate form of Chagas disease; Tc-Card, sera from chagasic patients in the cardiac stage of Chagas disease; CL, sera from patients with

cutaneous leishmaniasis; VL, sera from patients with visceral leishmaniasis; C-, uninfected individuals. Squares represent sera from chagasic patient in the initial cardiac stage. The dotted line represents the cutoff value that was obtained based on the ROC curve. The solid line corresponds to the mean values.

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ilar accuracy compared to ELISA-IMT, Chagas III (BIOSChile) ELISACruzi (bioMérieux Brasil SA), Chagatest (bioMérieux Brasil SA) Chagatest Rec v3.0 (Wiener) and Pathozyme Chagas (Omega), which have sensitivities ranging from 75 to 100% and specificities ranging from 82.84 to 100% [15].

It is well established that new potential antigens selected for the diagnosis of chronic Chagas disease must meet three criteria: (i) expression in *T. cruzi* isolates from different DTUs and absence in other infectious disease pathogens. (ii) high immunogenicity regardless the clinical form of Chagas disease and (iii) stability and adaptability to quality-control tests to guarantee reproducibility [13,49,50]. We believe that the antigens identified in this study meet all these criteria. First, our data suggests that they were able to discriminate sera from patients infected with *T. cruzi* from sera from patients with cutaneous, visceral leishmaniasis or healthy donors, with specificity of 98.18 for *rTc\_11623.20*, 94.55 for *rTc\_N\_10421.310* and 98.18 for a pool of both proteins. Second, the antigens were recognized by 14 out of the 15 sera from patients infected with *T. cruzi* strains belonging to different DTUs. Third, both antigens were reactive with sera from patients with different clinical forms of Chagas disease, with the exception of one indeterminate sera for the protein *rTc\_N\_10421.310*. Forth, the expression protocol used in this study yielded approximately 17 mg of protein/culture liter, which allows nearly 22,500 sera trials to be performed with *rTc\_11623.20* and 11,300 trials with *rTc\_N\_10421.310*.

Further studies using a larger sera panel from negative and positive individuals with different clinical forms and from distinct endemic areas throughout Latin America where different *T. cruzi* DTUs are found will be necessary to better characterize the antigens identified in this study. Additionally, we are currently expanding the genome wide approach for the antigen selection described in this work to include new sequenced *T. cruzi* genomes [51,52] as an attempt to define an highly effective antigenic panel for the Chagas disease serodiagnosis.

## References

- Hotez PJ, Bottazzi ME, Franco-Paredes C, Ault SK, Periago MR (2008) The neglected tropical diseases of Latin America and the Caribbean: a review of disease burden and distribution and a roadmap for control and elimination. *PLoS Negl Trop Dis* 2: e300.
- Coura JR, Dias JC (2009) Epidemiology, control and surveillance of Chagas disease: 100 years after its discovery. *Mem Inst Oswaldo Cruz* 104 Suppl 1: 31–40.
- Martins-Melo FR, Alencar CH, Ramos AN, Jr., Heukelbach J (2012) Epidemiology of mortality related to Chagas' disease in Brazil, 1999–2007. *PLoS Negl Trop Dis* 6: e1508.
- WHO (2012) Research Priorities for Chagas Disease, Human African Trypanosomiasis and Leishmaniasis. Technical Report of the TDR Disease Reference Group on Chagas Disease, Human African Trypanosomiasis and Leishmaniasis. (Technical report series; no. 975). Available at: [http://apps.who.int/iris/bitstream/10665/77472/1/WHO\\_TRS\\_975\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/77472/1/WHO_TRS_975_eng.pdf)
- Bern C, Kjos S, Yabsley MJ, Montgomery SP (2011) *Trypanosoma cruzi* and Chagas' Disease in the United States. *Clin Microbiol Rev* 24: 655–681.
- Benjamin RJ, Stramer SL, Leiby DA, Dodd RY, Fearon M, et al. (2012) *Trypanosoma cruzi* infection in North America and Spain: evidence in support of transfusion transmission. *Transfusion* 52: 1913–1921; quiz 1912.
- Schmunis GA, Yadon ZE (2010) Chagas disease: a Latin American health problem becoming a world health problem. *Acta Trop* 115: 14–21.
- Perez-Molina JA, Norman F, Lopez-Velez R (2012) Chagas disease in non-endemic countries: epidemiology, clinical presentation and treatment. *Curr Infect Dis Rep* 14: 263–274.
- Dias JC, Silveira AC, Schofield CJ (2002) The impact of Chagas disease control in Latin America: a review. *Mem Inst Oswaldo Cruz* 97: 603–612.
- Junqueira C, Caetano B, Bartholomeu DC, Melo MB, Ropert C, et al. (2010) The endless race between *Trypanosoma cruzi* and host immunity: lessons for and beyond Chagas disease. *Expert Rev Mol Med* 12: e29.
- Prata A (2001) Clinical and epidemiological aspects of Chagas disease. *Lancet Infect Dis* 1: 92–100.
- Teixeira AR, Nascimento RJ, Sturm NR (2006) Evolution and pathology in chagas disease—a review. *Mem Inst Oswaldo Cruz* 101: 463–491.
- Bern C, Coura JR, Goldenberg S, Guhl F, Junqueira ACV, Loura M, Luquetti AO, Ribeiro I, Sáez-Alquezar A, et al. (2008) International meeting: new diagnostic tests are urgently needed to treat patients with Chagas disease. *Rev Soc Bras Med Trop* 41: 315–319.
- Ferrer E, Lares M, Vietri M, Medina M (2013) [Comparison between immunological and molecular techniques for the diagnosis of Chagas disease]. *Efemer Infec Microbiol Clin* 31: 277–282.
- Caballero ZC, Sousa OE, Marques WP, Saez-Alquezar A, Umezawa ES (2007) Evaluation of serological tests to identify *Trypanosoma cruzi* infection in humans and determine cross-reactivity with *Trypanosoma rangeli* and *Leishmania* spp. *Clin Vaccine Immunol* 14: 1045–1049.
- Peralta JM, Teixeira MG, Shreffler WG, Pereira JB, Burns JM Jr., et al. (1994) Serodiagnosis of Chagas' disease by enzyme-linked immunosorbent assay using two synthetic peptides as antigens. *J Clin Microbiol* 32: 971–974.

## Supporting Information

**Figure S1 ROC curves obtained from the ELISA with the recombinant antigens and the sera from C57BL/6 mice infected with *T. cruzi*, *T. rangeli* or non-infected mice.** (DOCX)

**Figure S2 ROC curves obtained from the ELISA with the recombinant antigens and sera from Chagasic and non-Chagasic human patients.** (DOCX)

**Table S1 Sequence of the peptides in the immunoblotting membranes.** (XLSX)

**Table S2 Primers used to amplify the entire coding region of the *Tc00.1047053511623.20* gene and the 5' end of the *Tc00.1047053510421.310* gene.** (DOCX)

**Table S3 Reactivity of the top ten peptides in the immunoblotting assays with the sera from mice chronically infected with distinct *T. cruzi* strains.** (DOCX)

**Table S4 Measure of diagnostic performance for *rTc\_11623.20*, *rTc\_N\_10421.310* and pooled antigens.** (DOCX)

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## Author Contributions

Conceived and designed the experiments: JLRC DCB. Performed the experiments: JLRC TAOM RAL DRSR RAMA. Analyzed the data: JLRC TAOM RAL DCB. Contributed reagents/materials/analysis tools: MOT DSL ACJC CCO ML LMCG RTF DCB. Contributed to the writing of the manuscript: JLRC DSL DCB.

17. da Silveira JF, Umezawa ES, Luquetti AO (2001) Chagas disease: recombinant *Trypanosoma cruzi* antigens for serological diagnosis. Trends Parasitol 17: 286–291.
18. Vega Benedetti AF, Cimino RO, Cajal PS, Juarez MD, Villalpando CA, et al. (2013) Performance of different *Trypanosoma cruzi* antigens in the diagnosis of Chagas disease in patients with American cutaneous leishmaniasis from a co-endemic region in Argentina. Trop Med Int Health.
19. de Moraes MH, Guarneri AA, Girardi FP, Rodrigues JB, Eger I, et al. (2008) Different serological cross-reactivity of *Trypanosoma rangeli* forms in *Trypanosoma cruzi*-infected patients sera. Parasit Vectors 1: 20.
20. De Marchi CR, Di Noia JM, Frasch AC, Amato Neto V, Almeida IC, et al. (2011) Evaluation of a recombinant *Trypanosoma cruzi* mucin-like antigen for serodiagnosis of Chagas' disease. Clin Vaccine Immunol 18: 1850–1855.
21. Bogliolo AR, Lauria-Pires L, Gibson WC (1996) Polymorphisms in *Trypanosoma cruzi*: evidence of genetic recombination. Acta Trop 61: 31–40.
22. Brisse S, Dujardin JC, Tibayrenc M (2000) Identification of six *Trypanosoma cruzi* lineages by sequence-characterised amplified region markers. Mol Biochem Parasitol 111: 95–105.
23. Vargas N, Pedroso A, Zingales B (2004) Chromosomal polymorphism, gene synteny and genome size in *T. cruzi* I and *T. cruzi* II groups. Mol Biochem Parasitol 138: 131–141.
24. Cerqueira GC, Bartholomeu DC, DaRocha WD, Hou L, Freitas-Silva DM, et al. (2008) Sequence diversity and evolution of multigene families in *Trypanosoma cruzi*. Mol Biochem Parasitol 157: 65–72.
25. Zingales B, Andrade SG, Briones MR, Campbell DA, Chiari E, et al. (2009) A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem Inst Oswaldo Cruz 104: 1051–1054.
26. Verani JR, Seitz A, Gilman RH, LaFuente C, Galdos-Cardenas G, et al. (2009) Geographic variation in the sensitivity of recombinant antigen-based rapid tests for chronic *Trypanosoma cruzi* infection. Am J Trop Med Hyg 80: 410–415.
27. Westenberger SJ, Barnabe C, Campbell DA, Sturm NR (2005) Two hybridization events define the population structure of *Trypanosoma cruzi*. Genetics 171: 527–543.
28. de Freitas JM, Augusto-Pinto L, Pimenta JR, Bastos-Rodrigues L, Goncalves VF, et al. (2006) Ancestral genomes, sex, and the population structure of *Trypanosoma cruzi*. PLoS Pathog 2: e24.
29. El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, et al. (2005) The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science 309: 409–415.
30. Miles MA, Llewellyn MS, Lewis MD, Yeo M, Baleela R, et al. (2009) The molecular epidemiology and phylogeography of *Trypanosoma cruzi* and parallel research on Leishmania: looking back and to the future. Parasitology 136: 1509–1528.
31. Zingales B, Miles MA, Campbell DA, Tibayrenc M, Macedo AM, et al. (2012) The revised *Trypanosoma cruzi* subspecific nomenclature: rationale, epidemiological relevance and research applications. Infect Genet Evol 12: 240–253.
32. Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B (1996) DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. Mol Biochem Parasitol 83: 141–152.
33. Burgos JM, Altcheh J, Bisio M, Duffy T, Valadares HM, et al. (2007) Direct molecular profiling of minicircle signatures and lineages of *Trypanosoma cruzi* bloodstream populations causing congenital Chagas disease. Int J Parasitol 37: 1319–1327.
34. Morales L, Romero I, Diez H, Del Portillo P, Montilla M, et al. (2002) Characterization of a candidate *Trypanosoma rangeli* small nucleolar RNA gene and its application in a PCR-based parasite detection. Exp Parasitol 102: 72–80.
35. Brasil PE, De Castro L, Hasslocher-Moreno AM, Sangenis LH, Braga JU (2010) ELISA versus PCR for diagnosis of chronic Chagas disease: systematic review and meta-analysis. BMC Infect Dis 10: 337.
36. Camara AC, Varela-Freire AA, Valadares HM, Macedo AM, D'Avila DA, et al. (2010) Genetic analyses of *Trypanosoma cruzi* isolates from naturally infected triatomines and humans in northeastern Brazil. Acta Trop 115: 205–211.
37. Dutra WO, Rocha MO, Teixeira MM (2005) The clinical immunology of human Chagas disease. Trends Parasitol 21: 581–587.
38. Camargo ME (1966) Fluorescent antibody test for the serodiagnosis of American trypanosomiasis. Technical modification employing preserved culture forms of *Trypanosoma cruzi* in a slide test. Rev Inst Med Trop Sao Paulo 8: 227–235.
39. WHO (2014) Chagas disease (American trypanosomiasis). Neglected tropical diseases. Available: [http://www.who.int/neglected\\_diseases/diseases/chagas/en/](http://www.who.int/neglected_diseases/diseases/chagas/en/). Accessed 02 June 2014.
40. Umezawa ES, Nascimento MS, Kespere Jr., Coura JR, Borges-Pereira J, et al. (1996) Immunoblot assay using excreted-secreted antigens of *Trypanosoma cruzi* in serodiagnosis of congenital, acute, and chronic Chagas' disease. J Clin Microbiol 34: 2143–2147.
41. Larsen JE, Lund O, Nielsen M (2006) Improved method for predicting linear B-cell epitopes. Immunome Res 2: 2.
42. Mendes TA, Reis Cunha JL, de Almeida Lourdes R, Rodrigues Luiz GF, Lemos LD, et al. (2013) Identification of strain-specific B-cell epitopes in *Trypanosoma cruzi* using genome-scale epitope prediction and high-throughput immunoscreening with peptide arrays. PLoS Negl Trop Dis 7: e2524.
43. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, et al. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31: 3497–3500.
44. Barona-Vilar C, Gimenez-Marti MJ, Fraile T, Gonzalez-Steinbauer C, Parada C, et al. (2012) Prevalence of *Trypanosoma cruzi* infection in pregnant Latin American women and congenital transmission rate in a non-endemic area: the experience of the Valencian Health Programme (Spain). Epidemiol Infect 140: 1896–1903.
45. Kessler DA, Shi PA, Avecilla ST, Shaz BH (2013) Results of lookback for Chagas disease since the inception of donor screening at New York Blood Center. Transfusion 53: 1083–1087.
46. Vexenat Ade C, Santana JM, Teixeira AR (1996) Cross-reactivity of antibodies in human infections by the kinetoplastid protozoa *Trypanosoma cruzi*, *Leishmania chagasi* and *Leishmania (viannia) braziliensis*. Rev Inst Med Trop Sao Paulo 38: 177–185.
47. Saez-Alquezar A, Sabino EC, Salles N, Chamone DF, Hulstaert F, et al. (2000) Serological confirmation of Chagas' disease by a recombinant and peptide antigen line immunoassay: INNO-LIA chagas. J Clin Microbiol 38: 851–854.
48. Marchini FK, de Godoy LM, Rampazzo RC, Pavoni DP, Probst CM, et al. (2011) Profiling the *Trypanosoma cruzi* phosphoproteome. PLoS One 6: e25381.
49. Gomes YM, Lorena VM, Luquetti AO (2009) Diagnosis of Chagas disease: what has been achieved? What remains to be done with regard to diagnosis and follow up studies? Mem Inst Oswaldo Cruz 104 Suppl 1: 115–121.
50. Coura JR, Borges-Pereira J (2012) Chagas disease. What is known and what should be improved: a systemic review. Rev Soc Bras Med Trop 45: 286–296.
51. Franzen O, Ochaya S, Sherwood E, Lewis MD, Llewellyn MS, et al. (2011) Shotgun sequencing analysis of *Trypanosoma cruzi* I Sylvio X10/1 and comparison with *T. cruzi* VI CL Brener. PLoS Negl Trop Dis 5: e984.
52. Franzen O, Talavera-Lopez C, Ochaya S, Butler CE, Messenger LA, et al. (2012) Comparative genomic analysis of human infective *Trypanosoma cruzi* lineages with the bat-restricted subspecies *T. cruzi* marinellei. BMC Genomics 13: 531.

# Identification of Strain-Specific B-cell Epitopes in *Trypanosoma cruzi* Using Genome-Scale Epitope Prediction and High-Throughput Immunoscreening with Peptide Arrays

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## Abstract

**Background:** The factors influencing variation in the clinical forms of Chagas disease have not been elucidated; however, it is likely that the genetics of both the host and the parasite are involved. Several studies have attempted to correlate the *T. cruzi* strains involved in infection with the clinical forms of the disease by using hemoculture and/or PCR-based genotyping of parasites from infected human tissues. However, both techniques have limitations that hamper the analysis of large numbers of samples. The goal of this work was to identify conserved and polymorphic linear B-cell epitopes of *T. cruzi* that could be used for serodiagnosis and serotyping of Chagas disease using ELISA.

**Methodology:** By performing B-cell epitope prediction on proteins derived from pairs of alleles of the hybrid CL Brener genome, we have identified conserved and polymorphic epitopes in the two CL Brener haplotypes. The rationale underlying this strategy is that, because CL Brener is a recent hybrid between the TcI and TcIII DTUs (discrete typing units), it is likely that polymorphic epitopes in pairs of alleles could also be polymorphic in the parental genotypes. We excluded sequences that are also present in the *Leishmania major*, *L. infantum*, *L. braziliensis* and *T. brucei* genomes to minimize the chance of cross-reactivity. A peptide array containing 150 peptides was covalently linked to a cellulose membrane, and the reactivity of the peptides was tested using sera from C57BL/6 mice chronically infected with the Colombiana (TcI) and CL Brener (TcVI) clones and Y (TcII) strain.

**Findings and Conclusions:** A total of 36 peptides were considered reactive, and the cross-reactivity among the strains is in agreement with the evolutionary origin of the different *T. cruzi* DTUs. Four peptides were tested against a panel of chagasic patients using ELISA. A conserved peptide showed 95.8% sensitivity, 88.5% specificity, and 92.7% accuracy for the identification of *T. cruzi* in patients infected with different strains of the parasite. Therefore, this peptide, in association with other *T. cruzi* antigens, may improve Chagas disease serodiagnosis. Together, three polymorphic epitopes were able to discriminate between the three parasite strains used in this study and are thus potential targets for Chagas disease serotyping.

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## Author Summary

Serological tests are preferentially used for the diagnosis of Chagas disease during the chronic phase because of the low parasitemia and high anti-*T. cruzi* antibody titers. However, contradictory or inconclusive results, mainly related to the characteristics of the antigens used, are often observed. Additionally, the factors influencing variation in the clinical forms of Chagas disease have not been elucidated, although it is likely that host and parasite genetics are involved. Several studies attempting to correlate the parasite strain with the clinical forms have used hemoculture and/or PCR-based genotyping. However, both techniques have limitations. Hemoculture requires the isolation of parasites from patient blood and the growth of these parasites in animals or *in vitro* culture, thereby possibly selecting certain subpopulations. Moreover, the level of parasitemia in the chronic phase is very low, hindering the detection of parasites. Additionally, direct genotyping of parasites from infected tissues is an invasive procedure that requires medical care and hinders studies with a large number of samples. The goal of this work was to identify conserved and polymorphic linear B-cell epitopes of *T. cruzi* on a genome-wide scale for use in the serodiagnosis and serotyping of Chagas disease using ELISA. Development of a serotyping method based on the detection of strain-specific antibodies may help to understand the relationship between the infecting strain and disease evolution.

## Introduction

Chagas disease, a zoonosis caused by the protozoan parasite *Trypanosoma cruzi*, affects approximately 10 million people in the Americas. Approximately 14,000 deaths occur annually, and 50,000–200,000 new cases are diagnosed each year [1]. During the acute phase of infection, diagnosis is based on parasitological methods [2]; however, in the chronic phase, such parasitological approaches have a low sensitivity, between 50–65%, because of low levels of parasitemia [3,4]. The chronic phase is also characterized by a strong and persistent humoral immune response, thus the measurement of IgG antibodies specific for parasite antigens should be performed for diagnosis [5]. However, serological methods from different laboratories have been observed to be inconclusive or contradictory [6–8]. These discrepancies are mainly related to technical errors and antigen composition because crude or semi-purified protein extracts of epimastigotes, a parasite stage not found in the mammalian host, are generally used [6,9]. Moreover, false-positive results are frequently observed because of the cross-reactivity of crude preparations of *T. cruzi* antigens with sera from individuals infected with *Leishmania* sp. and *T. rangeli* [10–12]. The use of recombinant antigens and synthetic peptides as a substitute for parasite lysates has increased reproducibility and, in addition, does not require the maintenance and processing of live parasites [13,14]. Despite recent advances in Chagas disease diagnostics, the methods available still have limitations related to low specificity and sensitivity [15,16]. Among the factors that compromise the performance of diagnostic tests, the genetic variability of the parasite is known to contribute to false-negative results in Chagas disease serodiagnosis [17].

Epidemiological, biochemical, and molecular studies have demonstrated that the *T. cruzi* taxon is extremely polymorphic [18–21]. Recently, *T. cruzi* strains were reclassified into six DTUs (discrete typing units) called TcI to TcVI [22], and there is much

speculation regarding whether this parasite variability could be associated with different disease prognoses. Although *T. cruzi* infection results in a broad spectrum of clinical forms as indeterminate, cardiac, and digestive forms, the determinant factors involved in the development of each clinical form have not been elucidated, though it is likely that genetic factors of the host and parasite are involved [23]. However, no study to date has found an unequivocal association between the infecting parasite DTU and the clinical forms of the disease. Nevertheless, this hypothesis has not been discarded because correlations between the geographic distribution profiles of different *T. cruzi* DTUs and a higher frequency of specific clinical forms have been reported [21]. Indeed, digestive manifestations are more common in the central region of Brazil and the southern part of South America, where infection by TcII, TcV, and TcVI predominates; in contrast, such manifestations are rare in the northern part of South America and in Central America, where infection caused by TcI is more common [24].

Correlation studies between the parasite DTU and clinical forms of Chagas disease are challenging because most of the techniques require parasite isolation from patient blood or parasite genotyping directly from infected tissues. Because many *T. cruzi* populations are polyclonal, hemoculture may select sub-populations of parasites more adapted to *in vitro* growth conditions [25]. Moreover, because of different tissue tropisms of some *T. cruzi* strains [26], in infections caused by polyclonal populations and/or co-infections, the clones circulating in the patient blood may not be the same as those found in tissue lesions. The current methodologies to genotype the parasite from tissue biopsies are laborious and expensive, thus limiting the number of samples that can be analyzed. Within this context, a parasite typing method based on the detection of strain-specific antibodies from patient sera could resolve many of these problems. Thus far, there is only one study that proposes the use of an antigen to discriminate among *T. cruzi* DTUs [17]. This study is based on an antigen named TSSA (tryommastigote small surface antigen), belonging to the TcMUC III protein family, which can differentiate between humans infected with TcI, TcIII, and TcIV and those infected with TcII, TcV, and TcVI.

In the present study, we performed a genomic screen to identify polymorphic and conserved linear B-cell epitopes in the predicted proteome of the CL Brener *T. cruzi* strain in an attempt to identify targets for the serotyping and serodiagnosis, respectively, of *T. cruzi*-infected patients. The results were validated using sera from experimentally infected mice and chagasic patients.

## Materials and Methods

### Ethics statement

The design and methodology of all experiments involving mice were in accordance with the guidelines of COBEA (Brazilian College of Animal Experimentation), strictly followed the Brazilian law for “Procedures for the Scientific Use of Animals” (11.794/2008), and were approved by the animal-care ethics committee of the Federal University of Minas Gerais (protocol number 143/2009).

The study protocol involving human samples from Bolivia was approved by the ethics committees of the study hospital, A.B. PRISMA, Johns Hopkins University and the U.S. Centers for Disease Control and Prevention. All subjects provided written informed consent before blood was collected. As for the Brazilian patients, written informed consent was obtained from the participants and was approved by the Ethics Committee of the

Federal University of Minas Gerais (UFMG), under protocol number No. 312/06.

### Mouse sera

Each experimental group was composed of six 2–4-week-old C57BL/6 male mice. The mice were infected with 50 Colombiana or 500 Y tryomastigotes. For the CL Brener clone, we used three mouse groups infected with 50, 100, or 500 tryomastigotes. Infection was confirmed by the observation of tryomastigote forms in blood collected from the tail at seven days after intraperitoneal inoculation. One additional group was infected with  $1 \times 10^5$  *T. rangeli* tryomastigotes, and the infection was confirmed by PCR [27]. Six un-infected mice were used as the control group. The chronic phase of infection was confirmed after approximately 3 months by negative parasitemia and the presence of anti-parasite IgG (as tested against *T. cruzi* and *T. rangeli* crude antigens) by ELISA [28]. Mouse blood was then obtained by cardiac puncture; coagulation was performed at room temperature for 30 minutes, and the serum was obtained after centrifugation at 4000×g for 15 minutes.

### Human sera

Blood samples from chagasic patients from Bolivia were collected in a public hospital in Santa Cruz de la Sierra. DNA was extracted from patient blood samples and parasite genotyping was performed as previously described [29]. Infection by TcI parasite lineage was confirmed for six samples (Supplementary Figure S1). Samples from 10 chagasic patients previously characterized to be infected with TcII [30] and 56 samples from chagasic patients infected with untyped parasites collected from Rio Grande do Norte State, Brazil, were also used. Samples from 14 patients infected with *L. braziliensis* and 14 patients with visceral leishmaniasis both known to be un-infected with *T. cruzi* and the sera from 24 un-infected humans were used as specificity and negative controls, respectively.

### Parasites

Epimastigotes of the Colombiana and CL Brener clones, and Y strain of *T. cruzi* and *T. rangeli* SC-58 were maintained in a logarithmic growth phase at 28°C in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 units/mL penicillin [31]. A total of  $1 \times 10^6$  *T. cruzi* epimastigotes/mL were incubated in triatomine artificial urine (TAU) medium for 2 hours at 28°C. L-proline (10 mM) was added to the medium, and the metacyclic forms were obtained after 72 hours at 28°C [32]. Trypomastigotes and amastigotes were obtained from rhesus-monkey epithelial LLC-MK2 cells infected with metacyclic forms cultured in RPMI medium supplemented with 2% fetal bovine serum at 37°C and 5% CO<sub>2</sub> [31]. Differentiation of *T. rangeli* epimastigotes to trypomastigotes was induced with 10<sup>6</sup> parasites/mL in DMEM medium (pH 8) for 6 days at 28°C [33].

### In silico prediction of linear B-cell epitopes

Linear B-cell epitopes were predicted for all the proteins of the CL Brener genome release 4.1 [34] using the Bepipred 1.0 program with a cutoff of 1.3 [35]. The Bepipred program assigns a score to each individual amino acid in a sequence, therefore only amino acids with prediction Bepipred score  $\geq 1.3$  were considered for the downstream analysis. Proteins encoded by the pair of Esmo and Non-Esmo alleles were aligned using the CLUSTALW program [36], and each pair of amino acids aligned received a polymorphism score according to the following scale: 0 for

identical amino acids; 1 for different amino acids with similar physical-chemical properties; 2 for a mismatch involving amino acids with dissimilar physical-chemical properties; and 3 for a gap position. A perl script based on a sliding window approach that uses a fixed window size of 15 amino acids and an increment of one amino acid identified all 15-mer subsequences in which each individual amino acid has a Bepipred score  $\geq 1.3$ . Those peptides with a polymorphism score above 6 (sum of the individual amino acid polymorphism scores) and a mean Bepipred score  $\geq 1.3$  were classified as polymorphic epitopes; those peptides identical between the Esmo and Non-Esmo haplotypes and with a mean Bepipred prediction score  $\geq 1.3$  were classified as conserved epitopes. The selected peptides were compared with the predicted proteins from the genomes of *L. infantum*, *L. major*, *L. braziliensis*, and *T. brucei* (release 4.1) [37] using the BLASTp algorithm [38]. Peptides with at least 70% similarity along 70% of the length were discarded. After elimination of peptides with potential cross-reactivity with *Leishmania* and *T. brucei*, 50 Esmo-like peptides, 50 Non-esmo-like peptides and 50 peptides conserved with the highest mean Bepipred score were selected.

### Spot synthesis and immunoblotting

Peptides were synthesized on pre-activated cellulose membranes according to the SPOT synthesis technique [39]. Briefly, Fmoc-amino acids were activated with 0.05 mM HOBt and 0.1 mM DIC and automatically spotted onto pre-activated cellulose membranes using the MultiPep SPOT synthesizer (Intavis AG). The non-binding sites of the membrane were blocked with 10% acetic anhydride, and the Fmoc groups were removed with 25% 4-methyl piperidine. These processes were repeated until peptide chain formation was complete. After synthesis, side-chain deprotection was performed by adding a 25:25:1.5:1 solution of trifluoroacetic acid, dichloromethane, triisopropylsilane, and water. The amino acid coupling and side-chain deprotection were monitored by staining the membrane with 2% bromophenol blue. The immunoblotting methodologies followed a previously described protocol [39]. First, the membrane containing peptides was blocked with 5% BSA and 4% sucrose in PBS overnight and incubated with infected and control mouse sera diluted 1:5,000 in blocking solution for 1 hour. After washing three times with PBS-T (PBS; 0.1% Tween 20), the membrane was incubated with the secondary HRP-conjugated anti-mouse IgG antibody (Sigma-Aldrich) diluted 1:10,000 in blocking solution for 1 hour. After a third wash, detection was performed using ECL Plus Western blotting (GE Healthcare), following the manufacturer's instructions, with the Gel Logic 1500 Imaging System (Kodak). The densitometry measurements and analysis of each peptide were performed using Image Master Platinum (GE), and the relative intensity ratio (RI) cutoff for positivity was determined at 2.0.

### Soluble peptide synthesis

The soluble peptides were synthesized in solid phase on a 30-µmol scale using N-9-fluorenylmethoxycarbonyl [40] with PSSM8 equipment (Shimadzu). Briefly, Fmoc-amino acids were activated with a 1:2 solution of HOBt and DIC. The active amino acids were incorporated into Rink amide resin with a substitution degree of 0.61. Fmoc deprotection was then performed using 25% 4-methylpiperidine. These steps were repeated until the synthesis of each peptide was complete. The peptides were deprotected and released from the resin by treatment with a solution of 9.4% trifluoroacetic acid, 2.4% water, and 0.1% triisopropylsilane. The peptides were precipitated with cold diisopropyl ether and purified by high-performance liquid chromatography (HPLC) on a C18 reverse-phase column using a gradient program of 0 to 25%

acetonitrile. The peptides were obtained with 90% purity, as confirmed by mass spectrometry using Autoflex Speed MALDI/TOF equipment.

### ELISA and affinity ELISA

Each well of flexible ELISA polyvinylchloride plates (BD Falcon) was coated with 2 µg of soluble peptide. After blocking with 5% BSA in PBS for 1 hour at 37°C, followed by three washing steps with PBS containing 0.05% Tween 20 (PBS-T), the plates were incubated with human or mouse serum (dilution 1:100). The plates were washed three times with PBS-T, and secondary HRP-conjugated anti-human or anti-mouse IgG antibody was added for 1 hour at 37°C, followed by four washes. A solution containing 0.1 M citric acid, 0.2 M Na<sub>2</sub>PO<sub>4</sub>, 0.05% OPD, and 0.1% H<sub>2</sub>O<sub>2</sub> at pH 5.0 was used for detection; the reaction was stopped with 4 N H<sub>2</sub>SO<sub>4</sub>, and the absorbance was measured at 492 nm. The mean optical density value at 492 nm plus three times the standard deviation of the negative serum was used as the cutoff value. For affinity ELISA, 6 M urea was added for 5 min at 37°C after incubation with the primary antibodies; the remainder of the protocol was the same [41]. The results are shown as an affinity index (AI) determined as the ratio between the absorbance values of the samples treated and not treated with urea. An AI value lower than 40% represented low-affinity antibodies, between 41 and 70% was classified as intermediate affinity and higher than 70% as high affinity.

### DNA extraction and sequencing

Genomic DNA extraction was performed using the GFXTM Genomic Blood DNA Purification kit (GE Healthcare) following the manufacturer's instructions. The DNA samples were quantified using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). The PCR products amplified with the primers listed in Supplementary Table S1 were subjected to sequencing at both ends using the ABI Prism 3730×1 DNA Analyzer (Applied Biosystems) by Macrogen Inc (Korea).

### RNA extraction and cDNA synthesis

Total RNA was isolated from 10<sup>8</sup> epimastigotes, 10<sup>6</sup> tryomas-tigotes, and 10<sup>8</sup> LLC-MK2 cells infected with approximately 10<sup>5</sup> intracellular amastigotes of the Colombiana, Y, and CL Brener strains using the NucleoSpin RNA II RNA extraction kit (Macherey-Nagel) following the procedures described by the manufacturer. RNA from 10<sup>8</sup> LLC-MK2 cells was also extracted and used as a negative control. The concentration and purity of the RNA samples were measured with a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). cDNA was synthesized using 10 ng of total RNA and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using random hexamer primers according to the manufacturer's instructions.

### Real-time PCR

Specific primers for each Esmo and Non-Esmo allele were designed, and the primer specificity was verified by electronic PCR using the entire parasite genome as a template. The primers used are listed in the Supplementary Table S2. Real-time PCR reactions were performed in an ABI 7500 sequence detection system (Applied Biosystems). The reactions were prepared in triplicate and contained 1 mM forward and reverse primers, SYBR Green Master Mix (Applied Biosystems), and 20 ng of cDNA. Standard curves were prepared for each experiment for each pair of primers using serially diluted *T. cruzi* CL Brener

genomic DNA to calculate the relative quantity (Rq) values for each sample. qRT-PCRs for the constitutively expressed GAPDH gene were performed to normalize the expression of the specific alleles.

### Statistical analysis

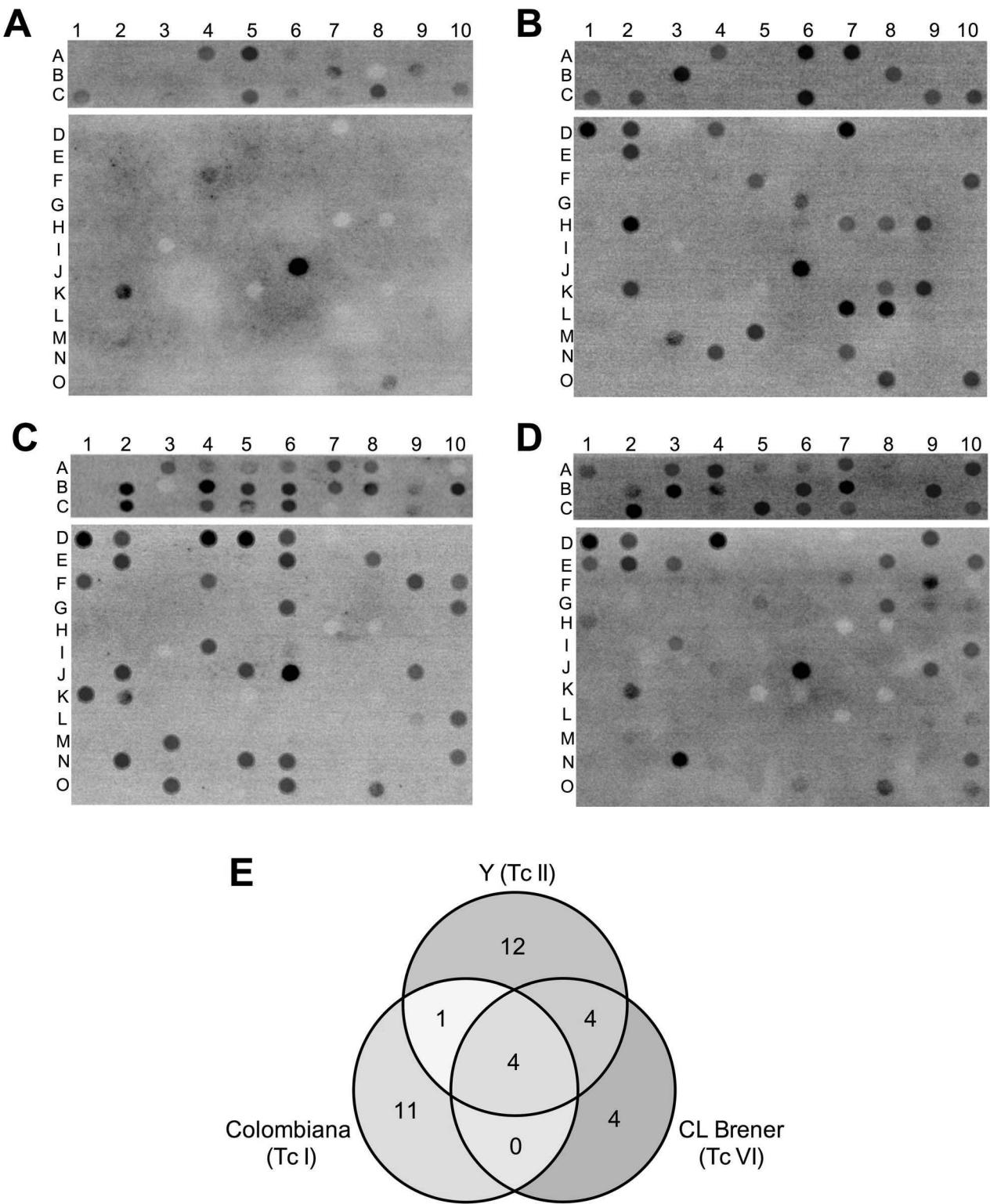
All statistical analyses were performed using Graph Prism 5.0 software. First, the normal distribution of data was evaluated by the Kolmogorov-Smirnov test; because all they showed a Gaussian profile, an unpaired t test was used for the comparative analysis between the two sets of data, and an ANOVA was used for three or more experimental groups. P-values lower than 0.05 were considered statistically significant. The sensitivity, specificity, and accuracy of the peptides were also calculated for the human samples. The sensitivity is represented by  $Se = TP/(TP+FN)$ , where TP (true positive) is the number of sera from individuals infected with *T. cruzi* above the cutoff value and FN (false negative) is the number of sera from infected individuals below the cutoff for the conserved peptide. For the polymorphic peptides, TP was defined as the number of sera from individuals infected with a specific strain above the cutoff value, and FN is the number of these sera below the cutoff for polymorphic peptides. The specificity is represented by  $Sp = TN/(TN+FP)$ , where TN (true negative) is the number of sera from individuals infected with *L. braziliensis* or un-infected individuals below the cutoff and FP (false positive) is the number of sera from these samples with reactivity for the conserved peptide. For the polymorphic peptides, TN was defined as the number of sera from individuals infected with a non-specific strain or *L. braziliensis* and uninfected individuals below the cutoff, and FP is the number of sera from these samples with reactivity. The accuracy is calculated as  $Ac = (TP+TN)/(TP+TN+FP+FN)$ .

## Results

### Epitope prediction using the *T. cruzi* CL Brener proteome and immunoblotting screening

We performed B-cell epitope prediction for 3,983 proteins derived from pair of alleles of CL Brener genome. We decided to restrict our analysis to this dataset because the CL Brener clone is a recent hybrid between the TcII and TcIII DTUs and evidence suggests that the latter is an ancient hybrid between TcI and TcII [42]. Therefore, it is likely that polymorphic epitopes in the pairs of alleles of CL Brener could also be polymorphic for its parental genotypes and other *T. cruzi* strains. In the CL Brener hybrid diploid genome, it is possible to identify two haplotypes: "Esmo", which is more similar to TcII; and "Non-Esmo", which is more similar to TcIII [34]. A total of 1,488 predicted epitopes were classified as conserved between the two haplotypes, and 428 were classified as polymorphic. We next excluded epitopes also present in *Leishmania major*, *L. infantum*, *L. braziliensis* and *T. brucei* to minimize the chance of cross-reactivity, because these parasites share many antigens with *T. cruzi* [10,12,16], thus reducing the number of conserved and polymorphic epitopes to 1,086 and 242, respectively.

A total of 50 conserved, 50 polymorphic Esmo-specific, and 50 polymorphic Non-Esmo-specific peptides with high epitope prediction scores were selected for the construction of peptide arrays. The reactivity of the peptides was tested using a pool of sera from six C57BL/6 mice chronically infected with Colombiana (TcI), Y (TcII), or CL Brener (TcVI) strains and un-infected mice as the control group (Figure 1). The quantification of the reactivity was performed by densitometric analysis (Supplementary Table S3). A peptide was considered reactive and antigenically



**Figure 1. Immunoscreening of peptide arrays with sera from mice infected with different *T. cruzi* strains.** (A) Sera from un-infected mice; sera from mice infected with Colombiana (TcI) (B), Y (TcII) (C), or CL Brener (TcVI) (D). (E) Venn diagram showing the specific and shared epitopes among *T. cruzi* strains.

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conserved if its intensity signal with all *T. cruzi* strains was two times higher than its signal with the sera from un-infected mice. A peptide was considered reactive and antigenically polymorphic if

its intensity signal with a specific strain was two times higher than the values with the other two strains and the un-infected mice. A total of 36 peptides were considered reactive with at least one

strain (Figure 1E). A conserved peptide with the highest reactivity with all *T. cruzi* strains (C6\_30\_cons) and three polymorphic peptides specific for Colombiana (A6\_30\_col), Y (B2\_30\_y), and CL Brener (B9\_30\_cl) were selected for soluble synthesis, and their reactivity was validated by ELISA.

### Epitope validation with ELISA and affinity ELISA

Because immunoblotting assays are semi-quantitative techniques, we validated the results with quantitative ELISA and affinity ELISA assays using individual sera from six C57BL/6 mice chronically infected with the Colombiana (TcI), Y (TcII), or CL Brener (TcVI) strains and the un-infected mice as a control group. For the conserved peptide C6\_30\_cons, no significant difference in the reactivity among the sera from animals infected with different *T. cruzi* strains was observed (Figure 2A). The sera from mice infected with the Colombiana strain had a higher antibody titer against the A6\_30\_col peptide compared to the sera from mice infected with the Y strain. More importantly, the affinity antibodies discriminated Colombiana infection from those caused by the other two strains (Figure 2B). An expected recognition profile was also observed for the peptide B2\_30\_y (Figure 2C): sera from mice infected with the Y strain had a significantly higher antibody titer than those from mice infected with Colombiana, and the highest affinity antibodies generated by the Y strain discriminated its infection from those caused by Colombiana and CL Brener. With regard to peptide B9\_30\_cl, conventional ELISA was able to discriminate CL Brener infection from that caused by Y and Colombiana (Figure 2D).

Because the infection caused by different *T. cruzi* strains has specific evolution and mortality rates in a mouse model [43], we infected mice with a distinct parasite inoculum for each strain to reach the chronic phase when the sera were collected. Thus, to evaluate whether the differences in reactivity observed in the ELISA experiments were dependent on the inoculum, we tested the reactivity of sera from mice infected with 50, 100, or 500 CL Brener trypomastigotes (Supplementary Figure S2). There was no significant variation among the different CL Brener inocula, suggesting that the antigenic variability among the parasite strains is the main factor responsible for the distinct recognition profile of the peptides tested in the ELISA experiments. The evaluation of cross-reactivity with sera from mice infected with *T. rangeli* and from Leishmaniasis patients demonstrated that the peptides are *T. cruzi* specific (Supplementary Figures S3 and S4).

### Prediction of specific epitope reactivity with different *T. cruzi* DTUs

We next analyzed the polymorphisms of the epitopes identified in this study and predicted their reactivity with sera from individuals infected with *T. cruzi* strains representative of each DTU (TcI to TcVI). To this end, we first subjected the peptide sequences to an AlaScan analysis [44] to identify the amino acid residues critical to antibody binding. We found that the pattern GXXXXXMRQNE in the carboxy-terminal region of conserved peptide C6\_30\_cons is important for the interaction with the antibodies generated in infection caused by the three *T. cruzi* strains (Figures 3A, B, and C). As for the polymorphic epitopes, the patterns PPDXDXSLXXP in peptide A6\_30\_col (Figure 3D), QPQPXPQXXXQP in B2\_30\_y (Figure 3E), and DEXXXXG in B9\_30\_cl (Figure 3F) are critical for binding with the antibodies generated by Colombiana, Y, and CL Brener infections, respectively.

We then sequenced the genomic DNA encoding these four epitopes in strains representative of the six *T. cruzi* DTUs to predict whether the peptides would be recognized in infections

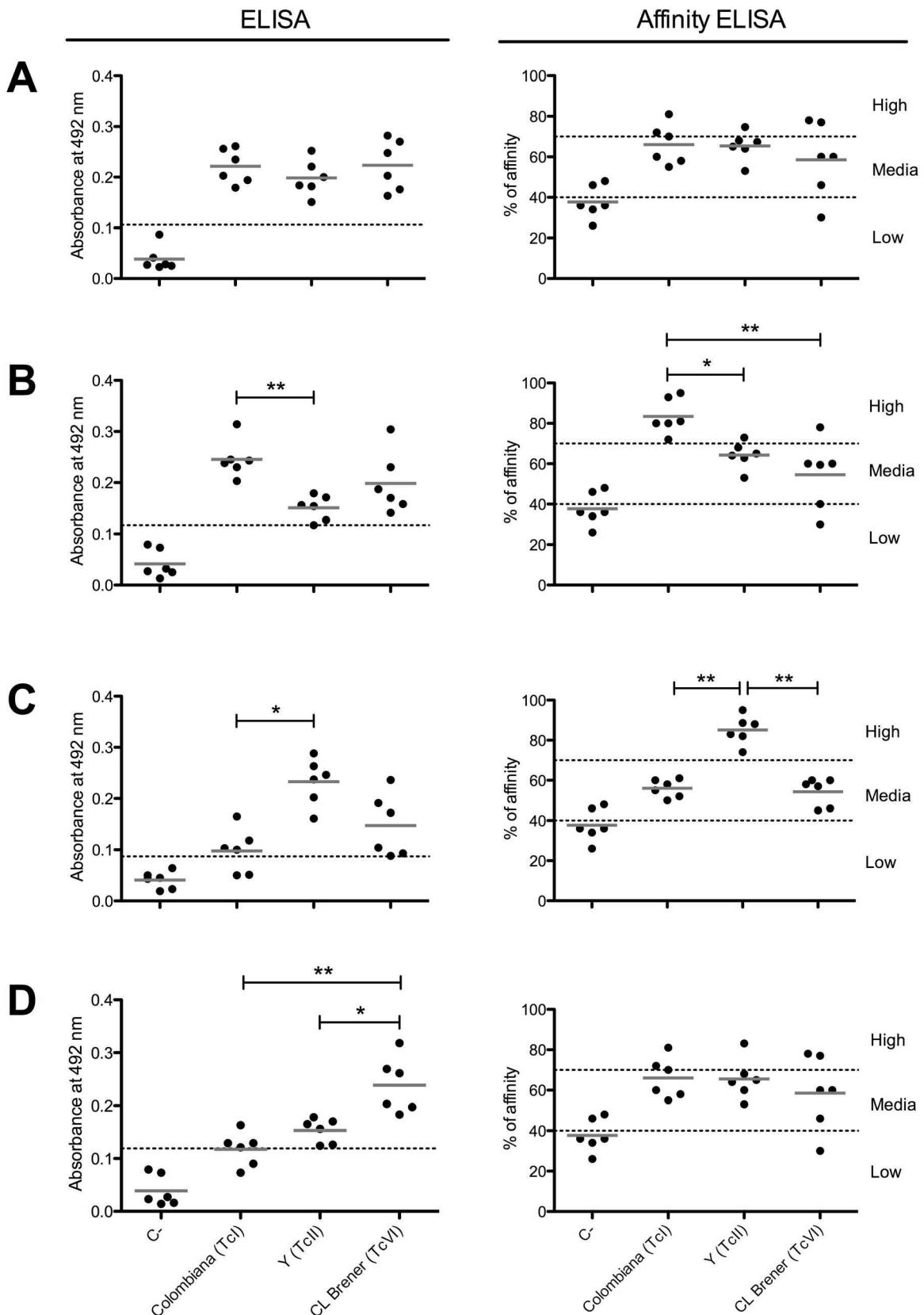
caused by different parasite DTUs. It is expected that a peptide would be recognized in infections caused by a specific strain if the amino acid residues critical for antibody recognition are encoded by its genome. Based on this criterion, we predicted that conserved peptide C6\_30 would be able to identify infection caused by four of the six *T. cruzi* DTUs (Figure 4A), whereas peptides A6\_30\_col and B2\_30\_y are expected to identify infections caused only by TcI and TcVI (Figure 4B) and TcII and TcVI (Figure 4C), respectively. Interestingly, the A6\_30\_col and B2\_30\_y epitopes are identical to the Non-Esmo- and Esmo-like CL Brener haplotypes, respectively, reinforcing the hypothesis that the nature of the CL Brener hybrid may have contributions of both the TcI and TcII genomes. B9\_30\_cl is predicted to identify patients infected with TcIII or TcVI (Figure 4D).

### Epitope expression at different parasite stages and strains

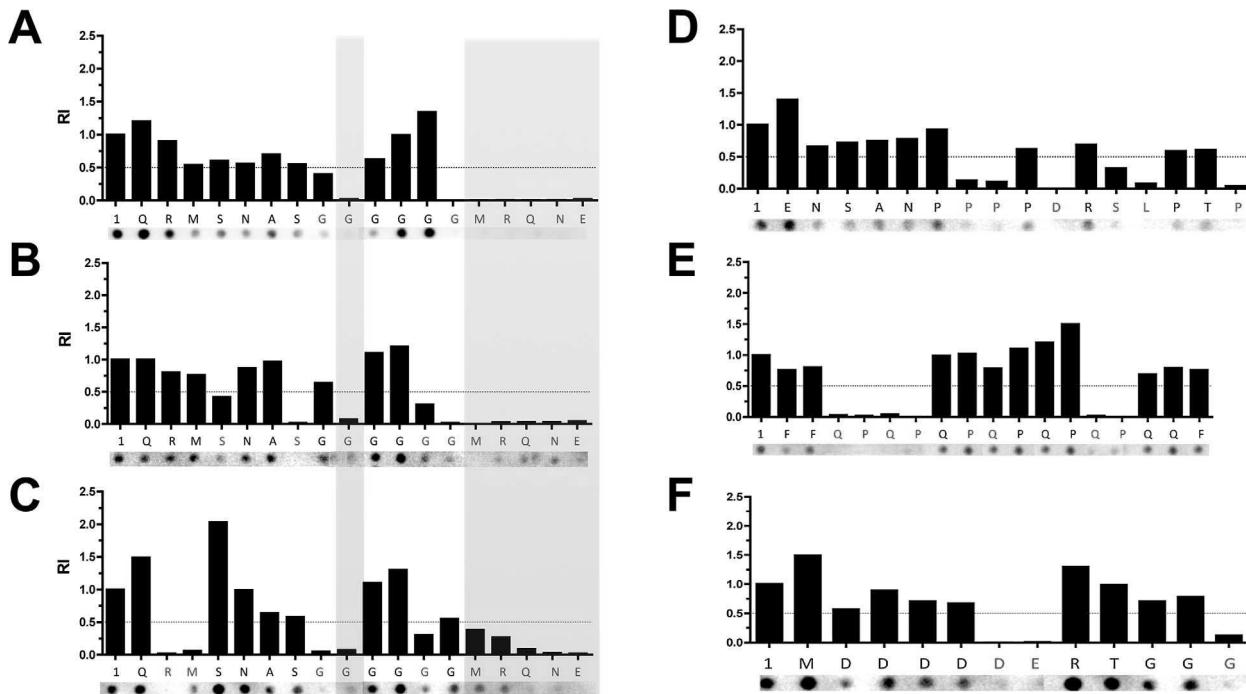
Although all peptides are derived from the CL Brener genome, the sera from mice infected with this strain had lower antibody affinities for the A6\_30\_col and B2\_30\_y peptides than did the sera from mice infected with the Colombiana or Y strain (Figure 2). Because CL Brener is a hybrid strain [34,42,45], the polymorphic epitopes encoded by its pairs of alleles may have distinct expression levels that could explain the differences in their reactivity. To investigate this further, we designed allele-specific primers for the genes that encode the epitopes to evaluate their expression levels in the trypomastigote and amastigote forms, the parasite stages found in mammalian hosts (Figure 5). As expected based on the *T. cruzi* phylogeny [46], Y expressed only the Esmo-like variants, and Colombiana expressed only the Non-Esmo variants, except for the B9\_30 transcript. CL Brener expressed both alleles of all genes, except for the B9\_30 transcript. The conserved peptide was expressed by both the Esmo and Non-Esmo haplotypes of CL Brener (Figure 5A). The polymorphic Non-Esmo peptide A6\_30\_col was expressed by the Colombiana and CL Brener strains (Figure 5B), and CL Brener also expressed the Esmo-like allele for this peptide. The opposite profile was observed for the polymorphic Esmo B2\_30\_y peptide, whereby only the Y and CL Brener strains expressed the Esmo-like allele and CL Brener also expressed the Non-Esmo allele of this peptide (Figure 5C). CL Brener only expressed the Esmo-like variant of the B9\_30\_CL epitope, and its level of expression was approximately 5 times higher than in the Y strain (Figure 5D).

### Potential use of epitopes for human serodiagnosis and serotyping

All previous results were based on a mouse model because the amount of the inoculum, infective strain, and time of infection can be adequately controlled. To test the potential application of these peptides for serodiagnosis and serotyping of human infection, we performed ELISA experiments with sera from chagasic patients with parasites genotyped as TcI or TcII, and healthy individuals. The conserved peptide C6\_30\_cons showed 95.8% sensitivity, 88.5% specificity, and 92.7% accuracy for the identification of chagasic patients, and no significant differences in the reactivity of sera from patients infected with TcI or TcII was observed (Figure 6). As expected, peptide A6\_30\_col showed much higher reactivity with the sera from patients infected with TcI (Figure 7A), with 100% sensitivity, 91.9% specificity, and 92.6% accuracy; peptide B2\_30\_y identified most of the individuals infected with TcII (Figure 7B), with 80% sensitivity, 94.8% specificity, and 92.6% accuracy. Additionally, none of the sera from patients infected with TcI recognized the B2\_30\_y peptide, and peptide



**Figure 2. Reactivity and affinity of sera from *T. cruzi*-infected mice against conserved and polymorphic epitopes.** (A) Peptide C6\_30\_cons. (B) Peptide A6\_30\_col. (C) Peptide B2\_30\_y. (D) Peptide B9\_30\_cl. The dotted line represents the cutoff value. The solid gray line represents the mean values. C, un-infected mice. Colombiana (TcI), mice infected with the Colombiana strain. Y (TcII), mice infected with the Y strain. CL Brener (TcVI), mice infected with the CL Brener strain. \* $p<0.05$  and \*\* $p<0.005$ .



**Figure 3. Mapping of antibody binding sites on the conserved and polymorphic epitopes by Ala-Scan.** Reactivity of peptide C6\_30\_cons against the sera from mice infected with Colombiana (A), Y (B), and CL Brener (C). Reactivity of peptide A6\_30\_col against the sera from mice infected with the Colombiana clone (D). Reactivity of peptide B2\_30\_y against the sera from mice infected with the Y strain (E). Reactivity of peptide B9\_30\_cl against the sera from mice infected with the CL Brener clone (F). 1, original peptide. RI, relative intensity calculated by the ratio between the reactivity of the peptide with a specific amino acid substitution and the original peptide. The dotted line indicates half of the intensity value obtained with the original peptide. The red letters represent amino acid substitutions that reduce the reactivity to at least half of the value obtained with the original peptide. The blue squares show the conserved amino acids critical for antibody binding with the sera from mice infected with different *T. cruzi* strains.

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B9\_30\_cl showed a low reactivity with both TcI and TcII (Figure 7C). All peptides were also *T. cruzi* specific because the majority of the sera from the patients infected with *L. braziliensis* were non-reactive (Supplementary Figure S4).

## Discussion

Despite efforts to identify new targets for the immunodiagnosis of Chagas disease, the impressive genetic variability of *T. cruzi* strains has imposed serious limitations on the development of high-sensitivity methods [47–50]. Additionally, serological cross-reactivity with *Leishmania* and *T. rangeli* infections [11,12,16] compromises the specificity of Chagas disease diagnosis. Therefore, the identification of new *T. cruzi*-specific antigens conserved among the parasite strains has been recognized as an important research area for Chagas disease diagnosis and control [47]. The polymorphic nature of *T. cruzi* isolates, on the other hand, opens new avenues for the development of serotyping methodologies to identify the parasite DTU causing infection based on a serological survey. For instance, this would allow large-scale epidemiological studies aimed at correlating the strain causing the infection with the clinical forms of Chagas disease, an open question that has been hampered by the limited number of samples that can be analyzed by the current genotyping methodologies [48]. To the best of our knowledge, only one study has identified a polymorphic epitope among the *T. cruzi* DTUs [49]. This marker is an immunodominant B-cell epitope of TSSA (tryomastigote small surface antigen), a representative of the TcMUC III gene family.

The TSSA-I and TSSA-II isoforms serologically discriminate between animals infected with *T. cruzi* I from those infected with *T. cruzi* II, according to the previous DTU classification (TcII-VI in the current classification), respectively. In a serological survey of chagasic patients from Argentina, Brazil, and Chile, anti-TSSA antibodies recognized only the TSSA-II isoform, suggesting that the TcII-VI DTUs are the cause of Chagas disease in those regions. In a more recent study, however, this same research group analyzed the diversity of the TSSA gene in several representatives of each of the six *T. cruzi* DTUs and found a complex pattern of sequence polymorphism. Based on their analysis, the epitope considered to be specific for TcII-VI was shown to identify the TcII, V, and VI DTUs. In addition, the peptide previously described as TcI specific shares key features with TcIII and IV. Therefore, there is no *T. cruzi* DTU-specific serological marker identified thus far.

The goal of this work was to identify conserved and polymorphic linear B-cell epitopes of *T. cruzi* for Chagas disease serodiagnosis and serotyping using ELISA. This technique was selected because it is a quantitative assay and easily automated, thus allowing the analysis of a large number of samples. In recent years, synthetic peptides used as antigens have shown high sensitivity and specificity in diagnostic tests [50]. Peptides have several advantages over chemically purified or recombinant antigens because their production does not involve the manipulation of living organisms and can be obtained with a high level of purity [51]. Recently, the use of peptide arrays has allowed the immunoscreening of a large number of epitope candidates [39].

<b>A</b>	Colombiana (TcI)	QRMSNAS-GGGGG--MRQNE
	Y (TcII)	KKKANGC-GGGGG--MRQNE
	231 (TcIII)	ORM---LGGGGGG--MRQNE
	CanIII (TcIV)	QRMPNASGGGGGGGETRQNE
	115 (TcV)	QRMSNASGGG-----MRQNE
	CL Brener Esmo (TcVI)	<b>QRMSNASGGGGGG--MRQNE</b>
	CL Brener Non-esmo (TcVI)	QRMSNASGGGGGG--MRQNE
<b>B</b>	Colombiana (TcI)	---ENSANPPPPDRSLPTP
	Y (TcII)	-----RRILPPPDR---TP
	231 (TcIII)	---ENGGNHPPPGRDHPRP
	CanIII (TcIV)	----KKPTPPPPPA--PTP
	115 (TcV)	KISANRVKTPHPPR--KTP
	CL Brener Esmo (TcVI)	---ENSANPP--DRSLPT-
	CL Brener Non-esmo (TcVI)	<b>---ENSANPPPPDRSLPTP</b>
<b>C</b>	Colombiana (TcI)	FFQPQPQP-----QPQQF
	Y (TcII)	FFQPQPQPQPQPQPQPQQF
	231 (TcIII)	FFQPQPQPQP--QPQQS
	CanIII (TcIV)	FFQPQPQP-----QPQQF
	115 (TcV)	SFQPQPQP-----QPQQF
	CL Brener Esmo (TcVI)	<b>FFQPQPQPQPQPQPQQF</b>
	CL Brener Non-esmo (TcVI)	FFQLQPQP-----QPFAQY
<b>D</b>	Colombiana (TcI)	MDDDDDDDDERTGGG
	Y (TcII)	YDKDDDD-ERTGGG
	231 (TcIII)	MDDDDDD--ETYRGG
	CanIII (TcIV)	MDDDDDDDDERTGGG
	115 (TcV)	MDDDDDDDDERTGGG
	CL Brener Esmo (TcVI)	<b>MDDDDDD--ETYRGG</b>
	CL Brener Non-esmo (TcVI)	MDDDDDDDDERTGGG

**Figure 4. Reactivity prediction of epitopes based on the sequences encoding the identified peptides in different *T. cruzi* strains.** (A) Peptide C6\_30\_cons. (B) Peptide A6\_30\_col. (C) Peptide B2\_30\_y. (D) Peptide B9\_30\_cl. The red letters represent key amino acids for antibody binding. The bold sequences represent the synthetic peptide tested.

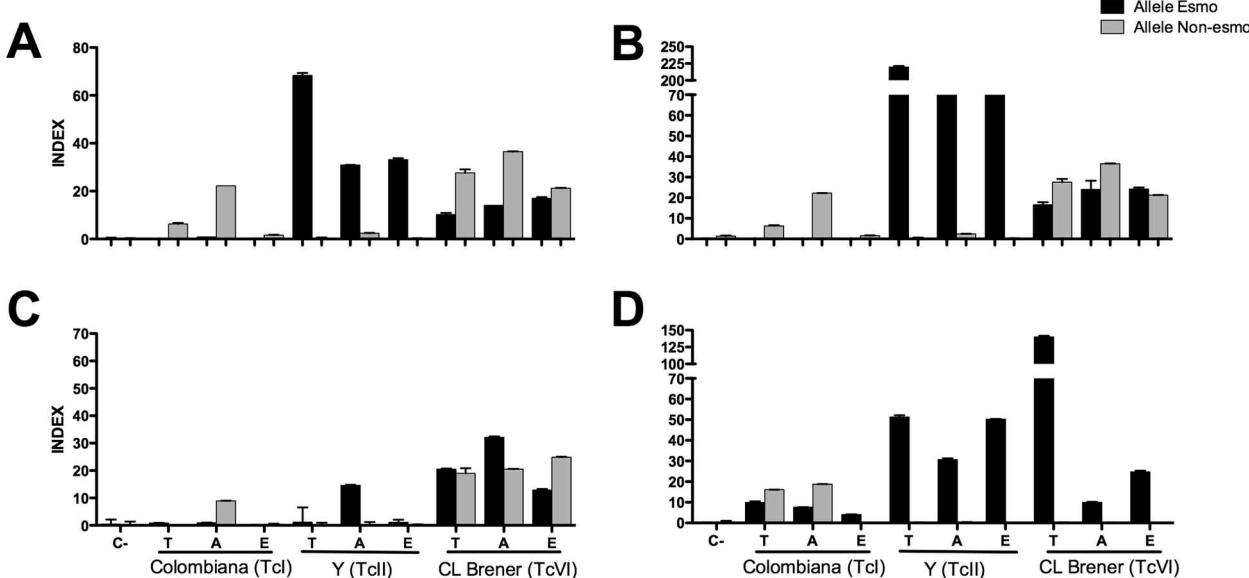
doi:10.1371/journal.pntd.0002524.g004

Thus, an approach based on a synthetic peptide array was chosen to screen of a large number of potential antigens by immunoblotting, followed by ELISA validation.

Initially, we screened the CL Brener genome to predict epitopes that are polymorphic and conserved between the Esmo and Non-Esmo haplotypes. The rationale underlying this strategy is that, because the CL Brener strain is a recent hybrid between the TcII and TcIII DTUs and there is evidence suggesting that the latter is an ancient hybrid between TcI and TcII [46], it is likely that the polymorphic epitopes between the CL Brener alleles would also be polymorphic among distinct *T. cruzi* strains. The Colombiana (TcI) and CL Brener (TcVI) clones and Y (TcII) strain were

selected for this study to evaluate the degree of polymorphism of epitopes in TcII, a direct representative of one CL Brener parental DTU, and TcI, a more distant DTU of CL Brener, along with CL Brener.

The immunoscreening of 150 high-scoring peptides resulted in the identification of 36 novel epitopes, indicating that our computational approach for the prediction and prioritization of epitope candidates was successful. Our rate of success (24%) was slightly higher than previously described (19.5%) for *T. cruzi* using a similar validation approach [50]. We found that only 11% (4/36) of the reactive peptides are shared among the three parasite strains (Figure 1E), highlighting the problem with identifying



**Figure 5. Expression levels of allele-specific genes encoding the identified conserved and polymorphic peptides.** (A) Peptide C6\_30\_cons. (B) Peptide A6\_30\_col. (C) Peptide B2\_30\_y. (D) Peptide B9\_30\_cl. INDEX was calculated by the copy number from the mRNA level of each gene normalized by the GAPDH mRNA level. C-, negative control. T, trypanomastigote. A, amastigote. E, epimastigote. The alleles were classified based on the annotation of the hybrid CL Brener genome.

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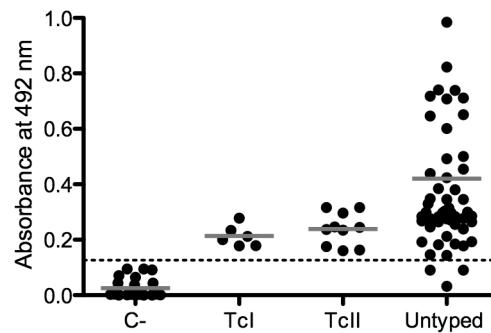
high-sensitivity antigens for the serodiagnosis of Chagas disease due to the high degree of *T. cruzi* polymorphism. One of the conserved epitopes identified in this study, peptide C6\_30\_cons, has proven to be a new conserved *T. cruzi* antigen with a potential application in Chagas disease serodiagnosis (Figures 2A, 6A, S2, and S3).

Together, the three polymorphic epitopes were able to discriminate among infections caused by the three different *T. cruzi* strains included in this study and, thus, have the potential to be used for the serotyping of infections caused by this parasite. ELISA experiments using human sera confirmed the predictive reactivity of A6\_30\_col and B2\_30\_y (Figure 6). A6\_30\_col was able to identify 100% of the patients infected with TcI. As expected, the serum samples obtained from Brazilian patients known to be infected with TcII were reactive only with the C6\_30 conserved and B2\_30\_y peptides. These results confirm the potential use of this peptide set for Chagas disease serotyping.

The peptide A6\_30\_col and B9\_30\_cl are derived from RNA binding proteins and RNA polymerase III, respectively (Supplementary Table S3). Both are predicted to have an intracellular localization. Indeed, humoral response against intracellular antigens is quite common in trypanosomatids as shown by the work described by da Rocha et al., 2002 [52] that performed immunoscreening of an amastigote cDNA library using sera from chagasic patients. About 70% of the amastigote antigens identified in this study is derived from intracellular parasite proteins. Similar to *Leishmania* infection, it is postulated that during *T. cruzi* infection a proportion of trypanomastigotes/amastigotes cells are destroyed, thus releasing substantial amounts of multicomponent complexes containing intracellular antigens [53]. This reactivity could be the result of high abundance of these antigens as circulating complexes during the parasite infection due to high and constant expression of nuclear and house-keeping genes; higher stability due to formation of nucleoprotein particles more resistant to degradation; and their increased capacity to be processed by antigen-presenting

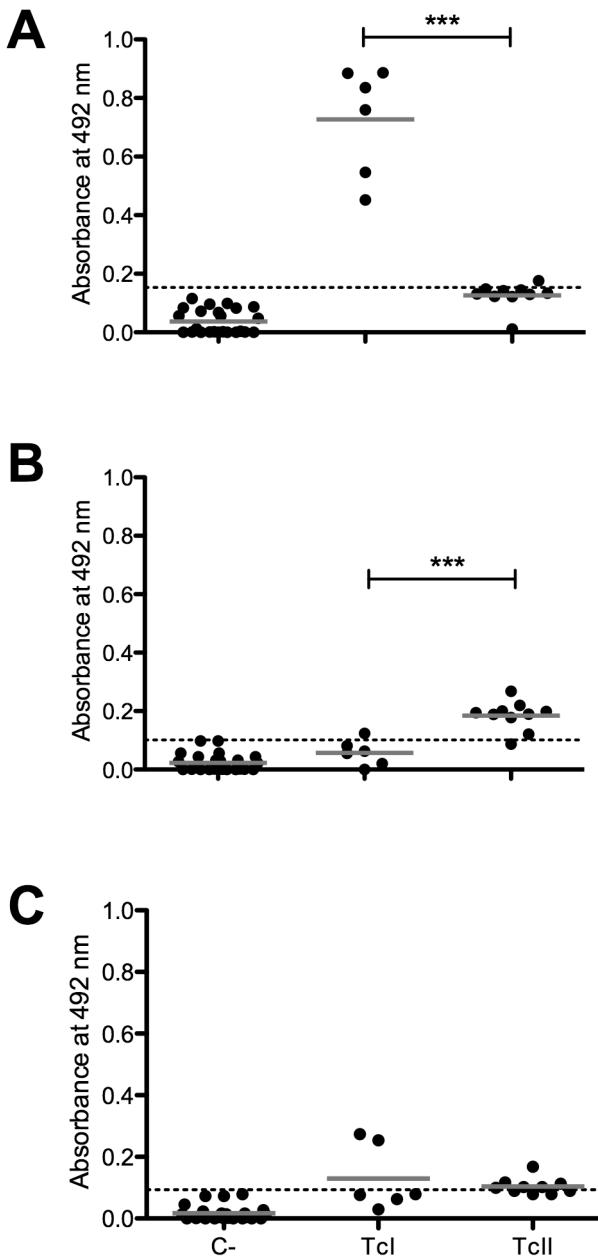
cells because multicomponent particles are taken into the cell more efficiently than soluble antigens [54].

It is worth noting that the conserved and polymorphic epitopes identified in this study encompass repetitive regions. Interestingly, two of these peptides have proline-rich regions (Figure 4) that may be involved in protein-protein interactions in prokaryotes [55] and eukaryotes [56]. It has been demonstrated that the overall immunogenicity of proteins harboring tandem repeats is increased, as is the antigenicity of epitopes contained within repetitive units [52,57]. Therefore, one expects that repeats receive a high B-cell epitope prediction score. Furthermore, the polymorphic epitopes containing repeats were top ranked for an additional reason: our polymorphic scale applied to the CL Brener pair of alleles attributes the highest score to a gap position in the alignment, a situation always present when the contraction or expansion of a



**Figure 6. Reactivity of sera from humans infected with *T. cruzi* against the conserved peptide C6\_30\_cons.** The dotted line represents the cutoff value. The solid gray line represents the mean values. C-, uninfected human. TcI, Chagasic patients infected with TcI DTU. TcII, Chagasic patients infected with the TcII DTU. Untyped, Chagasic patients infected with untyped parasites.

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**Figure 7. ELISA with sera from humans infected with TcI and TcII *T.cruzi* DTUs against the polymorphic peptides.** (A) Peptide A6\_30\_col. (B) Peptide B2\_30\_y. (C) Peptide B9\_30\_cl. The dotted line represents the cutoff value. The solid gray line represents the mean values. C-, uninfected human. TcI, Chagasic patients infected with TcI DTU. TcII, Chagasic patients infected with the TcII DTU. \*\*\*p<0.001. doi:10.1371/journal.pntd.0002524.g007

repetitive region occurs in one sequence but not in another. This criterion was used because it is well known that repetitive sequences evolve faster than other regions of the genome [58], hence it is expected that they display a high level of polymorphism among distinct parasite strains. Additionally, because it is known that the number of repetitive antigenic motifs may affect antibody binding affinity [59], we hypothesized that polymorphic repetitive epitopes would be differentially recognized by the sera of animals and human infected with distinct parasite DTUs, an assumption that was reinforced by our results.

The cross-reactivity of the epitopes among the sera from mice infected with distinct parasite strains is in agreement with an origin hypothesis of the different *T.cruzi* DTUs. In two-way comparisons, the CL Brener and Y strains, the two more phylogenetically related strains, shared a higher number of epitopes (4) compared to Y and Colombiana (1), whereas CL Brener and Colombiana did not share any epitope (Figure 1E).

Interestingly, despite the fact that all of the peptides are derived from the CL Brener genome, a smaller number of epitopes were identified in this strain compared with Y and Colombiana (Figure 1E). We speculate that the co-expression of alleles that encode the polymorphic epitopes in CL Brener may affect the titer of the antibody and/or its affinity for the variant epitopes. For example, the pattern of expression and the reactivity of the polymorphic peptides A6\_30\_col and B2\_30\_y (Figures 4B, 4C, 5B, and 5C) suggest that the co-expression of polymorphic epitopes in CL Brener could induce low-affinity antibodies. A similar phenomenon has been described for the polymorphic *T.cruzi* trans-sialidase (TS) multigene family, whereby TS displays a network of B-cell cross-reactive and polymorphic epitopes that delays the generation of high-affinity neutralizing antibodies and hamper an effective elicitation of a humoral response against these proteins [60]. Whether this is a more general adaptive immune evasion strategy that affects antibody affinity maturation, particularly in the case of hybrid strains, remains to be investigated.

Altogether, the results demonstrated that peptide C6\_30\_cons is a new *T.cruzi* antigen conserved in the majority of DTUs of this parasite. Using this peptide, in association with other *T.cruzi* antigens, may improve the serodiagnosis of Chagas disease. The three polymorphic epitopes identified were able to discriminate among infections caused by the three different *T.cruzi* strains included in this study and, thus, have the potential to be used for the serotyping of infections caused by this parasite. This is the first study on the genomic scale to identify DTU-specific antigens. The genome sequencing of other *T.cruzi* strains will help identify new strain-specific and conserved epitopes and increase the number of antigen candidates for Chagas disease serodiagnosis and serotyping. The development of a robust panel of strain-specific epitopes may allow large-scale epidemiological studies aimed at correlating the infective strain with the variability in clinical outcomes observed in chagasic patients.

## Supporting Information

**Figure S1 Genotyping of *T.cruzi* isolates infecting chagasic patients from Bolivia.** DNA extracted from blood of chagasic patients was amplified using primers specific to the *T.cruzi* mitochondrial COII gene followed by digestion with the *Ahd*I restriction enzyme, as previously described (33), and separation by electrophoresis in 8% polyacrylamide gel. DNA bands were visualized by silver staining. MW, molecular weight; C-, negative control; 1, Genomic DNA of Colombiana clone (TcI); 2, Genomic DNA of Y strain (TcII); 3, Genomic DNA of CL Brener clone (TcVI); 4–9, DNA extracted from blood of chagasic patients. DNA fragments of 264-, 212- and 294-pb characterize TcI, TcII and TcIII-TcVI DTUs, respectively (33). (TIFF)

**Figure S2 ELISA with the sera from mice infected with different CL Brener strain inocula against conserved and polymorphic peptides.** (A) Peptide C6\_30\_cons. (B) Peptide A6\_30\_col. (C) Peptide B2\_30\_y. (D) Peptide B9\_30\_cl. The dotted line represents the cutoff value. The solid gray line represents the mean values. C-, uninfected mice. CL Brener 50, mice infected with 50 trypanomastigotes of the CL Brener. CL

Brener 100, mice infected with 100 trypomastigotes of the CL Brener. CL Brener 1000, mice infected with 1000 trypomastigotes of the CL Brener.

(TIFF)

**Figure S3 Cross-reactivity evaluation of conserved and polymorphic peptides against the sera from mice infected with *T. rangeli*.** (A) Peptide C6\_30\_cons. (B) Peptide A6\_30\_col. (C) Peptide B2\_30\_y. (D) Peptide B9\_30\_cl. The dotted line represents the cutoff value. The solid gray line represents the mean values. C-, uninfected mice. *T. rangeli*, mice infected with *T. rangeli*.

(TIFF)

**Figure S4 Cross-reactivity of conserved and polymorphic peptides against sera of patients with cutaneous and visceral leishmaniasis.** (A) Peptide C6\_30\_cons. (B) Peptide A6\_30\_col. (C) Peptide B2\_30\_y. (D) Peptide B9\_30\_cl. The dotted line represents the cutoff value. The solid gray line represents the mean values.

## References

- Martins-Melo FR, Alencar CH, Ramos AN Jr, Heukelbach J (2012) Epidemiology of mortality related to Chagas' disease in Brazil, 1999–2007. *PLoS Negl Trop Dis* 6(2):e1508.
- Bern C, Montgomery SP, Herwaldt BL, Rassi A, Jr., Marin-Neto JA, et al. (2007) Evaluation and treatment of chagas disease in the United States: a systematic review. *JAMA* 298: 2171–2181.
- Chiari E, Dias JC, Lana M, Chiari CA (1989) Hemocultures for the parasitological diagnosis of human chronic Chagas' disease. *Rev Soc Bras Med Trop* 22:19–23.
- Ávila HA, Pereira JB, Thiemann O, Paiva E, Degrave W, et al. (1993) Detection of *Trypanosoma cruzi* in blood specimens of chronic chagasic patients by polymerase chain reaction amplification of kinetoplast minicircle DNA: comparison with serology and xenodiagnosis. *J Clin Microbiol*, 31: 2421–2426.
- Prata A (2001) Clinical and epidemiological aspects of Chagas disease. *Lancet Infect Dis* 1: 92–100.
- Gomes YM (1997) PCR and sero-diagnosis of chronic Chagas' disease. Biotechnological advances. *Appl Biochem Biotechnol* 66: 107–119.
- Furucho CR, Umezawa ES, Almeida I, Freitas VL, Bezerra R, et al. (2008) Inconclusive results in conventional serological screening for Chagas' disease in blood banks: evaluation of cellular and humoral response. *Trop Med Int Health* 13: 1527–1533.
- Ramirez JD, Guhl F, Umezawa ES, Morillo CA, Rosas F, et al. (2009) Evaluation of adult chronic Chagas' heart disease diagnosis by molecular and serological methods. *J Clin Microbiol* 47: 3945–3951.
- Tarleton RL (2001) Parasite persistence in the aetiology of Chagas disease. *Int J Parasitol* 31: 550–554.
- Andrade CR, Andrade PP, Wright EP (1988) Leishmania donovani donovani antigens recognized by kala-azar patient sera and identification of cross-reacting antigens to Chagas' disease. *Braz J Med Biol Res* 21: 511–515.
- Guhl F, Hudson L, Marinkelle CJ, Morgan SJ, Jaramillo C (1985) Antibody response to experimental *Trypanosoma rangeli* infection and its implications for immunodiagnosis of South American trypanosomiasis. *Acta Trop* 42: 311–318.
- Vexenat Ade C, Santana JM, Teixeira AR (1996) Cross-reactivity of antibodies in human infections by the kinetoplastid protozoa *Trypanosoma cruzi*, *Leishmania chagasi* and *Leishmania (viannia) brasiliensis*. *Rev Inst Med Trop Sao Paulo* 38: 177–185.
- Meira WS, Galvao LM, Gontijo ED, Machado-Coelho GL, Norris KA, et al. (2002) Trypanosoma cruzi recombinant complement regulatory protein: a novel antigen for use in an enzyme-linked immunosorbent assay for diagnosis of Chagas' disease. *J Clin Microbiol* 40: 3735–3740.
- da Silveira JF, Umezawa ES, Luquetti AO (2001) Chagas disease: recombinant *Trypanosoma cruzi* antigens for serological diagnosis. *Trends Parasitol* 17: 286–291.
- Afonso AM, Eboll MH, Tarleton RL (2012) A systematic review of high quality diagnostic tests for Chagas disease. *PLoS Negl Trop Dis* 6: e1881.
- Caballero ZC, Sousa OE, Marques WP, Saez-Alquezar A, Umezawa ES (2007) Evaluation of serological tests to identify *Trypanosoma cruzi* infection in humans and determine cross-reactivity with *Trypanosoma rangeli* and *Leishmania* spp. *Clin Vaccine Immunol* 14: 1045–1049.
- Bhattacharya T, Brooks J, Yeo M, Carrasco HJ, Lewis MD, et al. (2010) Analysis of molecular diversity of the *Trypanosoma cruzi* trypomastigote small surface antigen reveals novel epitopes, evidence of positive selection and potential implications for DTU-specific serology. *Int J Parasitol* 40: 921–928.
- Brisse S, Verhoef J, Tibayrenc M (2001) Characterisation of large and small subunit rRNA and mini-exon genes further supports the distinction of six *Trypanosoma cruzi* lineages. *Int J Parasitol* 31: 1218–1226.
- de Freitas JM, Augusto-Pinto L, Pimenta JR, Bastos-Rodrigues L, Gonçalves VF, et al. (2006) Ancestral genomes, sex, and the population structure of *Trypanosoma cruzi*. *PLoS Pathog* 2: e24.
- Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B (1996) DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. *Mol Biochem Parasitol* 83: 141–152.
- Ackermann AA, Panunzi LG, Cosentino RO, Sanchez DO, Aguero F (2012) A genomic scale map of genetic diversity in *Trypanosoma cruzi*. *BMC Genomics* 13: 736.
- Zingales B, Andrade SG, Briones MR, Campbell DA, Chiari E, et al. (2009) A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. *Mem Inst Oswaldo Cruz* 104: 1051–1054.
- Bellini MF, Siliستino-Souza R, Varella-Garcia M, de Azeredo-Oliveira MT, Silva AE (2012) Biologic and genetics aspects of chagas disease at endemic areas. *J Trop Med* 2012: 357948.
- Buscaglia CA, Di Noia JM (2003) *Trypanosoma cruzi* clonal diversity and the epidemiology of Chagas' disease. *Microbes Infect* 5: 419–427.
- Macedo AM, Pena SD (1998) Genetic Variability of *Trypanosoma cruzi*: Implications for the Pathogenesis of Chagas Disease. *Parasitol Today* 14: 119–124.
- Andrade LO, Machado CR, Chiari E, Pena SD, Macedo AM (1999) Differential tissue distribution of diverse clones of *Trypanosoma cruzi* in infected mice. *Mol Biochem Parasitol* 100: 163–172.
- Morales L, Romero I, Diez H, Del Portillo P, Montilla M, et al. (2002) Characterization of a candidate *Trypanosoma rangeli* small nucleolar RNA gene and its application in a PCR-based parasite detection. *Exp Parasitol* 102: 72–80.
- Brasil PE, De Castro L, Hasslocher-Moreno AM, Sangenio LH, Braga JU (2010) ELISA versus PCR for diagnosis of chronic Chagas disease: systematic review and meta-analysis. *BMC Infect Dis* 10: 337.
- DeFreitas JM, Augusto-Pinto L, Pimenta JR, Bastos-Rodrigues L, Gonçalves VF, et al. (2006) Ancestral genomes, sex, and the population structure of *Trypanosoma cruzi*. *PLoS Pathog* 2(3):e24.
- Cámera AC, Varela-Freire AA, Valadares HM, Macedo AM, D'Avila DA, et al. (2010) Genetic analyses of *Trypanosoma cruzi* isolates from naturally infected triatomines and humans in northeastern Brazil. *Acta Trop* 115(3):205–211.
- Bartholomeu DC, Silva RA, Galvao LM, el-Sayed NM, Donelson JE, et al. (2002) *Trypanosoma cruzi*: RNA structure and post-transcriptional control of tubulin gene expression. *Exp Parasitol* 102: 123–133.
- Bonaldo MC, Souto-Padron T, de Souza W, Goldenberg S (1988) Cell-substrate adhesion during *Trypanosoma cruzi* differentiation. *J Cell Biol* 106: 1349–1358.
- Koerich LB, Emmanuelle-Machado P, Santos K, Grisard EC, Steindel M (2002) Differentiation of *Trypanosoma rangeli*: high production of infective Trypomastigote forms in vitro. *Parasitol Res* 88: 21–25.
- El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, et al. (2005) The Genome Sequence of *Trypanosoma cruzi*, Etiologic Agent of Chagas Disease. *Science* 309:409–415.
- Larsen JE, Lund O, Nielsen M (2006) Improved method for predicting linear B-cell epitopes. *Immunome Res* 2: 2.
- Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, et al. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31: 3497–3500.

represents the mean values. C-, uninfected humans. CL, patients with cutaneous leishmaniasis. VL, patients with visceral leishmaniasis. (TIFF)

**Table S1 List of primers used to amplify the sequences encoding the epitopes.** (DOCX)

**Table S2 List of allele-specific primers used in the real-time RT-PCR analysis.** (DOCX)

**Table S3 Reactivity of the sera from mice infected with different *T. cruzi* strains against the peptides.** (XLSX)

## Author Contributions

Conceived and designed the experiments: TAdOM DCB. Performed the experiments: TAdOM JLRC RdAL GFRL LDL ARRdS. Analyzed the data: TAdOM DCB. Contributed reagents/materials/analysis tools: ACJdC LMdCG CB RHG RTF RTG DCB. Wrote the paper: TAdOM DCB.

37. Aslett M, Aurrecochea C, Berriman M, Brestelli J, Brunk BP, et al. (2010) TriTrypDB: a functional genomic resource for the Trypanosomatidae. *Nucleic Acids Res* 38: D457–462.
38. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403–410.
39. Frank R (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports—principles and applications. *J Immunol Methods* 267: 13–26.
40. Wellings DA, Atherton E (1997) Standard Fmoc protocols. *Methods Enzymol* 289: 44–67.
41. Hedman K, Hietala J, Tiilikainen A, Hartikainen-Sorri AL, Raiha K, et al. (1989) Maturation of immunoglobulin G avidity after rubella vaccination studied by an enzyme linked immunosorbent assay (avidity-ELISA) and by haemolysis typing. *J Med Virol* 27: 293–298.
42. Westenberger SJ, Barnabe C, Campbell DA, Sturm NR (2005) Two hybridization events define the population structure of *Trypanosoma cruzi*. *Genetics* 171: 527–543.
43. Monteiro WM, Margioto Teston AP, Gruendlung AP, Dos Reis D, Gomes ML, et al. (2013) *Trypanosoma cruzi* I and IV Stocks from Brazilian Amazon Are Divergent in Terms of Biological and Medical Properties in Mice. *PLoS Negl Trop Dis* 7: e2069.
44. Wells JA (1991) Systematic mutational analyses of protein-protein interfaces. *Methods Enzymol* 202: 390–411.
45. Porcile PE, Santos MR, Souza RT, Verbisck NV, Brandao A, et al. (2003) A refined molecular karyotype for the reference strain of the *Trypanosoma cruzi* genome project (clone CL Brener) by assignment of chromosome markers. *Gene* 308: 53–65.
46. Zingales B, Miles MA, Campbell DA, Tibayrenc M, Macedo AM, et al. (2012) The revised *Trypanosoma cruzi* subspecific nomenclature: rationale, epidemiological relevance and research applications. *Infect Genet Evol* 12: 240–253.
47. Gomes YM, Lorena VM, Luquetti AO (2009) Diagnosis of Chagas disease: what has been achieved? What remains to be done with regard to diagnosis and follow up studies? *Mem Inst Oswaldo Cruz* 104: 115–121.
48. Llewellyn MS, Miles MA, Carrasco HJ, Lewis MD, Yeo M, et al. (2009) Genome-scale multilocus microsatellite typing of *Trypanosoma cruzi* discrete typing unit I reveals phylogeographic structure and specific genotypes linked to human infection. *PLoS Pathog* 5: e1000410.
49. Di Noia JM, Buscaglia CA, De Marchi CR, Almeida IC, Frasch AC (2002) A *Trypanosoma cruzi* small surface molecule provides the first immunological evidence that Chagas' disease is due to a single parasite lineage. *J Exp Med* 195: 401–413.
50. Carmona SJ, Sartor PA, Leguizamon MS, Campetella OE, Aguero F (2012) Diagnostic peptide discovery: prioritization of pathogen diagnostic markers using multiple features. *PLoS One* 7: e50748.
51. Aguirre S, Silber AM, Brito ME, Ribone ME, Lagier CM, et al. (2006) Design, construction, and evaluation of a specific chimeric antigen to diagnose chagasic infection. *J Clin Microbiol* 44: 3768–3774.
52. DaRocha WD, Bartholomeu DC, Macedo CD, Horta MF, Cunha-Neto E, et al. (2002) Characterization of cDNA clones encoding ribonucleoprotein antigens expressed in *Trypanosoma cruzi* amastigotes. *Parasitol Res* 88: 292–300.
53. Soto M, Requena JM, Garcia M, Gomez LC, Navarrete I, et al. (1993) Genomic organization and expression of two independent gene arrays coding for two antigenic acidic ribosomal proteins of *Leishmania*. *J Biol Chem* 268(29):21835–21843.
54. Requena JM, Alonso C, Soto M. (2000) Evolutionarily conserved proteins as prominent immunogens during *Leishmania* infections. *Parasitol Today* 16(6):246–250.
55. Gu W, Helms V (2005) Dynamical binding of proline-rich peptides to their recognition domains. *Biochim Biophys Acta* 1754(1–2):232–238.
56. Ball IJ, Kühne R, Schneider-Mergener J, Oschkinat H (2005) Recognition of proline-rich motifs by protein-protein-interaction domains. *Angew Chem Int Ed Engl* 44(19):2852–2869.
57. Pais FS, DaRocha WD, Almeida RM, Leclercq SY, Penido ML, et al. (2008) Molecular characterization of ribonucleoproteic antigens containing repeated amino acid sequences from *Trypanosoma cruzi*. *Microbes Infect* 10: 716–725.
58. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, et al. (2008) Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* 455: 757–763.
59. Valiente-Gabioud AA, Veaut C, Perrig M, Galan-Romano FS, Sferco SJ, et al. (2011) Effect of repetitiveness on the immunogenicity and antigenicity of *Trypanosoma cruzi* FRA protein. *Exp Parasitol* 127: 672–679.
60. Pitcovsky TA, Buscaglia CA, Mucci J, Campetella O (2002) A functional network of intramolecular cross-reacting epitopes delays the elicitation of neutralizing antibodies to *Trypanosoma cruzi* trans-sialidase. *J Infect Dis* 186: 397–404.

## Chimeric synthetic peptides as antigens for detection of antibodies to *Trypanosoma cruzi*

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### Abstract

Six chimeric synthetic peptides (QCha-1, QCha-2, QCha-3, QCha-4, QCha-5, and QCha-6) incorporating antigenic sequences of two immunodominant repeat B-cell epitopes of *Trypanosoma cruzi* were synthesized by conventional solid-phase peptide synthesis. The antigenic activity of these peptides was evaluated by UltramicroEnzyme-linked immunosorbent assay (UMELISA) by using panels of positive Chagasic sera ( $n = 82$ ), while specificity was evaluated with samples from healthy blood donors ( $n = 44$ ) and patients with other infectious diseases ( $n = 86$ ). The antigenicity of the chimeric peptides in solid-phase immunoassays was compared with that of the monomeric peptides. Data demonstrated that the chimeric peptide QCha-5 was the most reactive because it detected antibodies to parasite efficiently. The results indicate that chimeric peptide as coating antigen is very useful for the immunodiagnosis of Chagas' disease.

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**Keywords:** Chimeric synthetic peptides; UMELISA; *Trypanosoma cruzi*; Chagas; Antigenicity

Chagas' disease or *American trypanosomiasis*, caused by the flagellate protozoan *Trypanosoma cruzi* (*T. cruzi*), is one of the most important endemic problems in Americas, particularly in South America [1–4]. Chagas' disease currently affects 16–18 million people. This illness is transmitted to humans and other mammals mostly by insect vectors of the subfamily *Triatominae* [5,6], during transfusion of individuals' blood infected with the protozoan [7], and through other transmission mechanisms: ingestion of food contaminated with parasites, organ transplantation, transmitted through the placenta, mother's milk, laboratory accidents, etc.

The disease affects the nervous system, digestive system, and heart. Chronic infections result in various neurological disorders, including dementia, damage to the heart muscle (cardiomyopathy, the most serious manifestation), and

sometimes dilation of the digestive tract (megacolon and megaesophagus), as well as weight loss.

The diagnosis of Chagas' disease is determined by means of the detection of the parasite in the blood samples by direct examination, hemoculture, or xenodiagnosis (direct methods) and/or for the detection of specific antibodies to *T. cruzi* antigens by immunological methods (indirect methods) [8].

Commercial ELISAs use, in solid surface, antigens obtained by epimastigote lysis or trypomastigote of the *T. cruzi* [9,10]. These tests present good sensitivity, but they are inconvenient in that they present cross-reactivity with *Leishmania* patient sera, for what they obtained frequently false positive results [11,12].

A solution to the problem of the serological diagnosis of the Chagas' disease is the use of recombinant [13] and synthetic peptides [14,15], designed, to obtain a diagnosis test that guarantees results with high levels of sensitivity and specificity. The antigenic sequences that used them more belong to repetitive regions of the parasite [16],

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shed-acute-phase-antigen (SAPA), cytoplasmic repetitive antigen (CRA), trypomastigote surface antigen (TSA), etc.

However, the main disadvantage of the short synthetic peptides is that these are poorly antigenic in solid-phase based immunoassays due to their weak binding to solid surfaces. Also, the synthetic peptides lose their antigenicity because of the masking of essential antigenic side chains following binding to a solid phase. For this reason, there is a tendency to use chimeric synthetic peptides to avoid this problem and to improve the sensitivity and specificity of the assays [17–21].

For this purpose, six chimeric synthetic peptides (QCha-1, QCha-2, QCha-3, QCha-4, QCha-5, and QCha-6) were synthesized. These peptides were designed by means of the combination of two immunodominant repeat B-cell epitopes from *T. cruzi*. The monomeric peptides (P1 and P2) were obtained.

## Materials and methods

*Solid-phase synthesis.* Based on the sequence for *T. cruzi* solid-phase peptide synthesis was performed by using the standard solid-phase method [18–22], as described previously. The monomeric and chimeric peptides (Table 1) were synthesized manually, using Boc chemistry. All solvents used for peptide synthesis were pure. Side-chain-protected amino acids used were from Bachem (Switzerland). As solid phase, the polymer *p*-methylbenzhydryl amine (Bachem, Switzerland) resin was used. The coupling reactions were carried out using DIPCDI (Merck, Germany)/HOBT (Sigma) in DMF. Following, each amino acid coupling one sample was taken and a qualitative ninhydrin assay was performed. After com-

pletion of synthesis, peptides were cleaved from the resin and the amino acid side chains were deprotected by acidic hydrolysis using HF pure for analysis (Fluka, USA) in the presence of scavengers (anisole, dimethyl sulfide, 1,2-ethanedithiol, and *p*-cresole) (Merck, Germany).

*Peptide purification and characterization.* The peptides obtained were analyzed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC, Pharmacia, LKB, Sweden), using a RPC18 protein/peptide column (Vydac, 4.6 × 150 mm) with a linear gradient from solution A (0.1% TFA in water) to solution B (0.05% TFA in acetonitrile), in 35 min. The peptides were detected by UV at  $\lambda = 226$  nm. Data were processed by the Biocrom program (CIGB, Cuba).

Peptide molecular weights were verified by electrospray ionization-mass spectrometry (ESI-MS) using mass spectrometer with orthogonal geometry QTOF-2 (Micromass, UK). The spectrum was processed with MassLinx v3.5 (Micromass, UK).

**Enzyme-linked immunosorbent assay.** UltramicroEnzyme-linked immunosorbent assay (UMELISA) combines the high sensitivity of current ELISA tests with the use of small volumes of samples and reagents. This assay was performed according to that described in [22]. Peptides were dissolved in 0.05 mol/L carbonate–bicarbonate buffer, pH 9.6, and the wells were coated using 15  $\mu$ L of a solution of the monomeric or chimeric peptides (4  $\mu$ g/mL).

All assays included positive and negative controls. The fluorescence reading from each sample was normalized as a relative value (FRV) on the fluorescence value of a positive control over the same plate. Samples were considered positive when FRV were equal to or higher than the cutoff value (0.30). All numeric results are means of duplicate.

The analyzed samples were human Chagasic sera ( $n = 45$ ) from Colombia and Brasil ( $n = 37$ ) defined as positive by indirect immunofluorescence assay. Specificity was assessed by using sera from the healthy blood donors ( $n = 44$ ) and 86 subjects with other infectious diseases: human immunodeficiency virus type 1 (HIV-1) ( $n = 15$ ), human immunodeficiency virus type 2 (HIV-2) ( $n = 5$ ), toxoplasmosis ( $n = 20$ ), hepatitis C virus (HCV) ( $n = 20$ ), human T-cell leukemia virus type I (HTLV-I) ( $n = 20$ ), and leprosy ( $n = 6$ ).

Cutoff value: The cutoff value (CO) was 0.30.

Table 1

Monomeric and chimeric synthetic peptides that correspond to immunodominant repeat B-cell epitopes from *Trypanosoma cruzi*

Peptides <sup>a</sup>	Sequence
P1	PSPFGQAAAGDK
P2	AEPKPAEPKS
QCha-1	P1-P2
QCha-2	P1-P2-P1
QCha-3	P2-P1-P2
QCha-4	P2-P1
QCha-5	P1-P1-P2
QCha-6	P2-P2-P1

<sup>a</sup> The peptide sequences were deduced from reported by Gruber A et al. [23] and Buschiazzo A et al. [24].

## Results

## *Synthesis, purity, and characterization of peptides*

Synthesis of our chimeric and monomeric peptides was based on the conventional solid-phase peptide methodology using Boc chemistry. The appropriately protected amino acids were incorporated into the peptide sequence using DIPCDI/HOBt as the coupling reagent. The peptides were obtained in good yield. The purity of the peptides was evaluated by analytical RP-HPLC, and all the peptides were

Table 2

Reactivity of the chimeric peptides, which sequences combine two immunodominant repeat B-cell epitopes from *Trypanosoma cruzi* and the monomeric peptides against the samples of the Chagasic sera from Colombia ( $n = 45$ ) and Brasil ( $n = 37$ ), and 86 subjects with other infectious diseases: human immunodeficiency virus type 1 (HIV-1) ( $n = 15$ ), human immunodeficiency virus type 2 (HIV-2) ( $n = 5$ ), toxoplasmosis ( $n = 20$ ), hepatitis C virus (HCV) ( $n = 20$ ), human T-cell leukemia virus type I (HTLV-I) ( $n = 20$ ), and leprosy ( $n = 6$ ).

found to be >85% pure. Peptides were successfully characterized by ESI-MS.

### Antigenicity

A summary of the antigenicities of the monomeric and chimeric peptides is presented in Table 2. To assess peptide specificity, 86 subjects with other infectious diseases: human immunodeficiency virus type 1 (HIV-1) ( $n = 15$ ), human immunodeficiency virus type 2 (HIV-2) ( $n = 5$ ), toxoplasmosis ( $n = 20$ ), hepatitis C virus (HCV) ( $n = 20$ ), human T-cell leukemia virus type I (HTLV-I) ( $n = 20$ ), and leprosy ( $n = 6$ ) were tested (Table 2). Samples from healthy blood donors were also evaluated ( $n = 44$ ), and all specimens were finally considered negative.

### Discussion

Two monomeric peptides (P1 and P2) and six chimeric synthetic peptides (QCha-1, QCha-2, QCha-3, QCha-4, QCha-5, and QCha-6), containing two immunodominant repeat B-cell epitopes, were obtained.

The antigenic activities of the new chimeric and monomeric synthetic peptides were evaluated. All peptides were assessed against the Chagas positive samples from Colombia and Brasil.

The monomeric peptides' performance with Chagas positive samples from Colombia ( $n = 45$ ) and sera from seropositive people from Brasil ( $n = 37$ ) is shown in Table 2, where: peptide P1 detected (31/45) (69%) positive sera, but detected (32/37) (86%) positive samples. Peptide P2 detected (22/45) (49%) positive sera and detected (33/37) (89%) positive samples.

Differences in reactivity to various chimeric synthetic peptides were observed (Table 2). Antibodies against peptides QCha-5 (45/45) (100%) and QCha-3 (40/45) (89%) were found at high levels in most serum samples from Colombia and peptides QCha-2 (37/37) (100%), QCha-3 (37/37) (100%), QCha-4 (36/37) (97%), QCha-5 (37/37) (100%), and QCha-6 (36/37) (97%) with positive sera from Brasil.

These results showed that the chimeric peptides are more antigenic than the monomeric peptides and these can be used to detect antibodies to more than one epitope simultaneously. Our results also showed that the order of location of the epitopes in the chimeric peptides is determinant in the antigenicity of these biomolecules. The P1-P1-P2 epitope orientation was found to be most suitable for an increased interaction with antibodies.

Similar results, regarding the order of location of the epitopes in the chimeric peptides, were reported by us in previous studies when the chimeric peptides of HTLV were evaluated [18–21]. This phenomenon should take place due to the space conformation of the molecule allowing an appropriate exposure to the antibodies.

In conclusion, we showed here that the chimeric peptide QCha-5, incorporating two immunodominant repeat B-cell epitopes of the *T. cruzi*, of this study was the most antigen-

ic peptide. Therefore, this peptide will be useful as antigen for the detection of antibodies to *T. cruzi* and for the control of disease transmission by blood transfusion.

### References

- [1] M.E. Jorg, *Tripanosomiasis cruzi* humana o enfermedad de Chagas-Mazza, Rev. Med. Trop. Sao Paulo 17 (1978) 185–187.
- [2] L. Mazzotti, E. Díaz, Resumen de los datos publicados sobre la enfermedad de Chagas en México, Rev. Soc. Mex. Hist. Nat. 10 (1949) 103–105.
- [3] R. Aras, M. Veiga, I. Gomes, G. Mota, B. Rodrigues, R. Rabelo, C. Guzman-Bracho, A. Melo, Prevalence of Chagas' disease in Mulungu do Morro northeastern Brazil, Arq. Bras. Cardiol. 78 (2002) 441–443.
- [4] J.C. Dias, A.C. Silveira, C.J. Schofield, The impact of Chagas disease control in Latin America: a review, Mem. Inst. Oswaldo Cruz 97 (2002) 603–612.
- [5] J. Tay, A.M.B. Biagi, Localidades nuevas de triatominos mexicanos y su infección natural por *Trypanosoma cruzi*, Rev. Fac. Med. 6 (1984) 305–308.
- [6] E. Dumonteil, S. Gourbiere, M. Barrera-Perez, E. Rodriguez-Felix, H. Ruiz-Pina, O. Banos-Lopez, M.J. Ramirez-Sierra, F. Menu, J.E. Rabinovich, Geographic distribution of *Triatomata dimidiata* and transmission dynamics of *Trypanosoma cruzi* in the Yucatan peninsula of Mexico, Am. J. Trop. Med. Hyg. 67 (2002) 176–183.
- [7] G.A. Schmunis, Prevention of transfusional *Trypanosoma cruzi* infection in Latin America, Mem. Inst. Oswaldo Cruz 94 (1999) 93–101.
- [8] M.A. Winkler, R.J. Brashear, H.J. Hall, J.D. Schur, A.A. Pan, Detection of antibodies to *Trypanosoma cruzi* among blood donors in the South western and western United States. II. Evaluation of a supplemental enzyme immunoassay and radioimmunoprecipitation assay for confirmation of seroreactivity, Transfusion 35 (1995) 219–225.
- [9] A. Ferreira, W.Z.R. Belem, M.E.G. Moura, M.E. Camargo, Aspectos da padronização de testes sorológicos para doença de Chagas: um teste imunoenzimático para a triagem de doadores de sangue, Rev. Inst. Med. Trop. Sao Paulo. 33 (1991) 123–128.
- [10] W.M.R. Oelemann, M.G.M. Teixeira, G.C. Verissimo Da Costa, Evaluation of three commercial enzyme-linked immunoadsorbant assays for diagnosis of Chagas' disease, J. Clin. Microbiol. 36 (1998) 2423–2427.
- [11] E.L. Malchiodi, M.G. Chiaramonte, N.J. Taranto, N.W. Zwirner, R.A. Margni, Cross-reactivity studies and differential serodiagnosis of human infections causal by *Trypanosoma cruzi* and *Leishmania* spp; use of immunoblotting and ELISA with a purified antigen (Ag163B6), Clin. Exp. Immunol. 97 (1994) 417–420.
- [12] C. Abramo, C.J. Fontes, A.U. Krettli, Cross-reactivity between antibodies in the sera of individuals with leishmaniasis, toxoplasmosis, and Chagas' disease and antigens of the blood-stage forms of *Plasmodium falciparum* determined by indirect immunofluorescence, Am. J. Trop. Med. Hyg. 53 (1995) 202–205.
- [13] A.W. Ferreira, Z.R. Belem, A.A. Lemus, S.G. Reed, A. Campos-Neto, Enzyme-linked immunosorbent assay for serological diagnosis of Chagas'disease employing a *Trypanosoma cruzi* recombinant antigen that consists of four different peptides, J. Clin. Microbiol. 39 (2001) 4390–4395.
- [14] O. Noya, M.E. Patarroyo, F. Guzman, B. Alarcon de Noya, Immunodiagnosis of parasitic diseases with synthetic peptides, Curr. Protein Pept. Sci. 4 (2003) 299–308.
- [15] G.N. Betonico, E.O. Miranda, D.A. Silva, R. Houghton, S.G. Reed, A. Campos-Neto, J.R. Mineo, Evaluation of a synthetic tripeptide as antigen for detection of IgM and IgG antibodies to *Trypanosoma cruzi* in serum samples from patients with Chagas disease or viral diseases, Trans. R. Soc. Trop. Med. Hyg. 93 (1999) 603–606.
- [16] C.F. Ibanez, J.L. Affranchino, R.A. Macina, M.B. Reyes, S. Leguizamon, M.E. Camargo, L. Aslund, U. Pettersson, A.C. Frasch, Multiple *Trypanosoma cruzi* antigens containing tandemly repeated amino acid sequence motifs, Mol. Biochem. Parasitol. 30 (1998) 27–33.

- [17] K. Shah, C. Davis, J. Wilson, B. Parekh, Chimeric synthetic peptides as antigens for detection of antibodies to HIV-1 and HIV-2, *East Afr. Med. J.* 73 (1996) 63–66.
- [18] M. Hernández, M.E. Selles, L. Pozo, I. Gómez, A. Melchor, Antigenicity of chimeric synthetic peptides based on HTLV-I antigens and the impact of epitope orientation, *Biochem. Biophys. Res. Commun.* 276 (2000) 1085–1088.
- [19] M. Hernández, L. Pozo, I. Gómez, A. Melchor, Chimeric synthetic peptide as antigen for immunodiagnosis of HIV-1 infection, *Biochem. Biophys. Res. Commun.* 272 (2000) 259–262.
- [20] M. Hernández Marin, P. Castellanos Pentón, Y. Márquez Bocalandro, L. Pozo Peña, J. Díaz Navarro, L.J. González López, Chimeric synthetic peptides containing two immunodominant epitopes from the envelope gp46 and the transmembrane gp21 glycoproteins of HTLV-I virus, *Biochem. Biophys. Res. Commun.* 289 (2001) 1–6.
- [21] M. Hernández Marin, P. Castellanos Pentón, Y. Márquez Bocalandro, L. Pozo Peña, J. Díaz Navarro, L.J. González López, Chimeric synthetic peptides from the envelope (gp46) and the transmembrane (gp21) glycoproteins for the detection of antibodies to human T-cell leukemia virus type II, *Biochem. Biophys. Res. Commun.* 289 (2001) 7–12.
- [22] M. Hernández Marin, Ch. Rodríguez-Tanty, D. Higginson-Clarke, Y. Marquez Bocalandro, L. Pozo Peña, Study of the peptide length and amino acids specific substitution in the antigenic activity of the chimeric synthetic peptides, containing the p19 core and gp46 envelope proteins of the HTLV-I virus, *Biochem. Biophys. Res. Commun.* 336 (2005) 983–986.
- [23] A. Gruber, B. Zingales, *Trypanosoma cruzi*: characterization of two recombinant antigens with potential application in the diagnosis of Chagas' disease, *Exp. Parasitol.* 76 (1993) 1–12.
- [24] A. Buschiazzo, O.E. Campetella, R.A. Macina, S. Salceda, A.C. Frasch, D.O. Sanchez, Sequence of the gene for a *Trypanosoma cruzi* protein antigenic during the chronic phase of human Chagas disease, *Mol. Biochem. Parasitol.* 54 (1992) 125–128.