
# Development of Rapid Diagnostic Tests for *Trypanosoma cruzi* Lineage-Specific Serology, Comparative Epidemiology and for Monitoring Efficacy of Chemotherapy

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## DECLARATION

I Niamh Murphy, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## ABSTRACT

*Trypanosoma cruzi*, the protozoan agent of Chagas disease, is split into six distinct genetic lineages TcI – TcVI, each associated with ecoepidemiological features and transmission cycles, and it has long been proposed that the differing lineages may contribute to the varying clinical forms of Chagas disease. The epidemiology is not fully understood as current knowledge is based on genotyping and may not give the full infection history due to the difficulty of isolating parasite during the chronic phase of infection.

In this thesis work I performed lineage-specific serological assays, on human and mammalian sera from the Gran Chaco in Northern Argentina. I show that a novel RDT incorporating the TSSA-II/V/VI synthetic peptide is more sensitive than the corresponding lineage-specific ELISA and can be used to help identify ecological and epidemiological associations in the Argentine Chaco. Prior to my research project there was no known serological marker for TcI. I demonstrated that a glycosylated TSSA-I recombinant protein, produced through the *L. tarentolae* system, has increased sensitivity with chagasic sera, compared to linear synthetic peptides due to the addition of sugars. In addition, I assayed paired pre-and post-treatment samples against TSSApep-II/V/VI and whole cell lysate to determine changes in antibody titre and assessed the decline of IgG1 to be a potential biomarker for cure.

Following the success above, I have established the *L. tarentolae* expression system at LSHTM and have successfully produced recombinant proteins of the TSSA peptides and demonstrated recognition by chagasic sera for glycosylated versions of TSSApep-II/V/VI and TSSApep-V/VI. On a second research visit to Argentina, I identified *T. cruzi* lineage by PCR from triatomine bugs from the Gran Chaco, the first documentation of this technique on direct rectal samples. Furthermore, I used bioinformatics to identify novel candidate epitopes for TcI serology.

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# CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| AIDS | Acquired immune deficiency syndrome |
| BLAST | Basic local alignment tool |
| Bp | Base pairs |
| CDC | Centre for Disease Control |
| ChLiA | Chagas chemiluminescent assay |
| DALY | Disability adjusted life years |
| DNA | Deoxyribonucleic acid |
| DTU | Discrete typing unit |
| ELISA | Enzyme-linked immunosorbent assay |
| FDA | Food and Drug Administration |
| HIV | Human immunodeficiency virus |
| IIF | Indirect immunofluorescence assay |
| IFAT | Indirect fluorescent antibody test |
| Ig | Immunoglobulin |
| IHA | Indirect hemagglutination assay |
| kDNA | Kinetoplast Deoxyribonucleic acid |
| LAMP | Loop-mediated isothermal amplification |
| LSHTM | London School of Hygiene and Tropical Medicine |
| MLEE | Multilocus enzyme electrophoresis |
| nts | Nucleotides |
| NTC | Noursceothricin |
| PAHO | Pan-American Health Organisation |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| Pol 1 RNA | Polymerase I Ribonucleic acid |
| qPCR | Quantitative Polymerase Chain Reaction |
| RDT | Rapid Diagnostic Test |
| RFLP | Restriction Fragment Length Polymorphism |
| rRNA | Ribosomal ribonucleic acid |
| SatDNA | Satellite Deoxyribonucleic acid |
| TcMUC | *T. cruzi* Mucin |
| TET | Tetracycline |
| TSSA | Trypomastigote Small Surface Antigen |
| UTRs | Untranslated regions |
| WHO | World Health Organisation |

## LIST OF TABLES



## LIST OF FIGURES

# 1. LITERATURE REVIEW

## 1.1 *Trypanosoma cruzi:* Parasite discovery and world impact

*Trypanosoma cruzi*, a protozoan parasite, is the causative agent of Chagas disease (American Trypanosomiasis). The first identification of the *T. cruzi* parasite was by Carlos Chagas in 1908. He had been made aware of large insects that infested houses and fed on humans, he caught and dissected them, identifying trypanosomes in their hindgut, he named these trypanosomes after his mentor, Oswaldo Cruz (1). Following this discovery, he sent a sample of these bugs to his laboratory and fed them on a marmoset, which became infected and trypanosomes were identified in the monkey's blood. The identification of *T. cruzi* infecting humans came slightly later, in 1909, Chagas identified the circulating parasite in a blood of a two-year-old girl from Brazil, who had a fever, and an enlarged spleen and liver (1). However, *T. cruzi* infection has been around for far longer, with the first documented case being found in a 9000-year-old mummy of the Chinchorro civilisation from the coastal region of Chile and Peru (2). The presence of *T. cruzi* DNA made it possible to estimate the prevalence rate of Chagas disease at this time at about 40.6 % (2).

Today, Chagas disease is endemic to the 21 continental countries of Latin America. The World Bank and World Health Organisation consider it the fourth most transmitted disease, after malaria, tuberculosis and schistosomiasis (3, 4) and currently estimate there are 6 – 7 million people infected, 25 million people at risk, and 10,000 deaths every year (5). Although most cases are within endemic areas it is increasingly becoming a global health issue, due to the migration of infected individuals out of Latin America (Figure 1), with an estimated 250,000 people infected within the USA, 100,000 and 12,000 people infected within Europe and the UK, respectively (6, 7). In 2019, Chagas disease was responsible for an estimated 9,490 deaths and 275,000 Disability-adjusted life years (DALYs) (8).

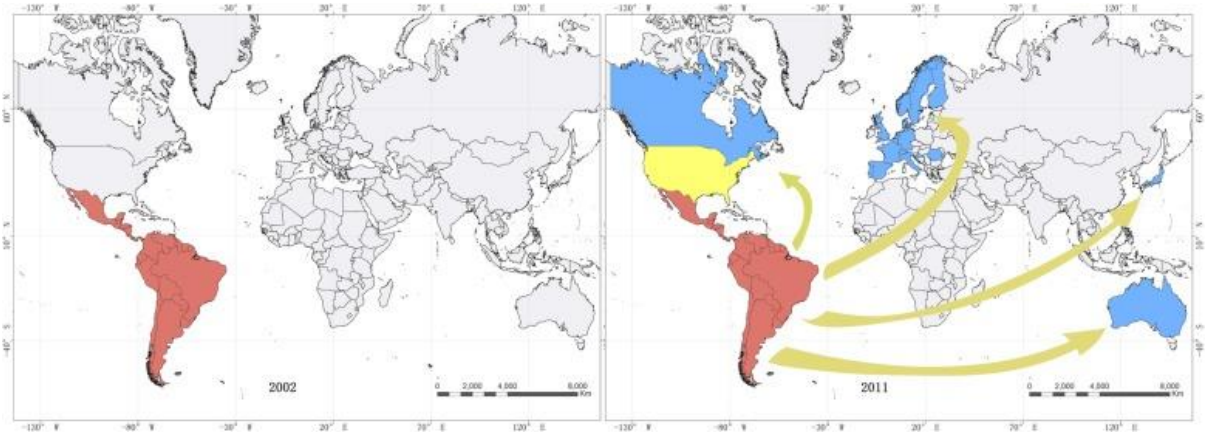


Figure 1. Global spread of Chagas disease from 2002 to 2011, taken from Liu et al., 2015 (4). Red: Endemic area of Chagas disease with vectorial transmission. Yellow: Endemic area with occasional vectorial transmission. Blue: Non-endemic area for Chagas disease with transmission via other routes.

## 1.2 Life cycle and vector transmission

Originally a zoonosis, the main route of transmission for *T. cruzi* is via the vector, the hematophagous triatomine bug (Kissing bugs). Formally maintained within a sylvatic cycle, the bugs nest within animal habitats, feeding on animals while they slept. The introduction of *T. cruzi* into domestic cycles would have occurred with the migration of humans into triatomines' natural habitats, disrupting the sylvatic cycle, it is hypothesised this first occurred in the Andean region (2). Some species of the triatomine bug have used this to their advantage, infesting poorly built houses, living within cracks and crevices of walls and feeding on people whilst they sleep, a typical feature of highly infested houses is the drip pattern of triatomine faeces down walls (faecal rain), deposited by the bugs as they return to their nesting spots after feeding (Figure 2). The main three genera associated with *T. cruzi* transmission are *Triatoma*, *Rhodnius* and *Panstrongylus*.


Figure 2. *T. cruzi* vector habitat and typical vector. A: Infested house in Bolivia, B: Faecal rain, deposits of faeces left on walls as the triatomine bugs return to nests after feeding C: Triatomine bug feeding and defecating on skin. (A, B Photos courtesy of Dr Louisa Messenger, LSHTM).

The parasites are passed from the triatomine vector to humans through contaminated bug faeces. When a bug takes a blood meal it defecates (Figure 2), metacyclic trypomastigotes within the faeces enter the host through the bite wound, abraded skin, or through mucosal membranes, such as the conjunctiva. Once within the host the metacyclic trypomastigotes invade host cells, differentiate into amastigotes and multiply by binary fission, eventually, they transform into trypomastigotes, burst from the cell and enter the bloodstream. They can either invade other cells within the host or are ingested by a feeding triatomine bug. Within the bug, the ingested trypomastigotes differentiate into epimastigotes and multiply in the midgut, differentiating into the infective metacyclic trypomastigotes in the hindgut (9) (Figure 3).

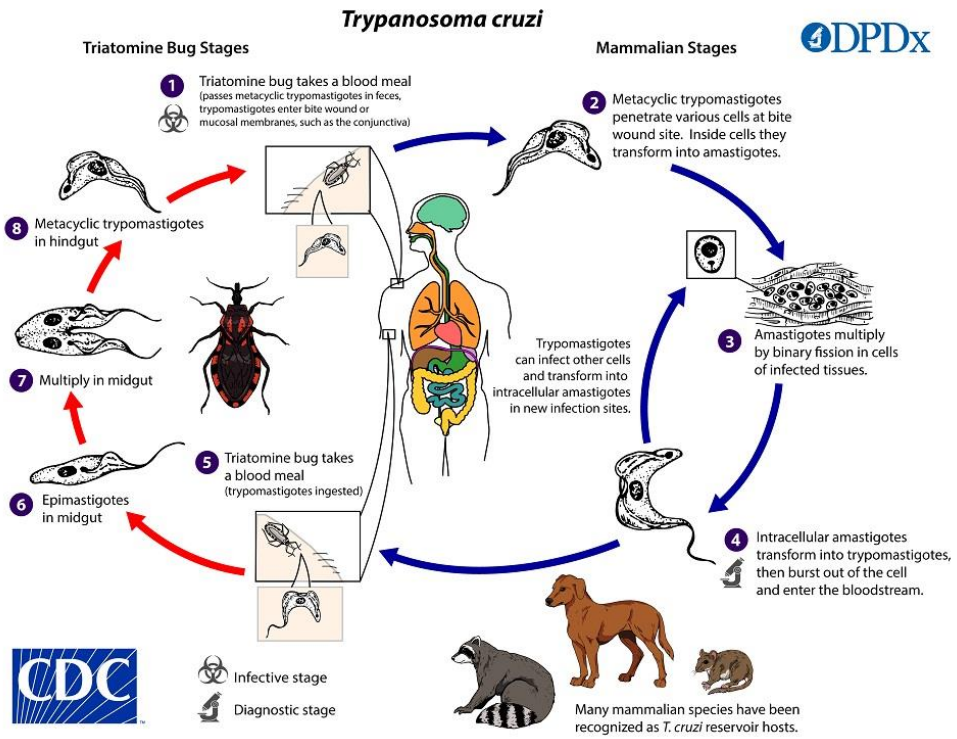


Figure 3. *Trypanosoma cruzi* life cycle. Taken from CDC (9).

## 1.3 Other routes of transmission

Vectoral transmission is the most common form of infection, but other routes can occur (Figure 4).

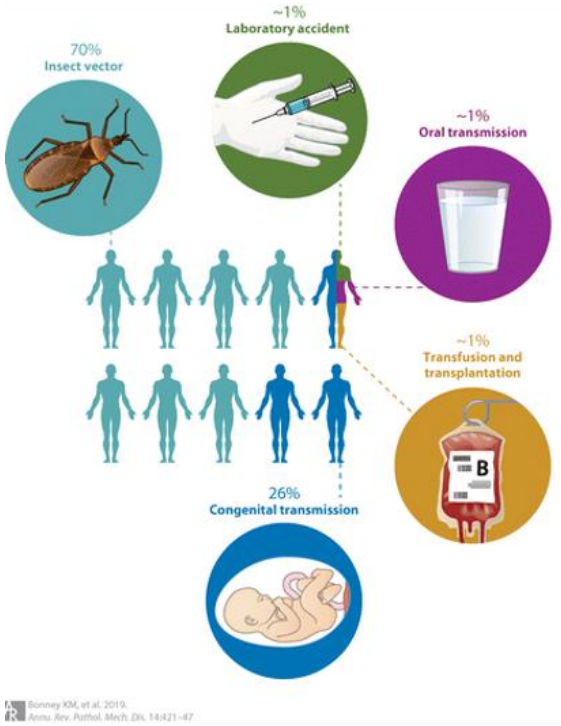


Figure 4. Transmission routes of *T. cruzi* infection, taken from Bonney et al., 2019 (10).

Oral transmission is through the ingestion of infected triatomine bugs, faeces or urine via contaminated food (wild animal meat and vegetables), or drinks (sugar cane extract and fresh pressed juice i.e., acai and guava juice). Outbreaks of oral transmission have been reported in Brazil, Venezuela, Colombia, French Guyana, Bolivia, Argentina and Ecuador (11), but is predominantly an issue in the Amazon region, where there has been a rise in cases of acute infection caused by oral transmission from 50 % in 2000 to 70 % between 2000- 2010 (12). It is also a key mode of transmission for sylvatic cycles (13) and it appears to be associated with more acute morbidity and higher mortality than vector-borne transmission (14).

Congenital transmission is emerging as a key route for transmission of *T. cruzi,* especially in non-endemic areas. An estimated 1 million women of reproductive age are infected and transmission occurs in around 5 % of infected mothers. It is thought to be responsible for 22 % of new cases in endemic areas, with approximately 9000 newborns being born infected with *T. cruzi* every year (15). It is the leading cause of infection in non-endemic areas and can be passed from one generation to another through multiple pregnancies (14).

Transmission through blood and organ donation has been recognised since 1952 and was considered the second most common route of infection in endemic countries (16). The total number of infections caused by transfusion in endemic areas is estimated between 300-800 cases. It is one of the leading causes of transmission in non-endemic areas, first reported in 1987 in North America, from bone marrow transfusion (17).

Laboratory accidents cause less than 1 % of new Chagas cases (10). Infection is usually caused by a puncture wound with a sharp instrument whilst handling *T. cruzi*- infected animals (18) and in 2006 only approximately 70 well-documented cases had been reported, mainly in Latin America (19).

Sexual transmission of Chagas disease is not well documented. Mouse studies have shown that infection is possible from infected male mice to naïve females (20). In humans, there has been a limited number of case reports of families with suspected sexual transmission (21). There have also been some scarce reports of transmission from mother to infant through breast feeding (22).

## 1.4 Control strategies

Since the discovery that the triatomine bug was the vector, control strategies have focused on its elimination from homes. Early attempts included dousing-infested household walls with

kerosine, or scalding water to the more extreme use of military flamethrowers or cyanide gas, had limited success (23). With the discovery of synthetic insecticides mass spraying campaigns began in several countries throughout the 1950s and 1960s, which were improved greatly by the development of pyrethroids in the 1980s.

The Southern Cone initiative was launched in 1991, with the combined ambition to control Chagas disease through the elimination of *T. infestans,* the main vector of *T. cruzi* in the six Southern Cone countries (Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay, with Peru joining later) and large-scale screening of blood donors (24, 25). The vector control consisted of two phases, the initial vertical phase, where all houses in endemic areas are treated with pyrethroids, followed by the horizontal phase in which residents reported any bugs found in their homes and retreatment was organised (25), between 1992 and 2001, more than 2.5 million homes were sprayed. Overall, the initiative was considered a wide success, in most areas that implemented the program infestation of households dropped to 5 % or below (26) and by 2001, disease transmission was interrupted in Uruguay, Chile, Paraguay and large parts of Brazil (23) (Figure 5). However, in the Gran Chaco region, covering Argentina, Bolivia and Paraguay *T. infestans* infestation persists, and control strategies encounter a few problems; fast reinfestation after spraying campaigns, lack of sustained surveillance and emergence of pyrethroid resistance bugs (27, 28).

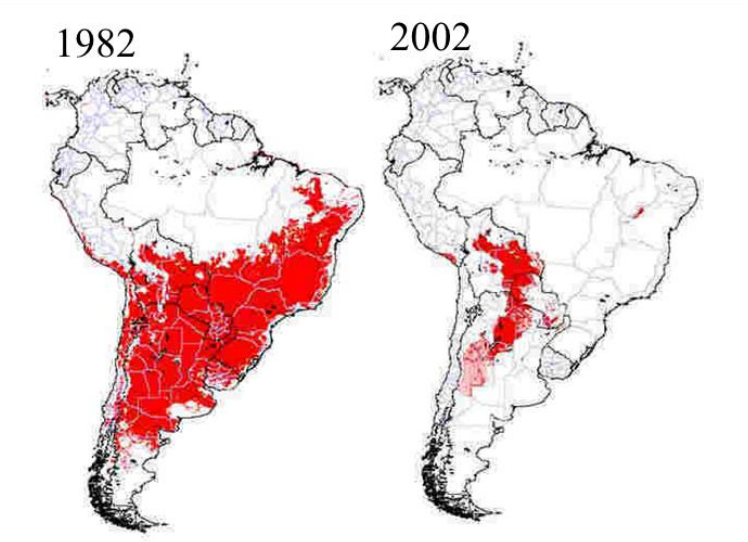


Figure 5. Distribution of *T. infestans* before and after the Southern Cone initiative was started (Provided by Chris Schofield).

Following suit, other vector control strategies were set up across Latin America. In 1997, the governments of Colombia. Ecuador, Peru and Venezuela established the Andean Initiative (29), targeting three main Triatomine bugs, *R. prolixus* in Colombia and Venezuela, *T. dimidiata* in Colombia and Ecuador and *R. ecuadoriensis* in Ecuador and Northern Peru (30). Also established in 1997, the Central American initiative, an agreement between the governments of Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama, which Mexico joined in 2012, targeted the control of *R. prolixus* and *T. infestans* (31). Lastly, the Amazon Initiative, which was set up in 2004, between Bolivia, Brazil, Colombia, Ecuador, Guyana, French Guyana, Peru, Suriname and Venezuela, focused on new methodologies for surveillance, vector control of domestic infestation and oral transmission routes (32).

The second pillar for control of Chagas disease is screening of donated blood and organs, as well as screening pregnant women. Screening of blood donors in Latin America began in 1990 and gained momentum during the AIDS epidemic. Since 2012, screening for blood donation has been mandatory in all endemic Latin American Countries (33). Guidelines for screening organ donors have also been published and are of great importance in countries that carry out heart organ transplantation. The United States screens all its blood donors for *T. cruzi* and European countries screen or exclude high-risk donors based on questionnaire assessment (29). Screening for congenital cases of Chagas disease would also help control the transmission from mothers to newborns and guidelines were released by WHO in 2002 (34).

## 1.5 Clinical manifestations and disease

Chagas disease occurs in two phases, acute and chronic. The acute phase is after initial infection, typically it lasts 2-3 months and is asymptomatic. However, in a small proportion of cases, non-specific symptoms occur, including fever, hepatosplenomegaly and lymphadenopathy. A typical specific symptom of the acute phase is swelling and redness around the infection site at the eye, known as Romaña's sign and elsewhere on the skin a Chagoma (Figure 6). In rare cases (~ 10 %) the acute stage can be fatal, due to acute myocarditis or meningoencephalitis, this tends to only be seen in congenital infections, young children or adults compromised with HIV infection.

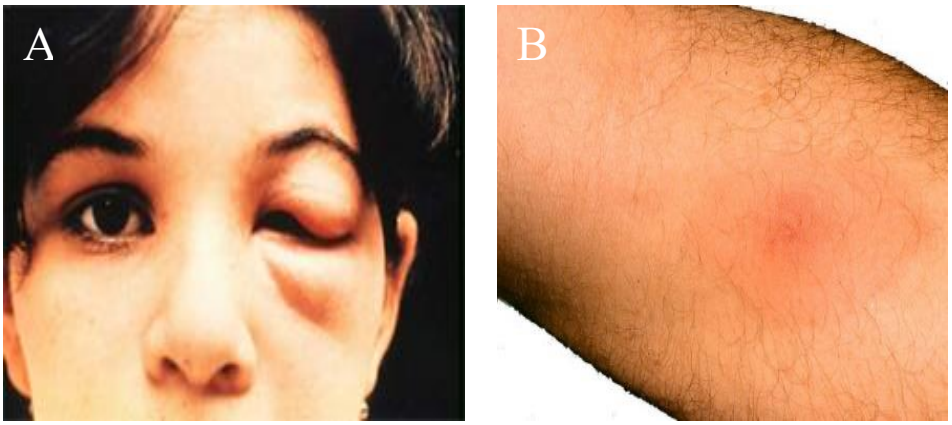

Figure 6. Typical specific symptoms of acute Chagas disease, swelling at the inoculation site A. Romaña's sign; B: Chagoma, taken from Rossi et al. 2003 (35).

If left untreated infection is life-long and the disease progresses to the chronic phase, which persists decades after the initial infection. However, most of those infected will remain asymptomatic, known as the indeterminate phase. Interestingly, Berenice, the young girl in which the parasites were first identified, never developed chronic symptoms and died at 73 from an unrelated issue (36). Roughly 30% of infected individuals develop symptomatic disease, which includes potentially fatal cardiomyopathy and in a smaller proportion mega-syndromes of the oesophagus and colon (Figure 7). The cause of these changes is still not fully understood, some hypothesise it is caused by an autoimmune response, based on antigenic mimicry in the form of an antibody targeting *T. cruzi* polypeptides, whilst more recently others suggest that it could be due to a persistence of parasites within the tissues (2). Experimentally infected mouse models using bioluminescent imaging have shown the gastro-intestinal tract is parasitic reservoir during chronic infections being the only site where parasites were always observed (37) and digestive chagas disease is likely due to a chronic inflammatory response associated with parasite persistence in the colon (38). In either case, there is no method of determining the prognosis for the development of chagasic symptoms.

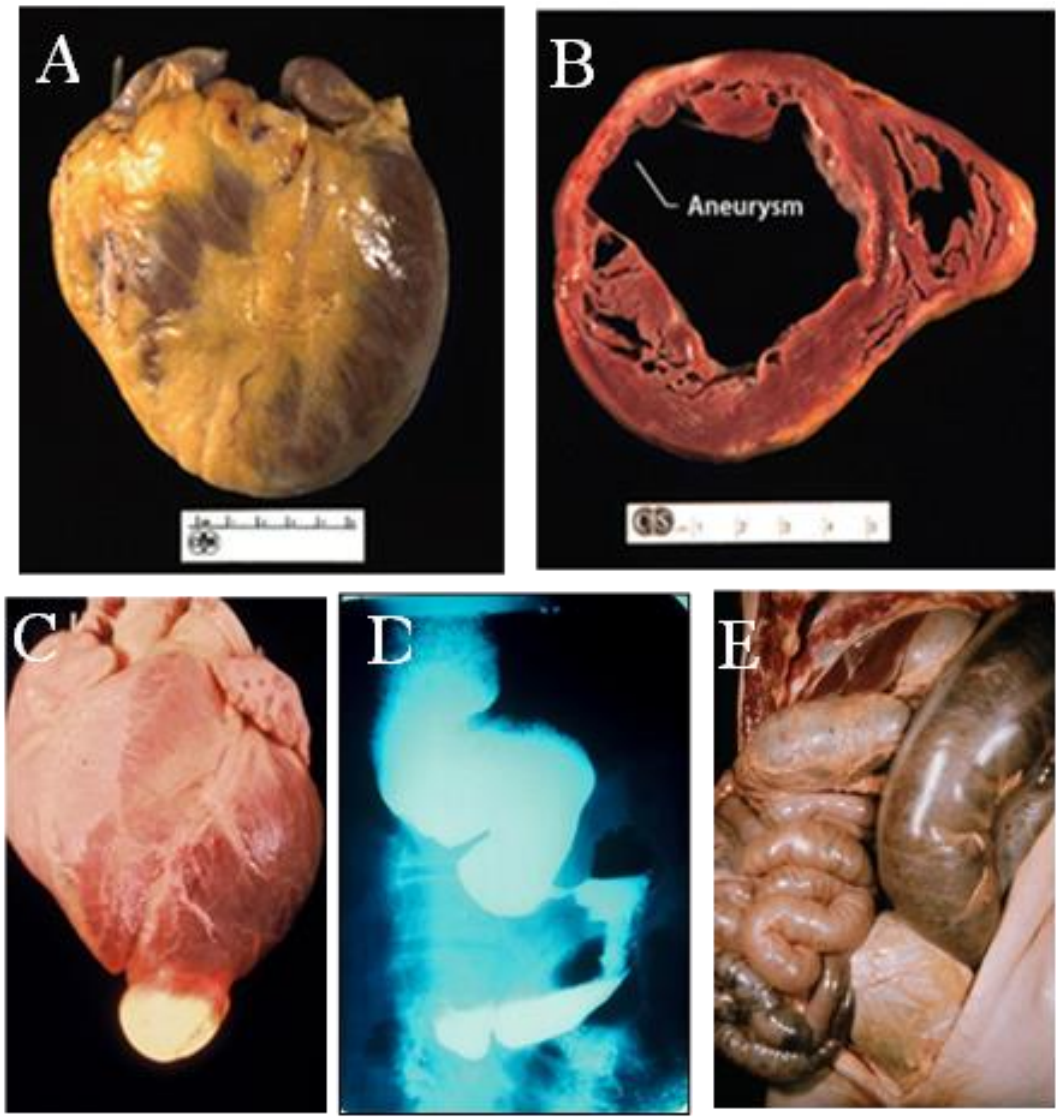


Figure 7. Chagas disease pathology. A: Cardiomegaly, multi-chamber dilation and epicardial scarring. B: Cross section of heart through ventricles showing aneurysm. (A & B taken from Bonney et al. 2019 (10)). C: Apical aneurysm. D: Megaoesophagus E: Megacolon (C, D and E photos provided by Professor Michael Miles).

## 1.6 Treatment

There are only two drugs available for the treatment of Chagas disease, nifurtimox and benznidazole (Figure 8), since the mid-1960s and early 1970s. Historically, treatment courses have been long (60- 90 days), with high dosages (5mg/kg/day benznidazole; 10mg/kg/day nifurtimox) (39), which have been attributed to causing severe adverse effects, such as dermatitis, anorexia, vomiting and nausea, resulting in a reported up to 20 % of patients, not completing treatment (40). Both drugs are tolerated better in younger age groups, with more adverse effects reported in the elderly (41) and in children, nifurtimox is better tolerated than benznidazole (42). Recently, shorter treatment courses and intermittent administration have been trialled and found to be effective with less severe adverse effects and a lower number of patients not completing treatment (43).

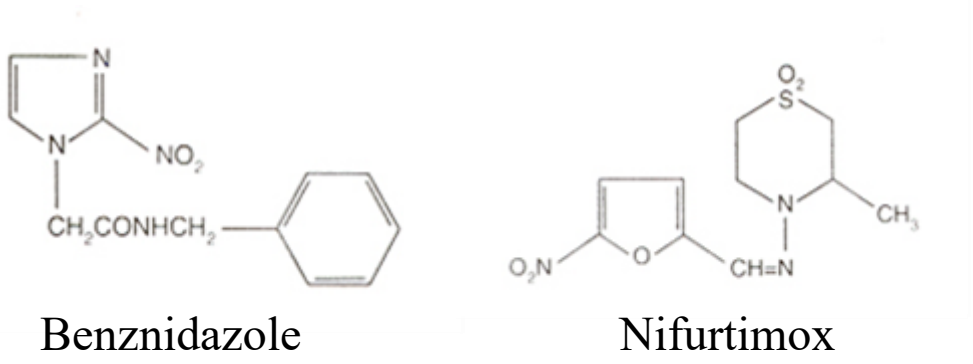


Figure 8. Chemical structure of Chagas disease drugs, Benznidazole and Nirfurtimox.

Although effective in the acute phase and early chronic phase, their effectiveness is less clear in the chronic phase due to the difficulty of diagnosis and there not being a sensitive enough test of cure, leaving patients without confirmation of treatment outcome. Additionally, there have been disparities in drug efficacy depending on the *T. cruzi* strain (44). This uncertainty can lead to issues with patient management, individuals may be less willing to start a treatment course with potential adverse effects, without the certainty of an improved prognosis (45, 46). Furthermore, with the increased migration of patients, long-term follow-up is difficult and impractical. Therefore, a sensitive test to indicate a cure after treatment is desperately needed.

## 1.7 Diagnosis

Different diagnostic methods are available for the diagnosis of *T. cruzi* infection, and these vary depending on the stage of the disease. During the acute stage, parasites can be found

circulating in the blood and at high numbers, therefore, parasitological methods can be used, including blood microscopy of direct wet blood films or thin/thick film smears stained with Giemsa, but with little sensitivity. If suspected acute infections have negative blood smears, concentration tests such as microhematocrit centrifugation and microscopy of the buffy coat layer or Strout's centrifugation and microscopy of serum are recommended, these methods have a sensitivity of 80-90 % in the acute phase.

In the chronic phase circulating blood parasites are rare, therefore other detection methods are employed:

Indirect parasitological methods, to identify live parasites by haemocultures or xenodiagnoses are possible but they are much slower, labour-intensive, require specific laboratory conditions, and have insufficient sensitivity for low levels of parasitaemia (34).

Molecular methods amplify parasitic DNA in the bloodstream, standard techniques are PCR or qPCR which typically amplify repetitive sequences of satellite DNA (SatDNA) or the minicircle of the kinetoplast (kDNA). LAMP is an alternative molecular method used, especially in congenital cases and is designed around the isothermal amplification of 18S rRNA (29). Although molecular methods are more sensitive than parasitological methods, all three have low sensitivity (20- 50 %). They can be useful in the chronic phase to detect therapeutic failure and reactivation in immunosuppressed individuals, but a negative test should not be relied on as an indicator or cure (47).

Serological methods identifying IgG antibodies are the gold standard for diagnosis during the chronic phase due to early seroconversion after initial infection (approx. 90 days) (48). However, no single test is considered the reference standard. Common commercial tests used are indirect immunofluorescence (IIF), indirect haemagglutination assays (IHA), enzyme-linked immunosorbent assays (ELISA) or commercially available rapid diagnostic tests (RDTs). These assays either incorporate whole-cell parasites and/or a mix of parasite antigens (conventional tests) or use recombinant antigens and synthetic peptides (recombinant or non-conventional tests). as detection molecules. These tests are time-consuming, require technically trained staff, specialised equipment, and vary in sensitivity and specificity. To make a definitive diagnosis the World Health Organisation recommends the use of two tests, using different detection methods and/or detecting different antibodies and potentially a third test if the results are conflicting (34).

## 1.8 Disparities in commercially available diagnosis tests

The sensitivity of tests varies greatly, and discordant results have been demonstrated with assays performed on patients from the same locality on chagasic patients from Ecuador (49), and Brazil (50).

Furthermore, the sensitivity of assays varies according to broader sample origin, Whitman et al (2019) looked into the performance of four FDA-cleared assays, three ELISAs (Hemagen, Ortho and Weiner) and one RDT (InBios), on blood donor samples in the US, originally from Latin America, tests varied in sensitivity between 99.3 – 87.5 %, and reactivity was highest with patient samples from the Southern Cone countries, followed by central America and lowest reactivity seen in samples from Mexico (51). The group later evaluated the performance of three latest generation assays, Weiner Lisado and Wiener v4.0 ELISAs and Abbott PRISM Chagas chemiluminescent assay (ChLiA), against the FDA-approved tests, they found increased sensitivity, but it still ranged depending on the test employed (98.9 % - 95.5 %) and similarly to what was found above, reactivity was highest in patients from South America and lowest in Mexico (52). Truyens et al (2021) assessed two rapid diagnostic tests (T detect and Stat-Pak) and one ELISA (Weiner) across a cohort of *T. cruzi* infected women from Argentina, Honduras and Mexico. Similarly, they found the reactivity to vary between tests and localities with the highest reactivity in Argentina and the lowest in Mexico. They found the difference was due to differences in antibody titres to *T, cruzi* antigens rather than parasitaemia, which can be influenced by geographical differences in *T. cruzi* strain and by population genetics (53).

## 1.9 Monitoring efficacy of chemotherapy

The efficacy of treatment for Chagas disease is difficult to determine and follow-up methods differ depending on the stage of the disease (acute or chronic). There are two main determinates of Chagas disease, the presence of parasites and anti-*T. cruzi* IgG antibodies. The humoral response to antigenic stimulation, if treatment is successful the parasite will clear, and specific IgG production will, decrease (to seronegativisation). Therefore, the criteria for cure are negative serological tests. However, depending on which stage the patient is in when being treated seroconversion of anti-*T. cruzi* IgG to negative on conventional serological tests can take decades, and a steady decline of antibody titre is generally accepted to be suggestive of cure (48). Monitoring of antibody titre should be performed every 6 – 12 months, and ideally

compared to a sample taken before treatment. A decline of titre should be witnessed within 6 – 12 months in the acute phase, within the 5 – 10 years in the chronic phase (54). In comparison, seronegativisation can take 5 years in the acute phase and up to 20 years in the chronic phase (48). The usefulness of monitoring antibody titres as indicators of disease progression as well as treatment outcome has also been suggested (55-57).

Parasitological (haemoculture, or direct microscopy of the blood or buffy coat) and molecular methods (PCR) may be used for the acute phase, early congenital or reactivated *T. cruzi* infection, as they have relatively high sensitivity for circulating parasites (58). During the chronic phase these tests should only be used to determine therapeutic failure, i.e., the result is positive. The estimated percentage of cure is also disease stage dependent, congenitally infected patients have the highest estimated cure rate between 90 % - 100 %, acute patients 60 – 85 % and chronic 8 – 60 % (48, 58, 59)

## 1.10 The humoral response to *T. cruzi* infection and isotype switching.

The immune response to *T. cruzi* infection consists of both an innate and acquired response. After initial infection, parasites circulating the blood are targeted by macrophages, dendritic cells and natural killer cells, causing the production of chemokines and cytokines (60). This is response can control parasite replication, and usually leads to host survival. However, it is not enough to clear the parasite completely, resulting in chronic infection (61). The adaptive immune response and the activation of B cell triggers a large and continuous humoral response. The antibodies produced by the humoral response are split into diagnostic and lytic antibodies. Diagnostic antibodies are used in serological tests, whilst lytic antibodies which cause parasite lysis *in vitro* lead to parasite clearance when injected alongside trypomastigotes in naive mice (62). The critical role of the humoral response has been shown in experimental studies, mice unable to produce a humoral response are unable to control parasite replication and die in the acute stage of the infection (63).

In human serum there are four main immunoglobulins, IgG, IgM, IgE and IgA. Typically, IgM produced by the mature stage of the differentiating B cell, is found in abundance during the acute stage of infection, as early as two weeks post infection in experimentally infected rhesus monkeys (64). In human infections, IgM antibodies have been detected at 17 days post-infection and peak between 17-34 days post infection (65). For serological diagnosis IgM is the best marker to identify an acute infection. Although IgG is also produced during this time,

it is at a lower level. As the disease develops, there is an isotype switch caused by the Th1 system and the production of interferon-gamma, to the production of IgG antibodies, the level of IgM depletes until it is no longer detectable, whilst the production of IgG increases and is maintained throughout the rest of infection (64). The early, intermediate, and late acute stages of Chagas disease can be tracked using the different levels of IgM, IgG and IgG (Gal) (66).

IgG is the most common immunoglobulin in human serum and is split into four subclasses IgG1, IgG2, IgG3 and IgG4. The most dominant IgG subclasses produced during *T. cruzi* infection are IgG1 and IgG2, IgG3 is produced at relatively lower levels. All three of these are associated with Th1 response, whilst IgG4 is associated with a Th2 response (67). The role of IgE in *T. cruzi* infection is not well studied and there is limited information, in other infections (i.e. helminths) IgE is associated with the Th2 response. Those with indeterminate Chagas disease have sustained production of IgE, whilst those with cardiac cardiomyopathy do not (68). Increased levels IgA have been linked to the digestive form of Chagas disease (69). However, differences in immune responses have been documented, populations genetics and the infecting lineage of *T. cruzi* can cause differences in immune responses, may cause the different clinical presentations witnessed (70).

## 1.11 *Trypanosoma cruzi* genetic diversity

*T. cruzi* is highly genetically diverse, currently thought to be split into six distinct lineages (TcI – VI) or discrete typing units (DTUs), with a possible seventh, TcBat. The identification and characterisation of these subgroups within *T. cruzi* have long been discussed, morphological differences are seen in the drawings by Carlos Chagas in 1909, with the earliest attempts of classification being based on multilocus enzyme electrophoresis (MLEE), which identified three distinct groups or zymodemes I, II, III (71, 72). However, due to the lack of standardized molecular typing methods and the use of multiple techniques, these subgroups have been categorised under many different nomenclatures, biodemes, clonets, clades and riboclades (14). To form a consensus, a meeting was held in 1999, where it was decided, based on biological and biochemical features (biodemes and zymodemes) and molecular techniques (random amplification of polymorphic DNA and nuclear loci), that *T. cruzi* should be classified into two groups, named *T. cruzi* I and *T. cruzi* II (73). Following this meeting and advances in the field, multilocus genotyping revealed six distinct lineages, and *T. cruzi* II was further split into five DTUs IIa-e (74, 75), which were later renamed TcI – TcVI (73), intra-lineage genetic diversity has also been identified. Several models for the evolution of these lineages have been proposed,

all approaches agree that TcI and TcII are ancestral lineages that evolved separately, with TcV and TcVI being more recent hybrids of TcII and TcIII, it is unclear how TcIII and TcIV evolved. Experimental crosses have shown that *T. cruzi* is capable of hybridisation via a parasexual mechanism (76-78). Each lineage is associated with ecoepidemiology features and transmission cycles, and it has long been proposed that the differing lineages may contribute to the varying clinical forms of Chagas disease in South America (79).

## 1.12 Lineage-specific epidemiology

Based on genotyping, TcI is the most widely distributed lineage, in both sylvatic and domestic cycles, ranging from North to South America, originally thought to be benign it is now recognised as the most common cause of Chagas disease in countries north of the Amazon basin (80), with the principal hosts being *Didelphis* species of opossum. Chagasic cardiomyopathy is common in TcI-infected areas, whilst megasyndromes are rarely seen (81).

TcII is the main cause of Chagas disease in the Southern Cone countries of Latin America, where megasyndromes of the oesophagus and colon are common. It is most common in domestic cycles, with *Triatoma* species being the principal vector, it is rarely recorded in sylvatic cycles. One exception to this is in transmission cycles in the Atlantic Coastal Rainforest of Brazil where TcII infection is common in golden lion tamarinds with high parasitaemia (82). However, sylvatic reservoir hosts of these strains are not fully elucidated (83), and in contrast to TcI *Didelphis* species are not the principal hosts, in fact, Maria Deane et al., showed that some *Didelphis* can control and eliminate infection when inoculated with a TcII strain (84).

TcIII is found throughout South America (North-western Venezuela to Argentina), with armadillos being the natural hosts (85). It is relatively rare in domestic transmission cycles and seldom causes Chagas disease. Nevertheless, it poses a risk as it has been identified infecting domestic dogs (86), and so has the potential of being a disease agent if transmission cycles evolve (81).

TcIV is the secondary cause of Chagas disease in Venezuela after TcI, and has been linked to oral outbreaks in Canudos, Belém (87). It circulates in the same area as TcI in wild primates, *Monodelphis* (opossum) and *Dasypus* (armadillo) species in the Amazon, and racoons and dogs in North America (81).

Like TcII, TcV and TcVI are mainly restricted to domestic transmission cycles south of the Amazon basin (87), with *T. infestans* being the principal vector. They are the main cause of Chagas disease in the greater Gran Chaco region and neighbouring countries Bolivia, Chile, northern Argentina, Paraguay and parts of Southern Brazil, where Chagas disease presents as both cardiomyopathy and megasyndromes (Figure 9).

TcBat is isolated from bat species across Panama, Brazil and Columbia, it is most closely related to TcI (88). It is potentially infectious to humans, although identification of TcBat is rare it has been identified in DNA from mummies and in one 5-year girl from Colombia (89).

However, the full epidemiology of each of the lineages is unknown, due to the difficulty of isolating the parasite, even genotyping may not give the full infection or ecological history because of possible lineage-dependent biased selection in culture or sequestration in host tissues, especially in the chronic phase.

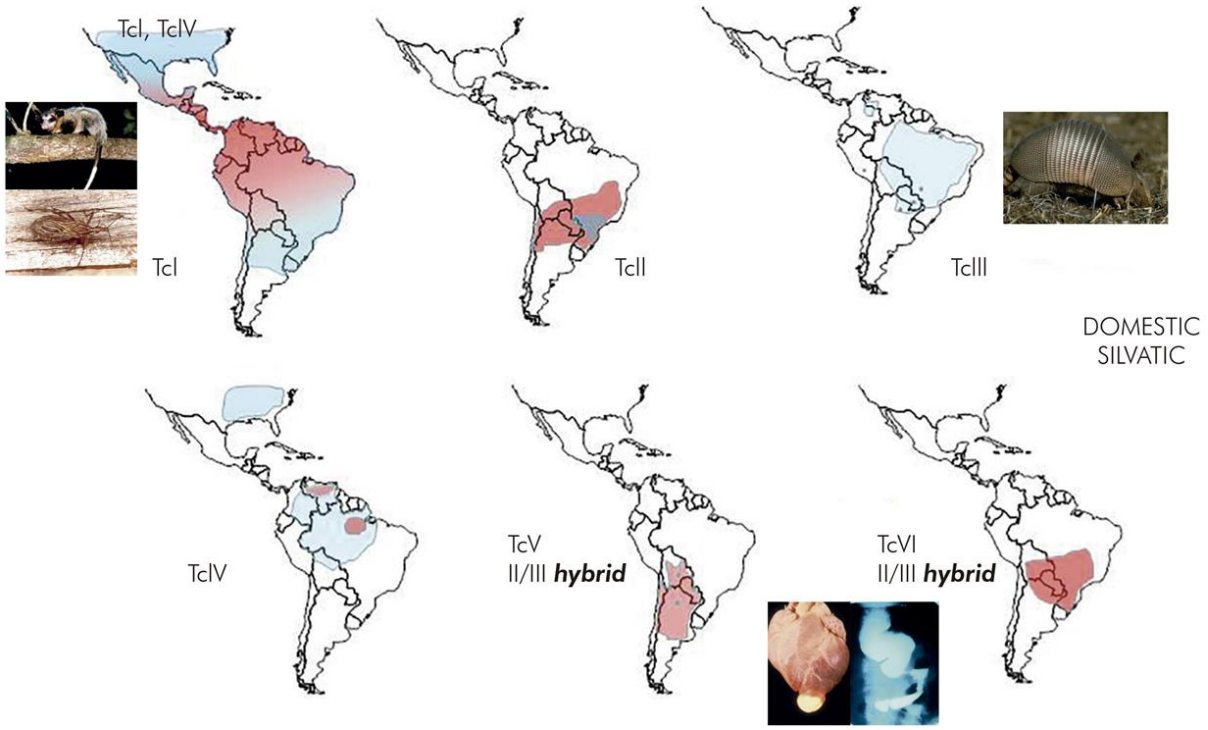


Figure 9. Approximate geographical distribution of *T. cruzi* lineages in domestic and sylvatic transmission cycles based on genotyping, taken from Miles et al. 2009 (81).

## 1.13 The trypomastigote small surface antigen and lineage-specific serology

A key research priority is to investigate an individual's infecting lineage history, to further knowledge and understanding of this complicated parasite, to elucidate transmission cycles and understand the pathology of the disease. To develop a test able to distinguish between the lineages serologically a sufficiently polymorphic antigen is required. The trypomastigote small surface antigen (TSSA) is so far the only known immunological marker able to distinguish between the lineages. TSSA is part of the *T. cruzi* mucin family, a major family of surface glycoproteins widely distributed over the cell surface, flagellum and flagellar pocket. They play a key role in parasite protection, infectivity, and modulation of the host immune response throughout the *T. cruzi* life cycle. *T. cruzi* mucins have been split into two distinct families TcMUC and TcSMUC, TcMUC has been subdivided into three groups TcMUC I-III. TcMUC I proteins are distributed on the surface of the amastigote form, whilst TcMUC II proteins are predominant in membrane lipid rafts of the trypomastigote blood form. TcMUC III consists of TSSA only, it is a glycoprotein displayed on the surface of *T. cruzi* during the mammalian stages. Di Noia et al (2002), identified TSSA as an immunological marker for *T. cruzi*, able to

distinguish between *T. cruzi* I (TcI) and *T. cruzi* II (subsequently classified into TcII - TcVI) due to a dimorphism (90). However, it has since been discovered that TSSA has greater inter-lineage diversity (91). The TSSA-II epitope is now known to be common to TcII, TcV and TcVI; the latter hybrid lineages have an additional distinct sequence that differs from TcII by one amino acid change. TSSA- I was found to be more closely related to TSSA-III and TSSA-IV, each of which has its own unique epitope which varies by two amino acid changes (Table 1) (91). Most recently, Romer et al (2023) have reported greater TSSA sequence diversity with the identification of a total of nine novel TSSA variants in TSSA-II, TSSA-III, TSSA-IV and TSSA-I (92).

**Table 1**. TSSA epitope lineage-specific amino acid sequences, later incorporated into synthetic peptides; the polymorphic residues are underlined.

| **Peptide** | **Amino Acid sequence** |
|---|---|
| TSSApep-I | GTDKKTAAGGTPSPSG |
| TSSApep-II/V/VI | GTENKPATGEAPSQPG |
| TSSApep-III | GTEKKAAAGEAPSPSG |
| TSSApep-IV | GTDKKTAAGEAPSPSG |
| TSSApep-V/VI | GTENKPAAGEAPSQPG |

Through Blast analysis these differing forms of TSSA were found to be conserved within the specific lineages and not identified in any other organism; this and the fact that these polymorphic residues are located in close proximity in the protein sequence make TSSA an exciting prospective antigen for lineage-specific serology (93). Table 2 lists milestones of TSSA serology.

**Table 2**. A history of the trypomastigote small surface antigen: Discovery, development and application.

| Author | Year | Reports. |
|---|---|---|
| Di Noia et al (90) | 2002 | Discovery of TSSA as the first immunological marker able to discriminate between lineages. Dimorphism able to distinguish between *T. cruzi* I and *T. cruzi* II (subsequently re-classified into TcII-VI) |
| Bhattacharyya et al (91) | 2010 | Greater intraspecific diversity identified lineage specific epitopes TSSA-I. TSSA-II/V/VI, TSSA-III, TSSA-IV, and TSSA-V/VI. |
| Burgos et al (94) | 2010 | Identification of infecting lineage through recombinant TSSA-I and TSSA-II western blot in chronic heart disease patients. |
| Risso et al (95) | 2011 | Western Blot with recombinant TSSA-I and TSSA-II showed good concordance with genotyping. Recognition of TSSA-II by sera from Argentina, Paraguay, Colombia, Venezuela, and Mexico. |
| Cimino et al (96) | 2011 | Evaluation of recombinant TSSA-II ELISA in humans and first report in natural mammal infection (dogs). |
| Bisio et al (97) | 2011 | Predominance of TSSA-II/V/VI in chronically infected pregnant women residing in Buenos Aires. |
| Canepa et al (98) | 2012 | Functional role of TSSA-II/V/VI in *T. cruzi* adhesion and invasion of mammalian cells. No function identified for TSSA-I. |
| Bhattacharyya et al (93) | 2014 | Development of lineage-specific TSSA peptide ELISA confirms prevalence of TSSA-II/V/VI in Southern Come countries, and unexpectedly in Ecuador, where these lineages had not commonly been reported. |
| Floridia-Yapur et al (99) | 2014 | Recognition of a fusion protein of SAPA/TSSA-VI by dogs from the Argentine Chaco. |
| Balouz et al (100) | 2015 | Identification of antigenic part of the B-cell epitope located in the central portion of TSSA sequence. |
| Kerr et al (101) | 2016 | First application of TSSA lineage-specific ELISA on sylvatic infection identified Brazilian Atlantic Forest lion tamarins as reservoir hosts of TcII. |

| | | |
|---|---|---|
| Camara et al (102) | 2017 | TSSA-II plays an important role in the infectivity and differentiation of *T. cruzi* trypomastigotes. |
| Balouz et al (103) | 2017 | Anti-TSSA-II/V/VI antibody titres decreased significantly faster compared to anti-whole parasite titres in paediatric patients undergoing effective treatment. |
| Bhattacharyya et al (104) | 2018 | Chagasic cardiac severity associated with reactivity to a novel Chagas Sero K-SeT RDT incorporating TSSApep-II/V/VI |
| Mc Clean et al (105) | 2020 | Chagas Sero K-SeT RDT identified TcII/V/VI infection in multiple mammalian orders from Brazil |
| Sanchez et al (106) | 2022 | TSSApep-ELISA identified 65.3% of Chagas-infected mothers in Bolivian cohort had TSSApep-II/V/VI recognition. |

## 1.14 *T. cruzi* genomes in the public domain

The *T. cruzi* genome is usually described as diploid and be an estimated length of 80 to 150 Mb and has somewhere between 19 - 46 chromosomes (107). However, this varies depending on the strain, with TcI isolates having on average smaller genomes than TcII, TcV and TcIV (108). In 2005, the first genome sequence of *T. cruzi* was obtained, CL Brener (TcVI), using whole genome shot-gun (WSG) technique, with an estimated genome size of between 106.4 and 110.7 Mb (109), as a hybrid strain TcVI, the CL Brener genome is comprised of two diverging haplotypes Esmeraldo-like (TcII) and Non-Esmeraldo-like (109). The second genome sequenced was Sylvio X10/1 (TcI) in 2011, this was found to be smaller than the CL Brener genotype (44 Mbp) and had greater similarity in coding regions with non-Esmeraldo-like TcIII (98.2 %) than Esmeraldo-like TcII (97.5 %) (110). Bug2148 and 231 are the first published sequenced genome of lineage TcIV and TcIII, respectively (107, 111). To date, there are available public genomes of lineages TcI, TcII, TcIII, TcV and TcVI (Table 3).

**Table 3.** Publicly available published *T. cruzi* genomes and assembly statistics.

| Strain | Lineage | Size Mb | BUSCO** | | | | | | | | | | Accession number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Complete | Single | Duplicated | Fragmented | Missing | N markers | Scaffold N50 | Contigs N50 | Percent Gaps | No. of Scaffolds | |
| Sylvio X10/1 | I | 38.6 | 99.2 | 99.2 | 0 | 0.8 | 0 | 130 | 2307 | 2307 | 0.00% | 27019 | PRJNA40815 |
| G | 1 | 25.17 | 97.7 | 97.7 | 0 | 2.3 | 0 | 130 | 74655 | 6701 | 5.25% | 1450 | PRJNA315397 |
| Dm28c | 1 | 53.3 | 93.9 | 86.2 | 7.7 | 6.2 | 0.1 | 130 | 317638 | 317638 | 0.00% | 636 | PRJNA433042 |
| Brazil | I | 45.56 | 99.2 | 99.2 | 0 | 0.8 | 0 | 130 | 914771 | 191353 | 0.07% | 402 | PRJNA512864 |
| Colombiana | 1 | 30.9 | 94.6 | 94.6 | 0 | 3.1 | 2.3 | 130 | 5189 | 4903 | 0.05% | 9338 | PRJNA274443 |
| JR cl. 4 | 1 | 41.48 | 96.9 | 96.9 | 0 | 3.1 | 0 | 130 | 83591 | 7407 | 3.30% | 15312 | PRJNA59941 |
| B7 | 1 | 34.23 | 100 | 100 | 0 | 0 | 0 | 130 | 2846 | 2846 | 0.00% | 23154 | PRJNA77843 |
| H1 | I | 27.3 | 99.2 | 99.2 | 0 | 0.8 | 0 | 130 | 19104 | 16437 | 1.67% | 10811 | PRJNA880352 |
| DM28 | 1 | 17.23 | 112 | 112 | 0 | 17 | 1 | 130 | 3000 | 3000 | 0.00 % | 6541 | PRJNA661283 |
| B.M Lopez | I | 18.51 | 94.6 | 94.6 | 0 | 5.4 | 0 | 130 | 5125 | 5125 | 0.00% | 5923 | PRJNA595079 |
| DM25 | 1 | 38.6 | 99.2 | 99.2 | 0 | 0.8 | 0 | 130 | 1228545 | 1228545 | 0.00% | 35 | PRJNA1039287 |
| STIB980 | 1 | 27.9 | 93.8 | 93.8 | 0 | 4.6 | 1.6 | 130 | 158042 | 158042 | 0.00% | 492 | PRJNA1009388 |
| Esmeraldo-like (cl3) | II | 38.08 | 95.4 | 95.4 | 0 | 3.8 | 0.8 | 130 | 66229 | 5353 | 8.18% | 15803 | PRJNA50493 |
| Y clone C6 | II | 47.22 | 98.5 | 97.7 | 0.8 | 0.8 | 0.7 | 130 | 889019 | 396944 | 0.05% | 266 | PRJNA554625 |
| Y | II | 126 | 99.2 | 99.2 | 0 | 0.8 | 0 | 130 | 11962 | 11962 | 0.00% | 9821 | PRJNA393091 |
| Ycl4 | II | 26.15 | 90 | 90 | 0 | 10 | 0 | 130 | 10716 | 2057 | 5.16% | 6664 | PRJNA421475 |
| Ycl6 | II | 25.78 | 90.8 | 90.8 | 0 | 6.2 | 3 | 130 | 10394 | 2049 | 5.17% | 6967 | PRJNA421475 |
| Ycl2 | II | 25.91 | 92.3 | 92.3 | 0 | 6.9 | 0.8 | 130 | 10600 | 2031 | 5.14% | 6884 | PRJNA421475 |
| S15 | II | 27.51 | 95.4 | 95.4 | 0 | 2.3 | 2.3 | 130 | 5722 | 1724 | 9.49% | 6946 | PRJNA421475 |
| S92A | II | 27.08 | 93.1 | 93.1 | 0 | 6.9 | 0 | 130 | 20493 | 1914 | 5.51% | 7134 | PRJNA421475 |
| S23b | II | 28.13 | 96.2 | 96.2 | 0 | 3.1 | 0.7 | 130 | 20992 | 1869 | 8.09% | 7145 | PRJNA421475 |
| S162 | II | 27.30 | 92.3 | 91.5 | 0.8 | 7.7 | 0 | 130 | 12390 | 1846 | 7.88% | 8588 | PRJNA421475 |
| S11 | II | 28.48 | 90.8 | 90.8 | 0 | 8.5 | 0.7 | 130 | 18630 | 1748 | 8.30% | 7855 | PRJNA421475 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S44A | II | 17.19 | 69.3 | 68.5 | 0.8 | 3.8 | 26.9 | 130 | 17818 | 2164 | 8.42% | 4971 | PRJNA421475 |
| S154a | II | 19.27 | 96.9 | 96.9 | 0 | 2.3 | 0.8 | 130 | 17779 | 2002 | 5.88% | 9197 | PRJNA421475 |
| Berenice | II | 40.8 | 98.5 | 98.5 | 0 | 0.8 | 0.7 | 130 | 156193 | 148957 | 0.03% | 923 | PRJNA498808 |
| Ikiakarora | III | 18.47 | 81.5 | 77.7 | 3.8 | 15.4 | 3.1 | 130 | 2193 | 2193 | 0.00% | 11096 | PRJNA595095 |
| Non-Esnbmeraldo-like | III | 32.53 | 77.7 | 76.9 | 0.8 | 3.8 | 18.5 | 130 | 870934 | 33375 | 14.69% | 41 | PRJNA11755 |
| 231 | III | 35.36 | 97.7 | 97.7 | 0 | 2.3 | 0 | 130 | 14202 | 5300 | 4.33% | 8469 | PRJEB9129 |
| Bug2148 | V | 55.16 | 96.9 | 91.5 | 5.4 | 3.1 | 0 | 130 | 200364 | 200364 | 0.00% | 929 | PRJNA393091 |
| SC43cl1 | V | 79.9 | 99.3 | 23.1 | 76.2 | 0.8 | 0.1 | 130 | 280948 | 238666 | 0.04% | 1235 | PRJNA633093 |
| SOL | V | 20.61 | 79.3 | 76.2 | 3.1 | 16.2 | 4.5 | 130 | 2171 | 2171 | 0.00% | 11944 | |
| Aerquipa | V | 19.05 | 52.3 | 52.3 | 0 | 23.1 | 24.6 | 130 | 1932 | 1912 | 0.09% | 10224 | PRJNA274442 |
| CL Brener * | VI | 89.94 | 99.2 | 61.5 | 37.7 | 0.8 | 0 | 130 | 88624 | 14669 | 0.36% | 29495 | PRJNA11755 |
| TCC | VI | 87.1 | 99.3 | 23.1 | 76.2 | 0.8 | 0.1 | 130 | 280948 | 238666 | 0.04% | 1235 | PRJNA432753 |
| CL | VI | 26.77 | 86.1 | 56.9 | 29.2 | 7.7 | 6.2 | 130 | 57182 | 3818 | 21.78% | 7764 | PRJNA315397 |
| Tula cl2 | VI | 83.51 | 85.4 | 47.7 | 37.7 | 11.5 | 3.1 | 130 | 7772 | 2193 | 11.38% | 45711 | PRJNA169675 |
| Tulahuen | VI | 48.5 | 88.5 | 86.2 | 2.3 | 8.5 | 3 | 130 | 872484 | 872484 | 0.00% | 75 | PRJNA1011856 |
| M05 | | 51 | 92.3 | 90.8 | 1.5 | 7.7 | 0 | 130 | 110314 | 110314 | 0.00% | 1029 | PRJNA812518 |

* Reference genome on NCIB database.

** BUSCO assessing completeness of genome against euglenozoan, methods Appendix 1.

## 1.15 Aims and objectives.

As detailed above *T. cruzi* is a highly genetically diverse parasite, lineages vary across locations, present different clinical manifestations and interact with different hosts and transmission cycles. However, the full epidemiology of the parasite is unknown due to the difficulty in isolating the parasite during the chronic stage, and varying sensitives of commercially available serological assays, that do not distinguish between the infecting lineages, meaning the full infection history is unknown.

Overall, the aim of my PhD project is to identify and validate *T. cruzi* lineage-specific antigens and adapt these to RDTs to track transmission pathways of *T. cruzi* lineages, and to investigate potential to design RDTs that indicate clinical prognosis and treatment outcome

To improve the lineage-specific serology of *T. cruzi* my specific research objectives are:

1) Establish the *L. tarentolae* expression system at LSHTM, to apply it to produce lineage-specific recombinant proteins, and to validate its efficacy for recognition by chagasic sera.
2) Utilise lineage-specific serological assays (ELISA and RDT), to clarify transmission pathways and mammalian hosts in the Chaco region of Northern Argentina.
3) Evaluate the antigenicity of recombinant gTSSA-I produced in a *L. tarentolae* expression system
4) Assess IgG and IgG1 as potential biomarkers for the development of a test to monitor the outcome of chemotherapy.

# CHAPTER 1 PRODUCTION OF RECOMBINANT LINEAGE-SPECIFIC *T. CRUZI* GLYCOPROTEINS THROUGH THE *LEISHMANIA TARENTOLAE* EXPRESSION SYSTEM.

## ABSTRACT

The development and deployment of rapid diagnostic tests able to distinguish between the *Trypanosoma cruzi* lineages is a key research priority. *T. cruzi* is split into at least six distinct lineages Tc I - Tc VI, with disparate geographic locations, transmission cycles, and clinical manifestations. However, the full epidemiology is not known, due to the intracellular nature of the parasites. The Trypomastigote Small Surface Antigen (TSSA) is the only known serological marker able to distinguish between the lineages, and assays incorporating synthetic linear peptides of these antigens have been able to, identify infecting lineage, clarify reservoir hosts, and associate lineage with disease severity. However, they lower than 100 % sensitivity or specificity, and the synthetic linear antigen specific to Tc I shows negligible recognition by Chagasic sera. An alternative to synthetic linear peptides is the production of recombinant proteins, expressed with post-translational modifications. The *Leishmania tarentolae* system, has been used to produce highly antigenic recombinant proteins, and I have previously shown how a glycosylated TSSA recombinant protein specific to Tc I has increased recognition by chagasic sera, compared to its linear counterpart. Here, I demonstrate how the *L. tarentolae* expression system can be utilized to produce recombinant TSSA proteins specific to *T. cruzi* lineages II/V/VI, -III, IV, and V/VI, and that antigens are recognised by chagasic sera.

## INTRODUCTION

Chagas disease (American Trypanosomiasis), caused by the protozoan parasite *Trypanosoma cruzi*, is endemic to the 21 continental countries of Latin America. Transmission is primarily via the parasite vector hematophagous triatomine bugs, an estimated 70 million people live in these vectoral transmission areas and are therefore at risk of contracting the disease [1, 2]. Other transmission routes, include oral, congenital and through organ and blood transfusions. Worldwide there are an estimated 6 - 7 million people infected and it is a growing health concern as migration to non-endemic areas increases [3]. The disease presents in two stages, the acute and chronic. The acute stage occurs after initial infection, when parasites can be found circulating the blood stream, it is usually asymptomatic but in rare cases can be fatal. If

untreated, infection is lifelong, most of those infected will remain asymptomatic (chronic intermediate), but an estimated 30 % will develop symptomatic chronic Chagas disease [4]. Treatment options are limited to benznidazole and nifurtimox, both have long treatment courses, can cause severe adverse reactions and their effectiveness during the chronic stage remains unclear [5].

The intracellular nature of the parasite during the chronic phase limits diagnosis by parasitological or molecular techniques and may not divulge the true infection history. Therefore, diagnosis is highly reliant on serological methods to detect anti-*T. cruzi* IgG. However, commercially available tests may be time consuming, require trained laboratory staff, are expensive and vary in sensitivity and specificity. Therefore, the World Health Organisation (WHO) recommends the use of two serological assays incorporating different antigens/ recombinant proteins for IgG detection, and a third if results are discordant [2].

The disparity witnessed by these serological tests may be due to the genetic diversity of the parasite. Currently, *T. cruzi* is split into 6 distinct lineages Tc I – Tc VI with a possible 7$^{th}$, Tc Bat [6, 7]. It has long been proposed that the different geographical distribution of these lineages may contribute to the difference in clinical presentations seen throughout Latin America [8]. The only known serological marker able to distinguish between the lineages is the Trypomastigote Small Surface Antigen (TSSA), originally thought to be dimorphic, it has since found to have greater inter-lineage diversity [9]. Tc I, Tc III and Tc IV have distinct epitopes, Tc II/V/VI share a common epitope and TcV/VI share a second, differing by two amino acid change [9]. TSSApep-II/V/VI and TSSApep-V/VI ELISA have identified infecting *T. cruzi* lineage in humans [10-12] and animals [13] and TSSApep-II/V/VI incorporated onto an RDT (Chagas Sero K-SeT) revealed associations between infecting lineage and disease severity in a Bolivian cohort [14]. However, sensitivity and specificity are lower than 100 %, TSSApep-III and - IV have seldom been recognised, and TSSApep-I (as a synthetic peptide) has generally not been recognised by Chagasic sera. Therefore, there is a great need to develop better and more sensitive lineage-specific antigens.

An alternative approach to using synthetic linear epitopes is to produce recombinant proteins. The production of proteins through expression systems is an incredibly useful and advantageous tool, rather than isolating native proteins. Several protein expression systems exist and range from bacteria, yeast, fungi, insect and mammalian cells, transgenic animals and

plants, each with advantages and disadvantages. The most common bacteria expression system is *E. coli*, it is cheap, quick to run and can produce a high yield of protein in a short time due to its quick replication times. However, it does not offer any post-translation modifications such as naturalistic protein folding and glycosylation, provided by eukaryotes, so may limit the diagnostic potential of eukaryotic recombinant antigens. On the other hand, yeast cells produce high expression yields and can produce post-translational modifications, such as glycosylation and peptide folding, however, the glycosylation pattern seen is very different to that in mammals. Other eukaryotic expression systems such as insect cells and mammalian cells, are costly, slow and require special equipment.

Although eukaryotes, trypanosomatids offer unique modification for general processes, the regulation of protein expression is usually done post-transcriptionally through intergenic untranslated regions (UTRs), making their recombinant proteins difficult to mimic through other machinery. The use of *Leishmania* spp. and *T. cruzi* for expression systems has been successful, *T. cruzi* trans-sialidase has been expressed in *L. major* [15] and a *T. cruzi* expression model has been used to produce mammalian cytokines (IL-2 and IFN-Y) [16], but with very low yields and these parasites are pathogenic to humans, making handling difficult and limited due to biosafety.

*Leishmania tarentolae* is a non-pathogenic species originally isolated from geckos. It shares 90 % of its genome with human pathogenic *Leishmania* species (*L. major, L. infantum and L. braziliensis*), lacking ~250 genes, which are mostly involved in the intracellular survival of the species [17]. This makes it an attractive candidate for protein expression, as it shares common characteristics with other trypanosomatids, whilst being non-pathogenic to humans, so can be handled in biosafety level one. Breitling et al (2002) developed the protein expression system, expressing human erythropoietin, which was biologically active and N-glycosylated [18]. Later, it was further developed to include a T7 promoter/TET operating machinery [19].

The *L. tarentolae* expression system has been utilised to produce human [20, 21], mammalian [22], viral [23], insect [24, 25], and trypanosomal, *T. cruzi* [26] and *Leishmania* [27] proteins, for vaccine targets, drug discovery and diagnostics. The first application of the *L. tarentolae* expression system to produce antigens for the serological diagnosis of trypanosomatids was by Rooney et al (2015), showing that the expression of *T. b. gambiensie* recombinant surface glycoproteins (ISG65, VSG LiTat 1.3 and VSG LiTat 1.5), had high recognition by infected

sera, sensitivity above 92.4 % and have the potential for use in diagnostic assays [28]. In 2019, the recombinant proteins rk39 and Lbk39 expressed through the *L. tarentolae* system were assessed for diagnostic potential for *L. infantum* [29] and *L. braziliensis* [30], respectively. Both showed good diagnostic potential and an alternative to current methods, indicating promising results for the use of this method to produce *T. cruzi* antigens [31].

I have previously demonstrated that the expression of a recombinant TSSA protein specific to the *T. cruzi* lineage Tc I, through the *L. tarentolae* expression system has increased recognition by Chagasic sera compared to its synthetic linear counterpart due to the addition of sugars (Chapter 3 [32]). Here, I show how the *L. tarentolae* system can be utilized to produce TSSA recombinant proteins specific to the other lineages (TSSA-II/V/VI, TSSA-III. TSSA-IV and TSSA-V/VI), how these are recognised by Chagasic sera, indicating their diagnostic potential.

# METHODS

## Prediction of glycosylation sites within recombinant TSSA proteins

To determine the predicted presence of O and N glycosylation sites on the lineage-specific TSSA constructs, their amino acid sequences, including the SUMO and HIS-tag components were submitted to NetOGlyc 4.0 (https://services.healthtech.dtu.dk/services/NetOGlyc-4.0/) and NetNglyc 1.0 (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/).

## *L. tarentolae* cell culturing conditions

### Reactivation.

Cryopreserved stocks were re-activated by thawing cells on ice for 20 minutes, then transferred to a 15 mL tube and centrifuged for 10 mins at 2000 g. The pellet was resuspended in 10 mL of fresh BHI medium containing Pen-Strep (10 U penicillin and 10 µg/mL streptomycin), 100 mg/mL NTC and Hygromycin, and 5 µg/mL hemin.

### Culture maintenance.

*L. tarentolae* cells were routinely maintained in 10 mL ventilated tissue culture flasks stored upright at 26 °C; passages were performed 1:50 on Monday and 1:20 on Friday into fresh BHI medium supplemented as described above.

**Cryopreservation.**

One millilitre of 100 % glycerol was added to 4 mL of culture (to achieve 20 % glycerol), mixed thoroughly and evenly distributed into sterile cryovials, kept for 10 minutes at room temperature before transfer in a cryocontainer at 4 °C for 10 mins, then – 80 °C overnight. Cryopreserved stocks were stored in liquid nitrogen.

## TSSA plasmid design (constructs) and creation

The TSSA lineage specific constructs designed for insertion into the pLEXSY plasmids were based on the GenBank entries for TSSA-II/V/VI (GU075675), TSSA-III (GU075673.1), TSSA-IV (GU075672.1) and TSSA-V/VI (GU075677). The TSSA-I sequence used varied from the one previously evaluated (123), by one amino acid change K to T (GU059922.1), called here gTSSA-I (T). The expression cassette, synthesised by Genewiz into a pUC-GW-Kan plasmid, incorporated a SUMO component to increase protein solubility and a 6 x His tag for purification. The expression cassette was flanked by 5' Xbal and 3' KpnI restriction sites for sub-cloning.

HIS-SUMO-TSSA cassettes were sub-cloned into the pLEXSY_I-blecherry3 vector (Jena Bioscience) (Figure 1). The pUC-GW-Kan-TSSA and pLEXSY plasmids were digested for one hour at 37 °C with XbaI and KpnI and the resulting products were excised under UV light from a 1% agarose gel using New England Biolabs gel purification kit, following manufacturer's instructions. Ligation reactions contained a 1: 5 molar ratios of insert to vector DNA, 1 µl DNA ligase (Promega), 2 µl 10 x buffer, dd$H_2O$ was added to bring the total volume to 20 µl. Ligation reactions were incubated overnight at 16 °C. Transformations were performed with NEB 5-alpha super competent *E. coli* cells: 5 µl of the ligation mix was added to 0.05 mL (one vial) of ice-thawed cells. Following 30 minutes on ice, heat shock was performed at 37 °C for 5 minutes after which the cells were returned to ice for 10 minutes. Transformed cells were mixed with 950 µl LB broth on a shaking incubator 30 °C for 1 hour before plating on agar plates containing ampicillin and incubated at 30 °C for 36 - 48 hours, according to manufacture guidelines. Single colonies were inoculated into LB medium with ampicillin selection, and DNA was purified from overnight cultures using Qiagen miniprep kit following manufactures instructions. To ensure correct insertion into pLEXSY plasmid premixed tubes were prepared with 5 µL purified plasmid (30- 100 ng/ µL) and 5 µL primer (5 pmol/ 5 µL) and sent to Genewiz for Sanger sequencing. Sequences were viewed on Chromas software and aligned using BioEdit software.

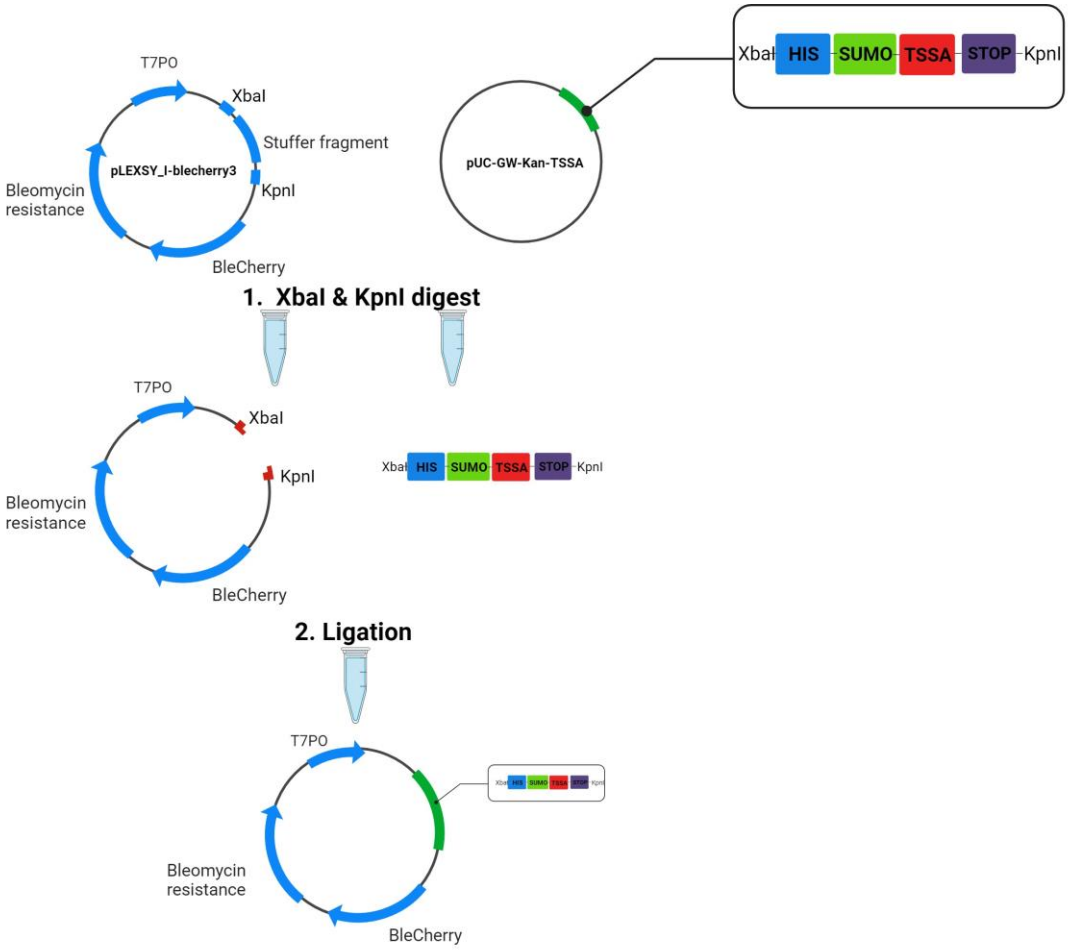


Figure 1. Subcloning of TSSA construct into pLEXSY plasmid. 1. The pUC-GW-Kan-TSSA and pLEXSY plasmids were digested XbaI and KpnI restriction enzymes. 2. The stuffer fragment was replaced in pLEXSY by the TSSA expression cassette. Created with BioRender.com

## pLEXSY-HIS-SUMO-TSSA transfection optimisation & confirmation of integration into *L. tarentolae*

In preparation for transfection, the pLEXSY-TSSA plasmids were linearized by overnight digestion at room temperature with SwaI. The following day, the desired 8 Kb band was excised from a 1% agarose gel electrophoresis under UV light and purified using New England Biolabs gel purification kit. The DNA was eluted in 12 µL $dH_2O$. For each plasmid 2 µL of the purified DNA was run on a 1 % gel to ensure the purification was successful.

The day before the transfection the cells were passaged 1: 10 into fresh BHI medium supplemented with Hemin, LEXSY NTC, LEXSY Hygro and PenStrep and incubated at 26 °C

overnight in a vented tissue flask, laid flat. The following day the cells were checked for morphology (a teardrop shape) via microscopy. Then, 5 mL of the *L. tarentolae* culture was pelleted by centrifugation 2000 g for 3 mins, and 3 mL of the supernatant removed. The cells were resuspended in the remaining supernatant and incubated on ice for 10 minutes, after which 350 µL was added to 10 µL of the desired recombinant plasmid. The cells were electroporated using GENE PULSE Xcell with PC module and a high voltage protocol, two 1500-volt pulses lasting 1.2 milliseconds delivered at 10 second intervals. The cells were replaced on ice for exactly 10 minutes before inoculation into fresh BHI culture medium, in 10 mL TC flask. Following an overnight incubation upright at 26 °C, as soon as the culture appeared turbid 100 mg/mL of bleomycin was added directly to the flask. The cultures were left upright at 26 °C and checked daily for changed in appearance and vitality. Three days after the drug challenge the cells were passaged 1: 20 into fresh BHI medium, with hemin and 100 mg/mL of bleomycin. The culture then underwent maintenance described above (Culture maintenance).

Genomic integration of the transfected cassette was checked by PCR using forward primer A708 (5'-GGATCCACCGCATGGCCAAGTTGACCAGTG-3') and reverse primer P1510 (5'-GTGCACCCATAGTAGAGGTGC-3'), under the following amplification conditions: 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 60 seconds. PCR products were analysed by gel electrophoresis for a confirmatory 2.7 kbp fragment. Genomic integration was secondarily confirmed by checking for the red fluorescence of the blecherry protein under a fluorescent microscope (Red C Emission wavelength 510). Transfection efficiency was estimated by a percentage of fluorescent cells compared to total cell number.

## TSSA recombinant protein expression induction

For protein expression 100 mL of BHI medium was inoculated 1:10 with the transfected cells and 10 mg/ mL tetracycline was added at the point of inoculation, to block the TET-repressor to induce the T7 RNA polymerase driven transcription. The culture was left to grow at 26 °C, 140 rpm for 70 hours.

## Concentration and purification of recombinant TSSA proteins

Harvesting of recombinant glycosylated TSSA proteins was performed approximately 70 hours after inoculation. The culture was centrifuged at 2000 g to 10 minutes to pellet the cells. The supernatant was retained, and 20 mL were concentrated in 10 kDa Spin-X UF Corning columns for 16 minutes at 4000 g, and the retained volume (non-filtrate) was collected. HIS-select

Nickel Affinity gel was prepared for use by centrifugation at 5000 x g for 5 minutes for removal of the storage ethanol. The pelleted beads were then washed in 10 x gel volume wash buffer (50mM sodium phosphate with 0.3 M sodium chloride) and centrifuged at 5000 x g for 5 minutes. Then, 1.5 mL of the size-concentrated culture supernatant was added to the affinity gel and then placed on an orbital shaker at 175 rpm for 15 minutes, before centrifugation at 5000 x g for 5 minutes and the supernatant removed. The beads were washed as above, and placed on the orbital shaker for 4 mins at 175 rpm, the supernatant was removed and protein concentration (at $A_{280}$, measured by nanodrop. This washing step was repeated until the protein concentration ($A_{280}$) stopped decreasing (reached < 0.05 mg/mL). Once the $A_{280}$ had reached a constant level, two gel volumes of elution buffer (wash buffer with 250 mM imidazole) was added to the beads and placed on an orbital shaker at 175 rpm for 10 mins, before centrifugation at 5000 x g for 5 minutes; the supernatant containing the His-tag purified recombinant protein was removed and stored at – 20 °C. This elution step was repeated once more, and the supernatants retained as separate fractions.

## SDS-page analysis for confirmation of recombinant TSSA protein production

Glycine SDS-PAGE minigels (4 % stacking, 12 % resolving) were made according to Laemmli (1970) [33] on a Mini Protean II system according to manufacturer's instructions (Mini-Protean ii, Bio-Rad UK), using a non-reducing buffer. Concentrated and His-tag purified TSSA recombinant protein was incubated 4:1 with non-reducing sample buffer at 37 °C for 30 minutes, 20 µL was then loaded into the gel. Gels were run at 30 V for 60 minutes then at 120 V for a further 120 minutes. Following this, the gels were removed from the apparatus and fixed using 100 ml of fix solution (50% methanol, 7% acetic acid and 43% water) for 30 minutes. This was repeated for another 30 minutes with fresh solution. The gels were stained with Sypro Ruby (S4942, Supelco, UK) according to manufactures protocol and the gels were visualised using Bio-Rad Gel doc.

## Assessing antigenicity of glycosylated TSSA recombinant proteins

Assays were carried out as described in Murphy et al 2020 with the following modifications [32]. The plates were coated with 50 µL/well of 10 µg/mL of *L. tarentolae* expressed glycosylated antigens in three different purities, namely: raw culture supernatant; concentrated but not purified; concentrated and purified. Serum samples were applied diluted 1:200; donkey

anti-human IgG-HRP was used at a dilution of 1:4000; reactions were developed with 50 µL of ABTS substrate and stopped with 50 µL 1% SDS after 15 minutes development in the dark; absorbance values were read at a wavelength of 405nm.

### Statistical analysis

ELISA plates were run in duplicate simultaneously and the cut-off was determined by first subtracting the background absorbance value from the mean reading of each sample, those with absorbance values three standard deviations greater than the mean of the absorbance values of the seronegative none-endemic controls were considered positive.

## RESULTS

### TSSA plasmid constructs and glycosylation prediction.

Figure 2 shows the lineage-specific TSSA recombinant proteins designed for production in the *L. tarentolae* system: His-tag in blue, SUMO sequence in green and TSSA lineage-specific sequence in red. The predicted O glycosylated amino acids are highlighted in yellow; no N-glycosylation was predicted for these sequences. The likelihood scores for the O-linked glycosylation predicted by bioinformatics are shown in Table 1.


Figure 2. *T. cruzi* TSSA recombinant proteins produced through the *L. tarentolae* expression system. His tag (Blue), SUMO sequence (Green), TSSA sequence (Red) with polymorphic residues underlined and predicted O glycosylated amino acids highlighted in yellow.

**Table 1**. Predicted O glycosylation and likelihood scores of the TSSA constructs to be expressed in *L. tarentolae*. Sites above 0.5 are considered glycosylated.

| TSSA residue Native | gTSSA residue | Amino Acid | O-glycosylation prediction score | | | | |
|---|---|---|---|---|---|---|---|
| | | | gTSSA-I (T) | gTSSA-II/V/VI | gTSSA-III | gTSSA-IV | gTSSA-V/VI |
| 5 | 119 | S | 0.80 | 0.83 | 0.59 | 0.56 | 0.65 |
| 6 | 120 | T | 0.64 | 0.80 | 0.65 | 0.57 | 0.62 |
| 7 | 121 | S | 0.64 | 0.78 | | | |
| 8 | 122 | S | 0.94 | 0.89 | 0.83 | 0.86 | 0.73 |
| 9 | 123 | T | 0.89 | 0.93 | 0.79 | 0.84 | 0.84 |
| 12 | 126 | S | | 0.90 | 0.84 | 0.88 | 0.77 |
| 13 | 127 | T | 0.87 | | | | |
| 14 | 128 | T | | 0.89 | 0.75 | 0.78 | 0.76 |
| 17 | 131 | T | 0.90 | | | | |
| 18 | 132 | T | | | | 0.88 | |
| 20 | 134 | T | | 0.89 | | | |
| 22 | 136 | T | 0.93 | | | | |
| 24 | 138 | S | 0.87 | | | | |
| 25 | 139 | S | | 0.76 | 0.84 | 0.91 | 0.58 |
| 26 | 140 | S | 0.85 | | | | |
| 27 | 141 | S | | | 0.77 | 0.75 | |

## PCR confirms integration of transfected TSSA plasmids into *L. tarentolae* genome

Plasmids for TSSA-I(T), -II/V/VI, -III, and -V/VI were successfully integrated into the *L. tarentolae* genome, as shown by the confirmatory 2.7kb amplicon (Figure 3).

## Visualization of cherry red protein production by transfected *L. tarentolae.*

The red fluorescent marker gene (Blecherry) was visualised under a fluorescent microscope for each of the transfected cultures (Figure 3), as a second method to confirm genomic integration and expression from the TSSA plasmids with *L. tarentolae.* Glycosylated TSSA-III had the highest estimated transfection success percentage (84.0 %), followed by gTSSA-V/VI (73.7 %), gTSSA-IV (69.7 %), gTSSA-I(T) (65.3 %), and gTSSA-II/V/VI (59.6 %)

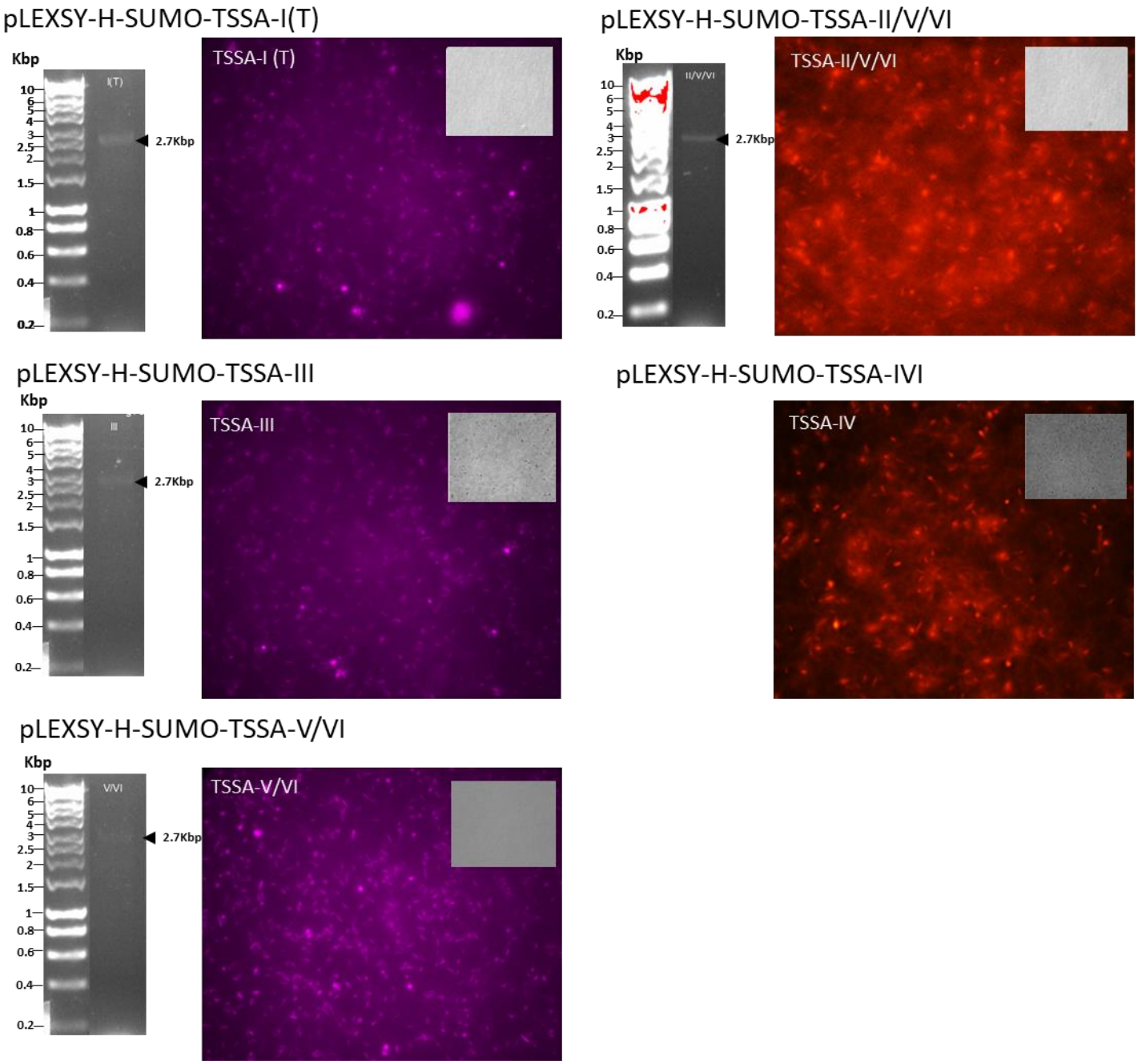


Figure 3. Successful genomic integration of the TSSA pLEXSY plasmids into the *L. tarentolae* genome demonstrated by confirmatory PCR and fluorescent microscopy of the BleCherry fluorescent marker gene. Transgenic *L. tarentolae* cultures TSSA-I(T), TSSA-II/V/VI, TSSA-III and TSSA-V/VI all contain the desired 2.7Kbp band (arrowed) and all five show BleCherry fluorescence. Phase contrast of the transfected *L. tarentolae* cultures in the same microscopy field is shown in the inset.

## Recombinant TSSA antigens produced through the *L. tarentolae* expression system

Figure 4 shows SYPRO Ruby stain of concentrated, His-Tag purified recombinant protein gTSSA-II/V/VI. The predicted size of the recombinant protein is 15kDa, however it appears as a smear at just above 25kDa, most likely due to the retarding effects of protein glycosylation on the electrophoretic mobility.

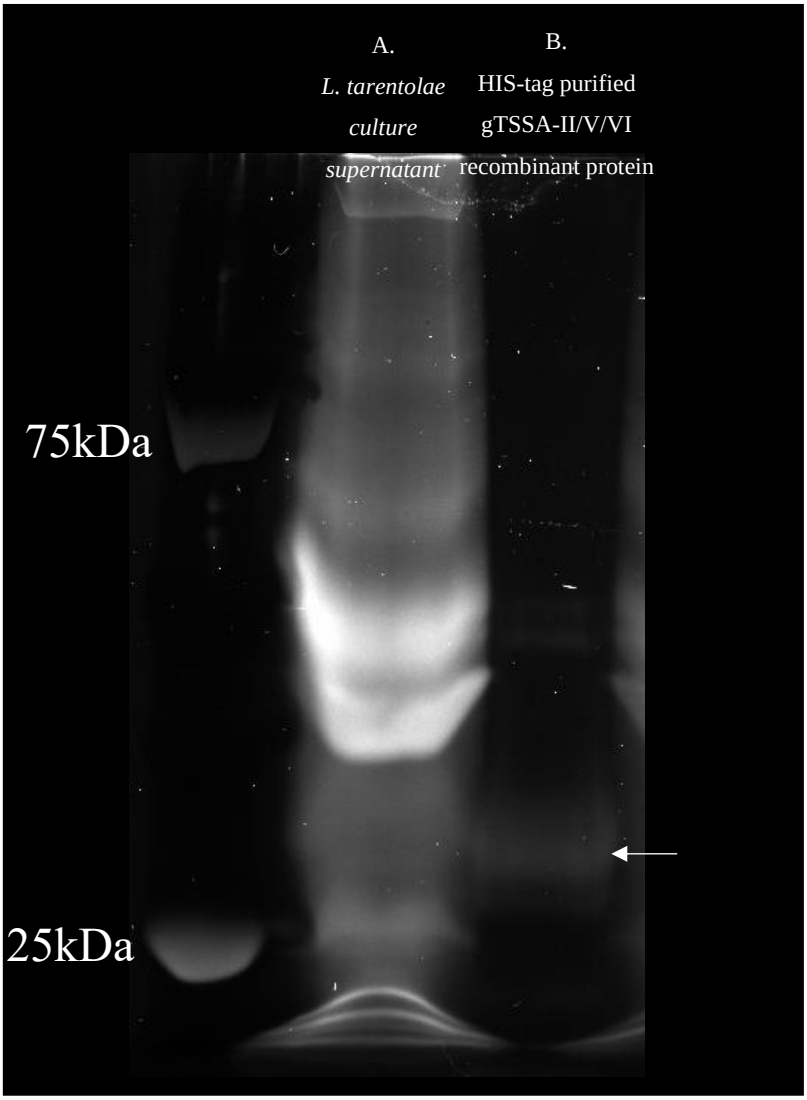


Figure 4. SYPRO Ruby stained gel of A: Concentrated gTSSA-II/V/VI supernatant, B: His-tag purified gTSSA-II/V/VI recombinant protein. The recombinant protein band is indicated by the arrow, it has a predicted weight of 15kDa, but a smear between 25 and 35 kDa is typical of glycosylated proteins due to the drag of the additional sugars.

## Recombinant gTSSA-V/VI from transfected *L. tarentolae* culture supernatant is recognised by chagasic sera

Supernatant from the gTSSA-V/VI *L. tarentolae* culture 70 hours post-inoculation and TET induction was assayed against chagasic sera from a TcV/VI endemic area (Brazil), demonstrating recognition of this antigen (Figure 5).

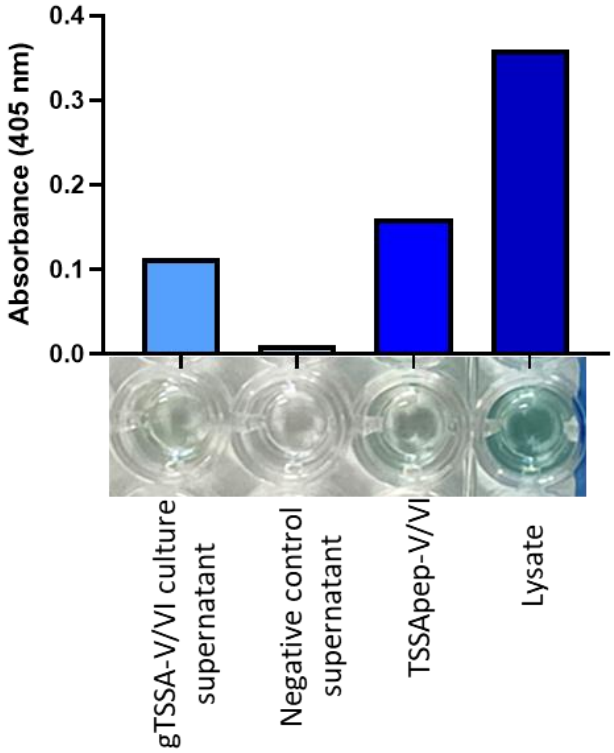


Figure 5. Transfected gTSSA-V/VI supernatant is recognised by chagasic sera. Chagasic sera from Brazil shows recognition of gTSSA-V/VI supernatant, TSSApep-V/VI and Lysate, there was no cross reaction with the negative control supernatant taken from the non-transfected *L. tarentolae* culture.

## gTSSA-II/V/VI transfected *L. tarentolae* culture supernatant had greater recognition by chagasic sera compared to the linear TSSApep-II/V/VI.

Figure 6 shows an example of gTSSA-II/V/V-I transfected *L. tarentolae* culture supernatant (yellow bars) recognition compared to the linear synthetic peptide TSSApep-II/V/VI (blue

bars). Chagasic samples from three different Latin American countries were assayed and the results summarised in Table 2.

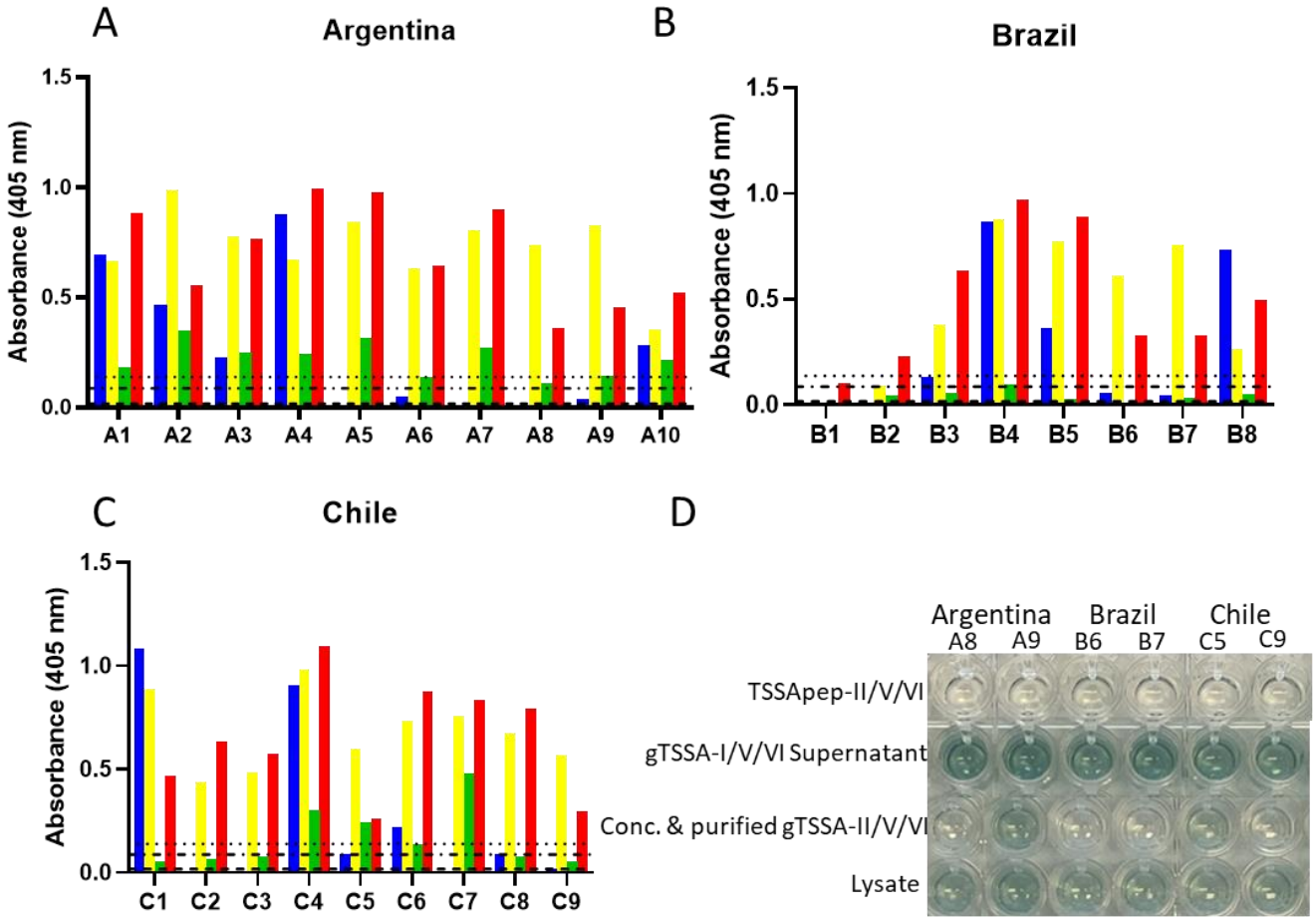


Figure 6. Antigenicity of gTSSA-II/V/VI supernatant compared to TSSApep-II/V/VI. A. Argentina, B. Brazil. C. Chile. BLUE, TSSApep-II/V/VI; Yellow, gTSSA-II/V/VI supernatant; GREEN, Concentrated and His-tag purified gTSSA-II/V/VI; RED, Lysate. Cut off values: Dash line TSSApep-II/V/VI (0.0165), Dotted line gTSSA-II/V/VI supernatant (0.1381), Dash and dotted line concentrated and purified gTSSA-II/V/VI (0.0871). D. ELISA plate illustrating the reactivity of TSSApep-II/V/VI compared to TSSA-II/V/VI-transfected *L. tarentolae* culture supernatant, and concentrated and purified gTSSA-II/V/VI. All six sera samples show a greater reaction with the supernatant collected from gTSSA-II/V/VI transfected cells than compared to the TSSA-peptide, irrespective of geographic location.

**Table 2.** Recognition of gTSSA-II/V/VI-transfected *L. tarentolae* culture supernatant by chagasic sera compared to TSSApep-II/V/VI.

| Origin of chagasic sera | TSSApep-II/V/VI positive | gTSSA-II/V/VI supernatant positive |
|---|---|---|
| Argentina (n = 10) | 7 (70.0 %) | 10 (100.0 %) |
| Brazil (n = 8) | 6 (75.0 %) | 6 (75.0 %) |
| Chile (n = 9) | 6 (66.7 %) | 9 (100 %) |

## DISCUSSION

The *Leishmania tarentolae* expression system offers a good alternative to produce recombinant proteins compared to bacterial, yeast or other eukaryotic expression systems, as it can combine the fast production of high yields at a relatively low cost associated with *E. coli*, with the post-translational glycosylation distinct to eukaryotic expression systems. Furthermore, it is safe to use in a biosafety 1 laboratory, as it is not pathogenic to humans, lacking the genes involved in the survival of pathogenic species, uniquely making it a promising system for trypanosomatids.

Trypanosomatids protein antigens for serological diagnosis have been expressed through the pLEXSY kit. Rooney et al (2015) that demonstrated the production of *Trypanosoma brucei* surface antigens for the use in RDT diagnosis of Human African Trypanosomiasis [28]. Production of *Leishmania* antigens, namely kinesin-related recombinant proteins for improved serodiagnosis of *L. infantum* [29] and *L. braziliensis* [30], have also been reported. Previously, I have shown that the production of a glycosylated protein specific to the *T. cruzi* lineage TcI has increased recognition compared to its linear synthetic counterpart due to the glycosylation (**Murphy et al., 2020**) [32].

In this work, I successfully established the *L. tarentolae* expression system and utilised it for the production of *T. cruzi* TSSA lineage-specific recombinant proteins (TSSA-I, TSSA-II/V/VI, TSSA-III, TSSA-IV, TSSA-V/VI). I designed the desired expression constructs, sub-cloned them into the pLEXSY plasmid with successful transfection and integration into *L. tarentolae* demonstrated by the confirmatory PCR and/or the production of the cherry red fluorescent marker protein.

The neat supernatant from the gTSSA-II/V/VI and gTSSA-V/VI-transfected cultures both showed recognition by chagasic sera from Argentina and Brazil, which was a promising result indicating the antigenicity of the recombinant proteins. Furthermore, once concentrated and purified, there was increased recognition of gTSSA-II/V/VI recombinant protein by Argentine and Chilean chagasic sera compared to the linear TSSApep-II/V/VI (Figure 6), whilst the same recognition pattern was shown with samples from Brazil. This demonstrates that the addition of sugars and/or protein conformation increases antigenicity. Further work on these *L. tarentolae*-produced recombinant proteins is required to assess their sensitivity and specificity.

A limitation of this study was the availability of equipment, resulting in me having to use the high-voltage transfection protocol instead of the recommended low-voltage one; this may have decreased the efficiency of the transfection. Also, initially I had difficulty culturing the parasite from the cryopreserved stock, attributed to the extended period of time the kit was kept before use due to the COVID-19 pandemic, which delayed this work substantially. Although the *L. tarentolae* expression system is a good alternative to other expression systems, especially for trypanosomatids, it requires specialised equipment and a cold-chain for transport and storage of *L. tarentolae*, limiting its wider application; a recent study has shown lyophilized version of *L. tarentolae* retain protein production and could allow for cheap transportation, making this more available across the globe [34].

In summary, I have demonstrated here how the *L. tarentolae* expression system can be used to express recombinant proteins specific to the *T. cruzi* lineages Tc II/V/VI, Tc III, Tc IV and Tc V/VI. I demonstrated when assayed against Chagasic sera samples that these recombinant proteins have increased antigenicity compared to synthetic linear versions, possibly due to the addition of sugars and/or protein confirmation. Although further work to assess the antigenicity and specificity of these antigens is needed, it is a promising initial finding, and indicates early potential for the development of a full library of diagnostic tests able to identify each *T. cruzi* lineage, which is vital to understanding this parasite further.

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# CHAPTER 2 LINEAGE-SPECIFIC RAPID DIAGNOSTIC TESTS CAN RESOLVE *TRYPANOSOMA CRUZI* TCII/V/VI ECOLOGICAL AND EPIDEMIOLOGICAL ASSOCIATIONS IN THE ARGENTINE CHACO.

## 2.1 Key points and implications.

- Pampa del Indio and Avia Teria are two municipalities within the Argentine Chaco, that remain endemic with Chagas disease despite a number of control strategies targeting the vector *T. infestans*. The two main ethnic groups are the Creole and Qom.
- Here, I performed the TSSA-II/V/VI lineage specific ELISAs and RDT on human, dog, cat and armadillo samples in order to better understand lineage distribution in the Gran Chaco region Argentina. This was the first time that lineage-specific serology had been applied to this endemic area.
- The Chagas Sero K-SeT RDT detected prevalence of TcII/V/VI was 69.5 % in humans and 65. 8 % for dogs, which is similar to what has previously been reported in Bolivian patients. Also, this supports findings indicated in other studies by artificial xenodiagnoses, *in vitro* culture, parasite isolation and PCR-based lineage identification that the hybrid lineages are prevalent within the Gran Chaco.
- For humans, analysis showed associations between TSSApep-II/V/VI recognition and location, and with increasing and decreasing age within the Qom and Creole populations, respectively.
- For dogs TSSApep-II/V/VI recognition was associated with being born before the mass spraying campaign and Qom household.
- Although TSSApep-II/V/VI ELISA results were positive by the RDT, not all RDT positives were reactive in the ELISA, showing that the RDT has greater sensitivity than the TSSApep-II/V/VI ELISA with humans and dogs. The Chagas Sero K-SeT can be used instead of the more technical and time-consuming ELISA, for humans and dogs.
- One armadillo was weakly positive by RDT, this individual had previously shown to be infected with TcIII via genotyping, strongly indicating a co-infection of TcIII and TcII, TcV or TcVI.
- However, the RDT is not suitable for the use of cat samples due to the detection molecule in Chagas Sero K-SeT RDT, protein G, not binding to feline IgG.

## 2.2 Candidate's contributions

- Research visit (laboratory and field work) to collaborating institute Universidad de Buenos Aires, Argentina.
- Plan and perform TSSA peptide lineage specific ELISAs, and Chagas Sero K-SeT RDTs.
- Generation and analysis of data, including univariate analysis.
- Prepared draft (text and figures) of manuscript and Corresponding Author for submission to journal *Parasites & Vectors*.


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| **Thesis Title** | Development of rapid diagnostic tests for Trypanosoma cruzi lineage-specific serology, comparative epidemiology and for monitoring efficacy of chemotherapy | | |
| **Primary Supervisor** | Michael Miles | | |

**If the Research Paper has previously been published please complete Section B, if not please move to Section C.**

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**SECTION E**

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| **Date** | 25/09/203 |

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# Lineage-specific rapid diagnostic tests can resolve *Trypanosoma cruzi* TcII/V/VI ecological and epidemiological associations in the Argentine Chaco

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## Abstract

**Background:** *Trypanosoma cruzi*, the protozoan agent of Chagas disease, is comprised of at least 6 genetic lineages (TcI-TcVI). Their geographical distribution, clinical associations and reservoir hosts are not fully elucidated, as genotyping is hampered due to the difficulty in isolating representative populations of organisms. Lineage-specific serological techniques may address these issues.

**Methods:** *Trypanosoma cruzi* lineage-specific serological assays were performed on human, canine, feline and armadillo sera from the Gran Chaco in northern Argentina, a region of ongoing transmission. Synthetic peptides representing lineage-specific epitopes of the trypomastigote small surface antigen (TSSA) were used in ELISA, and the TcII/V/VI shared epitope peptide (TSSApep-II/V/VI) was used in the Chagas Sero *K*-SeT rapid diagnostic test (RDT).

**Results:** Chagas Sero *K*-SeT RDT, using Protein G to detect human and canine IgG, was at least as sensitive as TSSApep-II/V/VI ELISA using specific secondary antibodies. For sera from humans TSSApep-II/V/VI seroprevalence by Chagas Sero *K*-SeT was 273/393 (69.5%), for dogs 48/73 (65.8%) and for armadillos 1/7 (14.3%); by ELISA for cats 5/19 (26.3%). The seroprevalence for humans was similar to that for Bolivian patients, amongst whom we previously observed an association of TSSApep-II/V/VI seropositivity with severity of cardiomyopathy. In humans, prevalence of TSSApep-II/V/VI recognition was associated with locality, and with increasing and decreasing age within the Qom and Creole populations, respectively. For dogs TSSApep-II/V/VI recognition was associated with being born before community-wide insecticide spraying ($P = 0.05$) and with Qom household ($P < 0.001$).

**Conclusions:** We show here that Chagas Sero *K*-SeT RDT can replace ELISA for TSSApep-II/V/VI serology of humans and dogs; for humans there were statistically significant associations between a positive Chagas Sero *K*-SeT RDT and being resident in Area IV, and for dogs association with Qom household or with being born before the mass spraying campaign; we also show that with cats the TcII/V/VI epitope can be detected by ELISA. We assessed the lineage distribution in an unprecedented 83% of the human *T. cruzi*-seropositive population. These results form the basis for more detailed studies, enabling rapid in-the-field surveillance of the distribution and clustering of these lineages among humans and mammalian reservoirs of *T. cruzi* infection.

**Keywords:** *Trypanosoma cruzi*, ELISA, Serology, Lineage-specific, Chagas disease, Argentina, Rapid diagnostic test

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## Background

Chagas disease, caused by infection with the protozoan parasite *Trypanosoma cruzi*, remains a major public health problem in endemic regions of Latin America. The initial acute phase of infection may be asymptomatic or have mild and non-specific symptoms but can be fatal, particularly in infants, young adults or the immunocompromised. Without successful treatment *T. cruzi* infection is life-long: the immune response reduces the level of infection but is unable to eliminate it, as is apparent from xenodiagnosis or PCR of seropositive patients, and recrudescent parasitaemia in the immunocompromised. In the chronic phase, around 30% of those infected will develop chagasic heart disease, and a proportion will also have gastrointestinal megasyndromes [1, 2]. Of the 1.5 million people in Argentina infected with *T. cruzi*, approximately 370,000 are estimated to have chagasic cardiomyopathy [3].

Ongoing transmission is primarily maintained by contamination with *T. cruzi* infected faeces of the predominant local triatomine insect vector, *Triatoma infestans*, which infests rural dwellings, especially in the Gran Chaco region, where vector control has had limited success [4]. Transmission can also be oral by consumption of triatomine faeces-contaminated food or congenitally, and *via T. cruzi* infected blood or organ donors. *Trypanosoma cruzi* infection is a zoonosis: dogs, cats and rodents associated with households are reservoir hosts, with evidence of a positive association between the number of infected dogs and the prevalence of human infection [5]. A wide range of sylvatic mammals carry *T. cruzi* infection [6].

*Trypanosoma cruzi* is currently understood to comprise six genetic lineages TcI-TcVI [7], with TcBat proposed as a seventh lineage, related to TcI [8]. Based on genotyping, TcII/V/VI lineages predominate in the domestic cycle in southern cone countries, including Argentina. However, genotyping may be biased by non-representative isolation of *T. cruzi*, which has sequestered intracellular replication and only scanty chronic blood infections, and by competitive selection *in vitro* between the lineages.

The polymorphic trypomastigote small surface antigen (TSSA), expressed on bloodstream trypomastigotes, has been the only antigen applicable for indirect, serological identification of lineage(s) carried by a patient or reservoir host [9]. TcI, TcIII and TcIV each have their own distinct potential TSSA epitope. At the same site a distinct amino acid sequence is shared by TcII/V/VI, and the hybrids TcV/VI also have a second sequence, as they are heterozygous and have two haplotypes at that locus [10]. Recombinant TSSA produced in *E. coli* or synthetic peptide epitopes (TSSApep) have been used with Argentine chagasic samples for *T. cruzi* lineage-specific serology [9, 11–21], particularly with the isoform common to TcII/V/VI; the recombinant form has also been used for canine serology [12, 22].

We recently developed the novel rapid diagnostic test (RDT) Chagas Sero *K*-SeT incorporating TSSApep-II/V/VI and found that response to this RDT was associated with severity of cardiomyopathy in Bolivian patients [23]. As Chagas Sero *K*-SeT uses Protein G to detect IgG, this same test should be directly applicable to both humans and diverse mammal species.

Here, our objectives were to apply *T. cruzi* lineage-specific TSSApep ELISA and the Chagas Sero *K*-SeT RDT to humans and mammals of the Chaco region of northern Argentina to gain further insight on ecological and epidemiological associations, focusing here on TcII/V/VI.

## Methods

Serum samples from seropositive patients and *T. cruzi*-infected animals were from archives stored at the University of Buenos Aires.

### Study sites

The two study sites were the municipalities of Pampa del Indio and Avia Terai in Chaco Province, northern Argentina. The majority of samples tested were from a larger ongoing project on the eco-epidemiology and control of Chagas disease, taking place in the rural area of Pampa del Indio (1600 $km^2$), consisting of 1446 inhabited households in 30 villages [24]. There are two main ethnic groups inhabiting the area, Creole and Qom; the latter make up half of the local population, but are unevenly distributed among the rural villages [25]. For logistic reasons we divided the rural area into 4 study areas (named Areas I-IV). Vector control activities included a baseline house infestation assessment, followed by a community-wide spraying with pyrethroid insecticides, which took place between 2007–2009, complemented by periodic entomological surveys and community-based surveillance to detect re-infestation [5, 26–28].

In 2015, research activities were expanded to include Avia Terai municipality (770 $km^2$), around 150 km from Pampa del Indio. This municipality comprises 307 rural households, inhabited by a Creole population. Figure 1 shows typical dwellings and environment of the study sites.

### Sample collection

#### *Human samples*

*Trypanosoma cruzi* seropositive human samples were obtained in different serosurveys that took place from August 2014 until July 2017. Serum samples were examined using conventional serology by means of two ELISAs

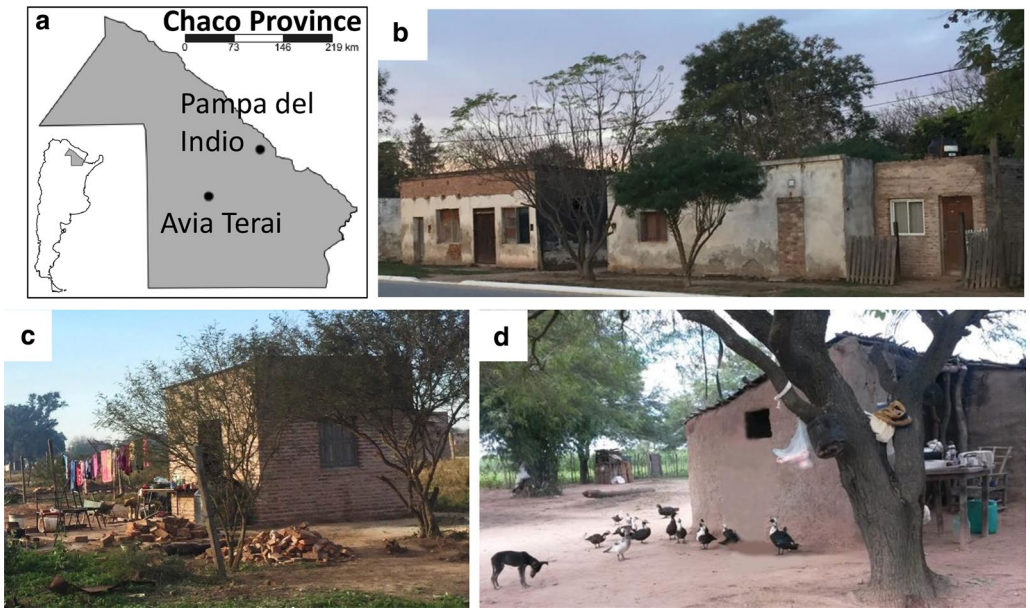


**Fig. 1** Study setting. **a** Location of Pampa del Indio and Avia Terai study sites in Chaco Province, Argentina. **b** Typical periurban dwelling. **c**, **d** Typical rural dwelling and environment in Avia Terai (**c**) and Pampa del Indio (**d**)

using either semipurified fractions of epimastigote lysate (Chagatest, Wiener lab, Argentina) or recombinant antigens (ELISA Rec V3.0, Wiener lab). A patient was considered Chagas seropositive if reactive in both tests. Serologically discordant samples were tested by an indirect immunofluorescence antibody test (IFAT) (Ififluor Parasitest Chagas, Laboratorio IFI, Buenos Aires, Argentina) or submitted to the reference diagnosis laboratory at the National Institute of Parasitology "Dr. Mario Fatala Chabén" (Buenos Aires, Argentina) for a final diagnosis. In addition, 10 *T. cruzi* seronegative human samples from Buenos Aires (a non-endemic area) presenting with other pathologies and 20 seronegative samples from the study sites were assayed by Chagas Sero *K*-SeT.

#### *Animal samples*

In 2008, cross sectional house-to-house surveys were carried out targeting all dogs and cats within 7 contiguous villages of Pampa del Indio considered to have a high infestation of *T. cruzi* infected triatomine bugs. Owners were interviewed *via* questionnaire and asked for further information on whether they had permanent residence in the study village or came from other villages outside the study area [25]. Additional samples were collected during a dog survey carried out in June 2016 (Cardinal et al., unpublished). Dogs and cats $\geq$ 4 months of age were examined by serology and younger animals and cats were examined by xenodiagnosis. Up to 7 ml of blood were taken from the animals by trained and experienced field personnel, and processed and stored as previously described [29]. A dog or cat was considered infected with *T. cruzi* if it was seroreactive with at least two serological tests (i.e. seropositive by ELISA and indirect haemagglutination test) or if it was xenodiagnosis-positive.

*Trypanosoma cruzi*-infected armadillos were captured using traps baited with beef or chicken strips soaked in fish sauce in different trapping surveys from August 2008 to August 2011. Traps were checked every morning and re-baited when needed. Full capture and sampling methods are described elsewhere [30]. Armadillos were examined for infection by xenodiagnosis as described [31] and not by conventional serology.

#### TSSA lineage-specific serology

A total of 393 human, 85 dog (*Canis familiaris*), 19 cat (*Felis catus*) and 7 armadillo (6 *Dasypus novemcinctus* and 1 *Tolypeutes malacus*) serum samples were tested here by TSSApep lineage-specific ELISA and/or the Chagas Sero *K*-SeT RDT. A subset of 38/393 human and 73/85 dog serum samples were tested by both TSSApep-II/V/VI ELISA and Chagas Sero *K*-SeT. All these human and dog samples tested by both lineage-specific serology methods were positive by conventional serology.

#### TSSApep lineage-specific ELISA

ELISAs were performed with synthetic peptides TSSApep-II/V/VI, -III, -IV and -V/VI representing residues 37–52 in the TSSA protein of those lineages (Additional

file 1: Table S1) and with a control reference *T. cruzi* TcII lysate (IINF/PY/00/Chaco23) as described previously [17], with the modifications described below for human, canine and feline samples. In all cases, two replica plates were run simultaneously. Cut-offs were determined by first subtracting the plate background (no antigen wells) absorbance values from the mean reading for each sample; those samples that were then greater than five standard deviations higher than seronegative controls were considered positive.

#### *Human samples*

This was performed as described previously [17], with the following modifications: 0.1 µg of each TSSApep was used per well; goat anti-human IgG-HRP (074-1006: SeraCare, USA) diluted 1:5000 was used; reaction wells were developed with 100 µl of ABTS substrate (50-62-00: SeraCare) and stopped with 50 µl of stop solution; absorbance values were determined at a wavelength of 405 nm.

#### *Dog and cat samples*

ELISA plates were coated directly with each TSSApep at 0.1 µg/100 µl / well in coating buffer overnight. After blocking and washing steps as described [17], 100 µl of 1:200 (dog) or 1:500 (cat) dilutions of sera were applied. Subsequently, 100 µl of goat anti-dog IgG-HRP (14-19-06, SeraCare) diluted 1:12,000, or goat anti-cat IgG-HRP (14-20-06, SeraCare) diluted 1:5000, was used, prior to addition of substrate.

### Chagas Sero *K*-SeT RDT

This novel RDT, manufactured at Coris BioConcept, employed TSSApep-II/V/VI as the antigen and Protein G as the detection molecule for IgG, as previously described [23]. Tests were visually assessed at 15 min maximum incubation time and considered valid if the control band was present; the additional presence of a test line band of any intensity was considered positive for TSSApep-II/V/VI recognition. The absence of test line band was considered a negative test. The presence of the test band was determined by visual inspection of the RDT, independently by two individuals.

### *Trypanosoma cruzi* genotyping

*Trypanosoma cruzi* lineage was determined by PCR of the genomic targets spliced-leader (SL) DNA, 24Sα ribosomal RNA genes and A10 from *T. cruzi* isolates [32, 33]. For humans only, a second PCR-based protocol targeting two nuclear genes (TcSC5D and TcMK) [34] was also employed [35] to allow for classification of lineages TcI-TcVI as well as TcBat and TcV/VI [34].

### Statistical analyses

Fisher's exact test (two tailed) was used to calculate odds ratios, 95% confidence intervals and *P*-values (StataCorp. 2019. Stata Statistical Software: Release 15. StataCorp LLC, Texas, USA). A $P$-value $\leq 0.05$ was considered significant. A Kappa test was used to determine the level of agreement between the TSSApep-II/V/VI ELISAs and Chagas Sero *K*-SeT RDT, the degree of agreement was qualified by Kappa and categorized as mild, moderate or severe and 95% confidence intervals calculated (GraphPad, San Digeo, USA). For seropositive humans from Area II and IV, we performed univariate and multivariate (generalized linear model, GLMs) analyses to detect factors associated with RDT reactivity by means of a logistic regression. The full model tested was: RDT reactivity ~ age at diagnosis *vs* ethnic group + study area + gender + occurrence of *T. infestans* in the household + another cohabitant with reactive RDT. Linear regressions were calculated for each ethnic group. For Creoles, we forced the origin in 100%. Univariate analysis of dog RDT reactivity was performed for animals examined for diagnosis in 2008.

## Results

A total of 373 human, 85 dog and 19 cat samples were seropositive as described in Methods. Seven armadillos were positive by xenodiagnosis. Additionally, 20 human samples were seronegative by conventional serology. Most (292/393, 74.3%) of the human samples belonged to 10 rural villages in Area II and Area IV from Pampa del Indio, where we aimed at full coverage of the detected seropositive population. In these villages a total of 1338 inhabitants were serodiagnosed and 332 (24.8%) found seropositive for *T. cruzi* (Macchiaverna et al., unpublished) with 88.0% (292/332) of these seropositive patients assayed by Chagas Sero *K*-SeT RDT.

### Chagas Sero *K*-SeT is more sensitive than TSSApep-II/V/VI ELISA for humans and dogs

Comparing the TSSApep-II/V/VI ELISA and the Chagas Sero *K*-SeT RDT, all human samples that were positive by TSSApep-II/V/VI ELISA were also positive by Chagas Sero *K*-SeT RDT for recognition of this peptide; Figure 2 shows examples of correspondence between these methods. However, this RDT additionally identified 10 human samples as positive that were negative by TSSApep-II/V/VI ELISA (Table 1), although seropositive by conventional serology. Thus, for human samples tested by both methods, 13/38 (34%) were TSSApep-II/V/VI ELISA positive whereas 23/38 (61%) were positive by Chagas Sero *K*-SeT. Consequently the Kappa statistic showed moderate agreement between the two tests (0.51; 95% CI:

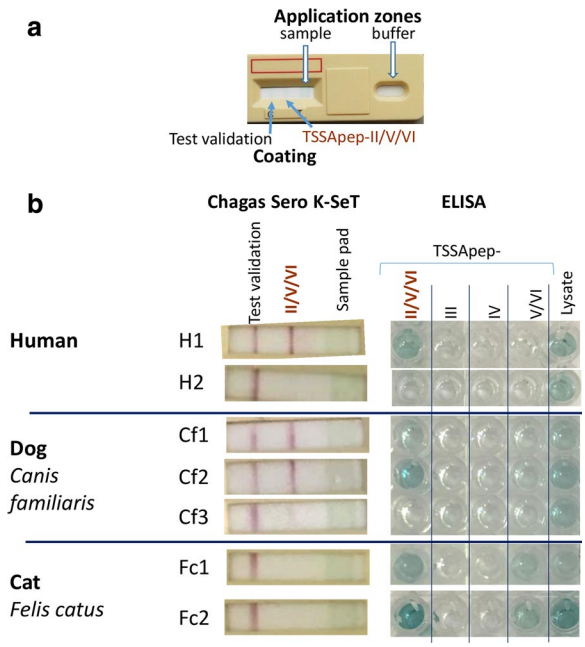


**Fig. 2** Comparison of Chagas Sero *K*-SeT RDT and ELISA for TSSApep-II/V/VI serology. **a** Chagas Sero K-SeT RDT, which uses Protein G for IgG detection, showing coating of nitrocellulose membrane and zones for application of sample then buffer. **b** Results were assessed visually and accorded with ELISA using specific secondary antibodies of both humans and dogs (samples H1 and Cf1, Cf2 positive by both tests; H2 negative by both tests), and was in some cases more sensitive than ELISA (sample Cf3: positive RDT, negative ELISA). Some dog and cat sera were positive by ELISA for TSSApep-V/VI in addition to TSSApep-II/V/VI (samples Fc1 and Fc2). Chagas Sero *K*-SeT was not able to detect feline IgG (samples Fc1, Fc2: positive ELISA, negative RDT). ELISA results were based on absorbance unit values and the cut off was determined by comparing to *T. cruzi* seronegative samples

0.28–0.74). Similarly, all dog samples that were positive by TSSApep-II/V/VI ELISA were also positive by Chagas Sero *K*-SeT RDT (Fig. 2); among these samples tested by both methods, 33/73 (45%) were TSSApep-II/V/VI ELISA positive whereas an additional 15 were positive by Chagas Sero *K*-SeT only (48/73; 66%). Here, the Kappa statistic found a good agreement between the two tests (0.60; 95% CI: 0.44–0.77). Furthermore, the Protein G conjugate in Chagas Sero *K*-SeT was highly effective in detecting binding of both human and canine IgG to TSSApep-II/V/VI, without the need for the specific secondary antibodies used in the ELISA.

### TSSApep ELISA

Table 1 shows the TSSApep lineage-specific ELISA results for human, dog and cat samples. For humans, ELISA reaction to TSSApep-II/V/VI occurred with 13/38 (34.2%) sera, whereas it was 33/85 (38.8%) for dog and 5/19 (26.3%) for cat. Interestingly, of these animal samples 12 dog and all 5 cat samples were additionally positive for TSSApep-V/VI, which differs from TSSApep-II/V/VI by a single amino acid substitution (Additional file 1: Table S1).

### Chagas Sero *K*-SeT RDT

In total across the two study sites the detected prevalences of TcII/V/VI infection by Chagas Sero *K*-SeT in humans (273/393, 69.5%) and dogs (48/73, 65.8%) were similar (Table 1). However, as expected due to the lack of Protein G efficacy with cats, Chagas Sero *K*-SeT was negative with sera of 2/2 cats strongly seropositive for TSSApep-II/V/VI by ELISA (Fig. 2). For armadillos, 1/7 (14.3%) was weakly positive with Chagas Sero *K*-SeT (Fig. 2).

### Comparison of lineage-specific serology with genotyping

Corresponding *T. cruzi* genotyping data were available for a subset of the human, dog, cat and armadillo samples tested by TSSApep lineage-specific serology (Additional file 1: Table S2). A total of 28 of 38 human serum samples with genotyping data were reactive by Chagas Sero *K*-SeT. Given that all human-infecting lineages were genotyped as TcV or TcVI, the sensitivity of Chagas Sero *K*-SeT was 73.7% (95% CI: 57.8–85.1%). None of the 10 *T. cruzi* seronegative serum samples from non-endemic patients with other pathologies was reactive by Chagas Sero *K*-SeT. However, 8 of 20 sera from the study sites that were negative with our conventional serology were reactive by Chagas Sero *K*-SeT. Overall, 8/30 samples

**Table 1** Human, dog and cat samples assayed by TSSApep ELISA and/or Chagas Sero *K*-SeT RDT

| Host | Assayed by both ELISA and RDT | | | | Individual assay positives | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | RDT and ELISA positive | RDT only positive | ELISA only positive | RDT and ELISA negative | II/V/VI ELISA | III ELISA | IV ELISA | V/VI ELISA | RDT |
| Human | 13/38 | 10/38 | 0 | 15/38 | 13/38 | 0 | 0 | 0 | 273/393 (69.5%) |
| Dog | 33/73 | 15/73 | 0 | 25/73 | 33/85 | 0 | 0 | 12/85[a] | 48/73 (65.8%) |
| Cat | 0 | 0 | 2/2 | 0 | 5/19 | 0 | 0 | 5/19[a] | 0/2 |

[a] Also positive by TSSApep-II/V/VI ELISA

Chagas seronegative by conventional serology were reactive by the RDT, thus estimated specificity was 73.3% (95% CI: 55.5–86.0%).

Of the 17 dogs for which the *T. cruzi* genotype was TcII/V/VI or TcVI, 11/17 were positive for TSSApep-II/V/VI by Chagas Sero *K*-SeT (7 were TSSApep-II/V/VI ELISA positive only, 4 were additionally TSSApep-V/VI positive, and 6 were TSSApep ELISA negative); 2/17 were negative by both lineage-specific serological methods. For the single dog from which TcIII was genotyped, the corresponding serum was Chagas Sero *K*-SeT positive but TSSApep ELISA negative.

Of the four cats for which *T. cruzi* was genotyped as TcII/V/VI or TcVI, all were negative by TSSApep ELISA. The single armadillo that was TSSApep-II/V/VI positive by Chagas Sero *K*-SeT had *T. cruzi* genotyped as TcIII, as were the remaining armadillos for which these genotyping data were available.

#### Hosts, clustering and ecological associations

Among the two sites (Pampa del Indio and Avia Terai), in Pampa del Indio 242/350 (69.1%) were positive by Chagas Sero *K*-SeT compared to 31/43 (72.1%) in Avia Terai, but this was not statistically significant (OR: 0.8; 95% CI: 0.4–1.7; $P = 0.69$).

Univariate associations of TSSA-II/V/VI seropositivity by Chagas Sero *K*-SeT within the Pampa del Indio study Areas II and IV (humans and dogs) are shown in Table 2. For humans, there were no significant associations between TSSApep-II/V/VI recognition and age, ethnicity, previously infested house, gender or having another householder TSSA-II/V/VI positive. A significantly higher Chagas Sero *K*-SeT reactivity was observed for patients inhabiting Area IV compared to Area II (OR: 2.07; 95% CI: 1.15–3.88; $P = 0.02$).

For dogs born before the mass insecticide spraying programme there was a significant association with TSSApep-II/V/VI seropositivity (OR: 8.70; 95% CI: 0.78–436.49; $P = 0.046$). Interestingly, unlike for humans, Qom household was also significantly associated with TSSApep-II/V/VI recognition in dogs (OR: 8.39; 95% CI: 1.73–78.91; $P = 0.003$). We found no evidence of significant associations between TSSApep-II/V/VI recognition and the roles and behaviour of dogs, such as sleeping inside or hunting (Table 2).

For the 19 cats that were assessed according to the available information, there were no significant associations between recognition of TSSApep-II/V/VI or TSSApep-V/VI, Qom or Creole ownership, hunting, domestication and sleeping habits (data not shown).

By means of multivariate regression analysis variables associated with Chagas Sero *K*-SeT seropositivity were identified. A significant interaction between age and ethnicity was observed: for Qom the reactivity increased with age, whilst for Creoles it decreased (Table 3 and Fig. 3). For Creoles, the percentage of RDT reactive persons decreased with age with a significant slope of − 0.72 * age (in years) ($R^2 = 70.34$, $P = 0.0003$) whereas for Qom the reactivity increased with a slope of 0.25*age, though it was marginally significant ($R^2 = 45.27$, $P = 0.098$). As observed in the univariate analysis, inhabitants from Area IV exhibited a higher reactivity than those from Area II. No significant associations were observed with the other variables evaluated (Table 3).

## Discussion

We have previously applied TSSApep lineage-specific ELISA to human chagasic sera [17] and to sylvatic primate hosts of *T. cruzi* [36], and adapted TSSApep-II/V/VI serology to the Chagas Sero *K*-SeT RDT [23]. Here, we deployed TSSApep serology as a rapid and efficient means for surveillance of *T. cruzi* lineage distribution among humans and animals in active transmission cycles in the Chaco region of northern Argentina.

Previous reports using TSSA serology on Argentine chagasic samples have been principally based on ELISAs and immunoblotting [9, 11–16, 18–21]. Here, we applied TSSApep-II/V/VI serology in a user-friendly, low cost RDT format, applicable at point-of-care to patients. We show excellent concordance between the performance of the Chagas Sero *K*-SeT and TSSApep-II/V/VI ELISAs in humans, as also seen with Bolivian sera [23]. However, more samples tested by both lineage-specific methods were positive with the RDT, suggesting potentially either a greater sensitivity or lower specificity. However, *in silico* analysis and sequencing of the TSSA gene from *T. cruzi* encompassing a range of hosts and geographical locations has not identified any novel epitopes (unpublished observations). Furthermore, none of the 30 seronegative samples was positive by this RDT, indicating that the RDT has greater sensitivity, as might be expected because the RDTs employ higher serum concentrations. Using the observed sensitivity and specificity we estimated that the prevalence of infection with TcII/V/VI in the seropositive human population of Pampa del Indio is 88.2% (95% CI: 76.4–99.0%), which provides further support for the prevalence of hybrid lineages in infected humans from the Chaco, as indicated by time-consuming artificial xenodiagnosis, *in vitro* culture, parasite isolation and PCR-based lineage identification [35].

Interestingly, the prevalence of Chagas Sero *K*-SeT RDT positives in these Argentine patients (69.5% for Pampa de lndio and Avia Terai combined) is similar to that seen in Bolivian patients (66.9%) amongst whom we observed an association with severity of cardiomyopathy [23]. Moreover, the significant differences observed in the

**Table 2** Univariate analyses of hosts, clustering and ecological associations with Chagas Sero *K*-SeT (Pampa del Indio)

| | Category | *n* | No. positive (%) | OR (95% CI) | *P*-value |
|---|---|---|---|---|---|
| Humans | Age | 292 | 199 (68.2) | 1.00 (0.98–1.01) | 0.62 |
| | Ethnicity | | | | |
| | Creole | 68 | 50 (73.5) | 1 | |
| | Qom | 224 | 149 (66.5) | 0.72 (0.38–1.29) | 0.28 |
| | Gender | | | | |
| | Female | 144 | 100 (69.4) | 1 | |
| | Male | 148 | 99 (66.9) | 0.89 (0.54–1.46) | 0.64 |
| | Study area | | | | |
| | II | 212 | 136 (64.2) | 1 | |
| | IV | 80 | 63 (78.8) | 2.07 (1.15–3.88) | 0.02* |
| | Presence of *T. infestans* in the household | | | | |
| | No | 59 | 43 (72.9) | 1 | |
| | Yes | 233 | 156 (67) | 0.75 (0.39–1.40) | 0.38 |
| | Cohabitant with reactive RDT | | | | |
| | No | 146 | 102 (69.9) | 1 | |
| | Yes | 146 | 97 (66.4) | 0.85 (0.52–1.40) | 0.53 |
| Dogs | Function | | | | |
| | Guardian | 41 | 26 (63.4) | 1 | |
| | Hunting | 32 | 23 (71.9) | 1.47 (0.49–4.59) | 0.47 |
| | Type of hunting | | | | |
| | Sylvatic animals | 34 | 23 (67.6) | 1 | |
| | Not hunting | 16 | 11 (68.8) | 1.05 (0.25–4.84) | 1.00 |
| | Place of birth | | | | |
| | In study area | 52 | 36 (69.2) | 1 | |
| | Not in study area | 12 | 9 (75) | 1.33 (0.28–8.63) | 1.00 |
| | Ethnicity of the household | | | | |
| | Creole | 57 | 31 (54.4) | 1 | |
| | Qom | 22 | 20 (90.1) | 8.39 (1.73–78.92) | 0.00* |
| | Born after mass spraying | | | | |
| | Yes | 5 | 1 (20) | 1 | |
| | No | 73 | 50 (68.5) | 8.70 (0.78–436.49) | 0.05* |
| | Place of sleeping | | | | |
| | Outside of the domicile | 18 | 12 (66.7) | 1 | |
| | Inside of the domicile | 14 | 12 (85.7) | 3.00 (0.41–35.07) | 0.41 |

*Note*: Areas II and IV only. In each case, only those samples where the examined information was known were included in the analysis

* Statistical significance ($P \leq 0.05$)

*Abbreviations*: OR, odds ratio; CI, confidence interval

Chagas Sero *K*-SeT RDT reactivity between study Areas II and IV of Pampa del Indio and the different trends for age in the two ethnic groups merits further study, given that this may be related to different incidence rates of cardiomyopathy.

One strength of this study is the level of coverage of the seropositive human population achieved. Overall, considering Area II and IV from Pampa del Indio, 59.9% of seropositive inhabitants yielded *T. cruzi* lineage identification. There is no precedent in the literature of such coverage in a well-defined human population. Most of the previous lineage identification studies comprised human samples collected in hospitals; therefore, the geographical or epidemiological context where the infection originated remains unclear [37–44]. Another constraint for mass lineage identification is the complexity of the traditional genotyping methods, which usually require *T. cruzi* isolation or large blood samples.

There was no association between ethnic group and overall human seroprevalence in Areas II and IV of Pampa del Indio. Nevertheless, Qom communities in Area III are predicted to have higher seroprevalence than

**Table 3** Multivariate analyses for associations with Chagas Sero *K*-SeT, Areas II and IV, Pampa del Indio

| Category | OR (95% CI) | *P*-value | RI |
|---|---|---|---|
| Age | 0.98 (0.93–1.02) | 0.31 | 0.48 |
| Ethnicity | | | 0.45 |
| Creole | 1 | | |
| Qom | 0.38 (0.05–3.06) | 0.36 | |
| Gender | | | 0.27 |
| Female | 1 | | |
| Male | 0.93 (0.56–1.55) | 0.78 | |
| Area | | | 0.82* |
| II | 1 | | |
| IV | 2.05 (1.08–3.88) | 0.03* | |
| Presence of *T. infestans* in the dwelling | | | 0.41 |
| No | 1 | | |
| Yes | 0.69 (0.36–1.32) | 0.26 | |
| Cohabitant with reactive RDT | | | 0.32 |
| No | 1 | | |
| Yes | 0.82 (0.49–1.38) | 0.45 | |
| Age *vs* ethnicity | | | 0.23 |
| Age *vs* Creole | 1 | | |
| Age *vs* Qom | 1.04 (1.00–1.09) | 0.04* | |

*Abbreviations*: OR, odds ratio; CI, confidence interval; RI, relative importance

* Statistically significant

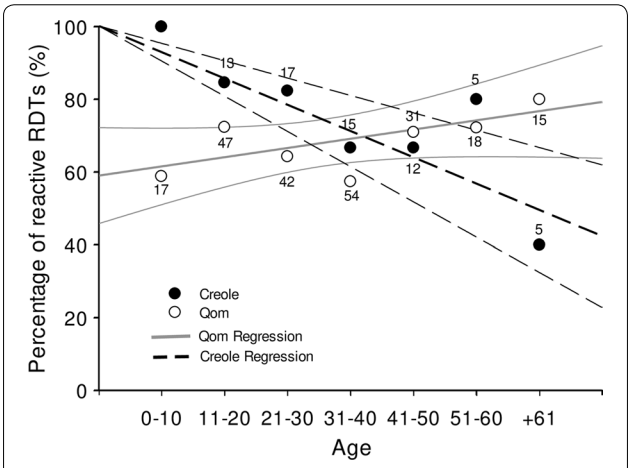


**Fig. 3** Chagas Sero K-Set seroreactivity by age and ethnicity, Areas II and IV, Pampa del Indio. Numbers above dots indicate serum samples analysed for each category. Lines represent linear regressions for each ethnicity

Creole communities because of their lower formal education level, tendency not to apply insecticides and lack of screened windows [45]. Creole households have been observed to have a substantially lower risk of triatomine bug and dog infection compared to Qom households in Area I [25].

We also demonstrate that Chagas Sero *K*-SeT is applicable, without modification, to dogs. In two previous studies on *T. cruzi* lineage-specific serology in Argentine dogs [12, 22], recombinant TSSA-II/V/VI protein was only used in ELISA. As with humans, we found that there was concordance between ELISA and Chagas Sero *K*-SeT RDT, and that a greater number of *T. cruzi* seropositive samples tested by both methods were positive with the RDT, confirming the greater sensitivity.

Although we tested a limited number of dogs born after the community-wide insecticide spraying, dogs born prior to this intervention were over eight times more likely to be TSSApep-II/V/VI seropositive, showing the substantially higher risk before the spraying campaign, as well as cumulative risk with age. Qom dog ownership, rather than Creole, was also associated with higher prevalence of TSSApep-II/V/VI seropositivity (Table 2). Furthermore, dogs were more frequently born in Qom communities rather than Creole, which influenced the age that the dogs entered the household, increasing the likelihood of the dog being exposed to triatomines [25]. Owners were asked if the dogs were hunters or guardians and whether the dogs slept inside or outside the domicile; in both of these categories there were not statistically significant differences in Chagas Sero-*K* SeT result, however, in both cases the categorical divisions may not be entirely definitive.

*Trypanosoma cruzi* infections in cats are not uncommon, and also occur in domestic mice, which are caught and eaten by cats [46]; however, to our knowledge this is apparently the first application of *T. cruzi* lineage-specific serology to cats. TSSApep-II/V/VI positive cats had no association with the environmental and behavioural variables listed in Table 2 (data not shown). The Chagas Sero *K*-SeT failed with cats, not unexpectedly; the utility of Protein A, produced naturally by *Staphylococcus aureus*, rather than Protein G, for binding feline IgG has been reported [47, 48].

Both lineage-specific serology and genotyping indicated the predominance of TcII/V/VI in this endemic region of the Gran Chaco. The Chagas Sero *K*-SeT RDT demonstrated similar prevalence and clustering in humans and dogs, with ELISAs showing prominent TcV/VI infections in dogs and cats. Half of the dogs tested here that reacted by ELISA with TSSApep-II/V/VI also reacted with TSSApep-V/VI. TcV and TcVI are the most common genotypes infecting dogs and cats in this area [33].

Genotyping confirmed the association of TcIII with armadillos [49, 50]. As with the single TcIII infected dog, the Chagas Sero *K*-SeT positivities imply that both that dog and this armadillo were co-infected with TcII, TcV or TcVI. There is clearly a need for more extensive sampling

among armadillos and sylvatic hosts generally. As with felines, the IgG-binding capacity of Protein A has been exploited in studies on armadilloes (*D. novemcinctus*), including the use of Protein A-sepharose columns to isolate Ig [51] and of HRP-conjugated Protein A in ELISA to recognise IgG [52].

There is as yet no reliably effective lineage-specific serology for TcI. Reasons for this are unclear, but may be due to the predicted low antigenicity of this isoform of TSSA [17] and perhaps associated with the lack of an ascribed function for TSSA-I, in contrast to TSSA-II/V/VI [53]. Thus, we cannot exclude some likely co-infections of TcI among the domestic and peridomestic transmission cycles at these study sites. There are relatively low sensitivity ELISAs for TcIII and TcIV [17, 36, 54], and more robust antigens for these lineages would greatly facilitate the study of ecological associations. However, for TcII/V/VI, we have proven here the practicality of deploying lineage-specific serology for surveillance and for enhancing understanding of transmission cycles, and the Chagas Sero *K*-SeT RDT, which is applicable in the field, can give a result in 15 minutes with minimal sample quantities (of whole blood, serum or plasma). Clearly, resolution of the molecular epidemiology of Chagas disease will also continue to benefit from further comparative genomics of *T. cruzi* isolates [55]. Nevertheless, the development of highly sensitive lineage-specific RDTs for all lineages, equally effective for both humans and a wide range of animals, with the aid of Protein G and Protein A detection, would be of great value. This would also allow the enigmatic issue of association of genetic lineage with pathology and prognosis of human Chagas disease to be re-addressed efficiently, and more widely [23].

We acknowledge that the samples used here represent single time-point sampling; however, they provide an antibody profile resultant from both historical and recent *T. cruzi* infections, although that profile may not be comprehensive.

## Conclusions

We have shown that lineage-specific serology can identify *T. cruzi* infecting lineage, without parasite isolation and genotyping. Furthermore, ELISA is replaceable by an at least equally sensitive RDT, the Chagas Sero *K*-SeT, which incorporates Protein G detection, and is thus directly applicable to humans and several other mammalian species. We assessed lineage distribution among 83% of the *T. cruzi*- seropositive human population, showing a statistically significant association of TSSApep-II/V/VI recognition with locality, and with increasing and decreasing age within the Qom and Creole populations, respectively. For dogs TSSApep-II/V/VI seroprevalence was linked to birth before the insecticide spraying programme and with Qom households. The Chagas Sero *K*-SeT is a low cost RDT, applicable for in-the-field surveillance, which can enhance understanding of the transmission pathways and clustering of the lineages, the epidemiology of Chagas disease and the risk of its further emergence from sylvatic cycles. Further research is required to produce corresponding lineage-specific RDTs for *T. cruzi* lineages TcI, TcIII and TcIV, particularly for TcI.

## Supplementary information

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s13071-019-3681-7.

**Additional file 1: Table S1.** *Trypanosoma cruzi* lineage-specific peptides (TSSApep), with polymorphisms underlined. **Table S2.** Available corresponding *T. cruzi* genotyping information from humans and animals tested by TSSA lineage-specific serology.

### Abbreviations

ELISA: enzyme-linked immunosorbent assay; GLM: generalized linear model; IFAT: immunofluorescence antibody test; IgG: immunoglobulin G; PCR: polymerase chain reaction; TSSA: trypomastigote small surface antigen; TSSApep: lineage-specific TSSA peptide; RDT: rapid diagnostic test.

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### Authors' contributions

NM, NPM and MVC generated the data. NM, NPM, MVC, TB and MAM analysed the data. PM, NZ, YG and QG developed and produced reagents. NM, NPM, MVC, TB, MAM and REG wrote the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

The dataset supporting the conclusions of this article are included within the article and its additional files.

### Ethics approval and consent to participate

Before venepuncture, patients or their parents or guardians provided written informed consent. The procedures for human serological diagnosis and etiological treatment (protocol no. TW-01-004) and the study of parasite diversity have been approved by the "Comité de Etica en Investigación Clínica" (Ethics Committee in Clinical Research) of Buenos Aires, Argentina. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Animal care and use were performed according to guidelines issued by the Animal Care and Use Committee at the Faculty of Exact and Natural Sciences, University of Buenos Aires, which is based on the International Guiding Principles for Biomedical Research Involving Animals, developed by the Council for International Organizations of Medical Sciences.Archived serum samples that had been obtained during ongoing field work by trained technical staff and stored at the University of Buenos

Aires were used. Further work and secondary data analysis on these samples was approved by the London School of Hygiene and Tropical Medicine Ethics Committee.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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## Publisher's Note

# CHAPTER 3 GLYCOSYLATION OF *TRYPANOSOMA CRUZI* TCI ANTIGEN REVEALS RECOGNITION BY CHAGASIC SERA

## 3.1 Key points and implications

- Repeated attempts have failed to develop a serological test for the TcI epitope, either using *E. coli* expressed recombinant protein or a synthetic peptide.
- Here, I evaluated recombinant trypomastigote small surface antigen gTSSA-I produced through the eukaryote expression system, *Leishmania tarentolae*, which allows for more naturalistic N- and O- linked glycosylation and protein structure of the antigen.
- gTSSA-I was recognised by chagasic sera from TcI endemic regions (Colombia), overall, 50.7 %. There was no reactivity with gTSSA-II/V/VI indicating no confounding cross reaction with the shared SUMO component of these recombinant antigens.
- To further investigate the antigenicity of gTSSA-I, the recombinant protein was heated to denature the protein structure, and periodate-treated to remove the sugar groups. Recognition of gTSSA-I by the Colombian sera decreased substantially when periodate-treated compared to heating, showing that antigenicity is dependent on glycosylation.
- Unexpectantly, recognition of gTSSA-I was observed with a small number of Gambian Malaria sera samples. This was abolished with heat denaturing, whilst periodate-treatment had little effect, indicating protein structure was important for the cross-recognition.

## 3.2 Candidate's contribution

- Laboratory: performing gTSSA-I ELISAs, including periodate-treatment and heat-denaturation
- Generation and analysis of data
- Prepared draft (text and figures) of manuscript and Corresponding Author for submission to journal *Scientific Reports*.

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| **Thesis Title** | Development of rapid diagnostic tests for Trypanosoma cruzi lineage-specific serology, comparative epidemiology and for monitoring efficacy of chemotherapy. | | |
| **Primary Supervisor** | Michael Miles | | |

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# Glycosylation of *Trypanosoma cruzi* TcI antigen reveals recognition by chagasic sera

Niamh Murphy[1,7]✉, Barrie Rooney[2,3,7], Tapan Bhattacharyya[1], Omar Triana-Chavez[4], Anja Krueger[5], Stuart M. Haslam[5], Victoria O'Rourke[1], Magdalena Pańczuk[1], Jemima Tsang[1], Jack Bickford-Smith[1], Robert H. Gilman[6], Kevin Tetteh[1], Chris Drakeley[1], C. Mark Smales[2] & Michael A. Miles[1]

**Chagas disease is considered the most important parasitic disease in Latin America. The protozoan agent, *Trypanosoma cruzi*, comprises six genetic lineages, TcI-TcVI. Genotyping to link lineage(s) to severity of cardiomyopathy and gastrointestinal pathology is impeded by the sequestration and replication of *T. cruzi* in host tissues. We describe serology specific for TcI, the predominant lineage north of the Amazon, based on expression of recombinant trypomastigote small surface antigen (gTSSA-I) in the eukaryote *Leishmania tarentolae*, to allow realistic glycosylation and structure of the antigen. Sera from TcI-endemic regions recognised gTSSA-I (74/146; 50.7%), with no cross reaction with common components of gTSSA-II/V/VI recombinant antigen. Antigenicity was abolished by chemical (periodate) oxidation of gTSSA-I glycosylation but retained after heat-denaturation of conformation. Conversely, non-specific recognition of gTSSA-I by non-endemic malaria sera was abolished by heat-denaturation. TcI-specific serology facilitates investigation between lineage and diverse clinical presentations. Glycosylation cannot be ignored in the search for immunogenic antigens.**

American trypanosomiasis (Chagas disease) is a neglected tropical disease caused by the protozoan parasite *Trypanosoma cruzi*, and is considered the most important human parasitic infection in Latin America. The infection is transmitted by blood sucking triatomine bugs through contamination of human mucous membranes, abraded skin or food, with *T. cruzi* infected triatomine faeces. Once acquired, unless successfully treated, *T. cruzi* usually persists as a life-long infection, which can also be transmitted congenitally from mother-to-child, and by transfusion or organ donation. Principal human tissues damaged are the heart and the intestinal tract, and symptoms may develop years after the early infection. An estimated 30% of *T. cruzi* infected individuals develop chagasic cardiomyopathy and a proportion of those develop chagasic gastrointestinal megasyndromes[1]. Only two drugs are currently available for treatment, benznidazole and nifurtimox. Due to severe dermatological side effects, treatment of adults frequently fails; a shorter, lower dose schedule for benznidazole and a paediatric formulation have recently been introduced[2,3].

Currently WHO estimates 5–6 million cases of *T. cruzi* infection worldwide[4]. Despite international control programmes, there is still a high prevalence and incidence in regions such as the Gran Chaco of Bolivia and Argentina. Triatomine vectors are spreading into periurban sites, and Chagas disease is becoming a global health issue among Latin American migrant populations, with an estimated 250,000 infected in the USA, more than 100,000 in Europe and 12,000 in the UK, with risk of global non-vector borne transmission, congenitally and via blood and organ donors[5,6].

*Trypanosoma cruzi* comprises six genetic lineages TcI-TcVI[7–9], with TcBat proposed as a seventh lineage related to TcI[10]. Based on genotyping, TcI is the predominant agent of Chagas disease north of the Amazon, with

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TcIV a secondary cause in Venezuela[11]. TcII, TcV and TcVI are prevalent among cases in the Southern Cone countries of South America (Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay); TcIII is uncommonly found in human infections[1].

In 1981[12] it was proposed that the different geographical distributions of the *T. cruzi* lineages may contribute to the disparate clinical presentations of Chagas disease in the Southern Cone countries, where megasyndromes are found, compared to northern South America, where they are not reported[1]. However, it is complex to prove such an association by parasite genotyping, because *T. cruzi* blood parasitaemia is scanty in chronic Chagas disease, does not necessarily represent lineages sequestered in the internal organs[13–16], and growth rate competition occurs between isolates grown in vitro.

One approach to surveillance of clinical, geographical and ecological distributions of the *T. cruzi* lineages is to develop lineage-specific serology, originally proposed by Di Noia et al.[17]. Specific epitopes of the *T. cruzi* trypomastigote small surface antigen (TSSA), a cell surface mucin, have been identified for all six genetic lineages, with the hybrid lineages TcV and TcVI having two epitopes encoded at the heterozygous locus, one of which is shared with TcII, as shown by Bhattacharyya et al.[18]. Lineage-specific serology with synthetic peptides representing the TcII/V/VI and TcV/VI epitopes enabled surveillance of chagasic patients[19], and the discovery of reservoir hosts[20,21]. Furthermore, TcII/V/VI serology, adaptable to rapid diagnostic test (RDT) format, demonstrated that among Bolivian patients stratified by severity of cardiomyopathy, TcII/V/VI seropositives were five-fold more prevalent in the severe versus no evidence of cardiomyopathy groups[22]. RDTs also identified TcII/V/VI seropositive sympatric humans and dogs in the Argentine Chaco[23].

A long-standing research objective is the validation of a robust and sensitive TcI-specific antigen that would enable the enigma of link between infective lineage and clinical prognosis to be more comprehensively investigated. Furthermore, this would enable systematic low-cost analysis of *T. cruzi* transmission cycles and evaluation of the risk of emergence of sylvatic lineages into the domestic environment. However, repeated attempts have failed to develop a lineage-specific serological test for the TcI specific epitope, either using an *E. coli*-expressed recombinant protein or a synthetic peptide[19,24–26].

Here, we have expressed the TSSA-I epitope within a related trypanosomatid, *Leishmania tarentolae,* which enables O-linked and N-linked glycosylation[27,28] to determine whether glycosylation and/or structural integrity impart serological recognition of this TcI antigen.

## Methods

**Ethics.** All human sera used here were archived, with consent for research, were anonymised, coded, and did not reveal patient identities. Informed consent was obtained from all subjects or, if subjects are under 18, consent was provided by a parent and/or legal guardian. No samples were collected specifically for this work.

Colombian (Bogotá), Venezuelan and Ecuadorean samples: these were collected as part of routine diagnostic examination, with local institutional ethical approvals Universidad de los Andes, Bogotá, Colombia; (Instituto de Medicina Tropical, Caracas, Venezuela; Pontificia Universidad Católica del Ecuador, Quito, Ecuador) and in accord with EC ethical standards, established as part of the ChagasEpiNet international collaboration (ethical approval from London School of Hygiene and Tropical Medicine, UK). Colombian (Medellín) ethical approval was provided by the Instituto de Biología, Universidad de Antioquia, Medellín, Colombia.

Peruvian and Bolivian samples: the following institutional review boards granted ethical approval: Johns Hopkins Bloomberg School of Public Health, USA; Hospital Universitario Japonés, Bolivia; Universidad Católica Boliviana, Bolivia; Universidad Peruana Cayetano Heredia, Peru; Asociación Benéfica Proyectos en Informatica, Salud, Medicina y Agricultura, Peru; and the Centers for Disease Control and Prevention, USA.

Gambian malaria sera were provided, with consent for further research on diagnostics, from London School of Hygiene and Tropical Medicine archives.

Non-endemic control sera were provided at the London School of Hygiene and Tropical Medicine and used with consent for further research on diagnostics.

**Sources of chagasic sera.** Chagasic sera were generously provided from the following sources: Colombia (Medellín, n = 55; Bogotá, n = 31); Ecuador (n = 14); Venezuela (n = 4), Peru (n = 42) and Bolivia (n = 10).

**Assessing serological recognition of synthetic peptides by ELISA.** Colombian (Medellín) sera were assayed by ELISA with synthetic peptides TSSApep-I, -II/V/VI, -III, -IV, -V/VI, according to protocols described previously[19]. Colombian (Bogotá), Ecuadorean and Venezuelan sera were previously assayed with synthetic peptides TSSApep-I, -II/V/VI, -III, -IV, -V/VI by ELISA; all were negative with TSSApep-I[19]; Peruvian and Bolivian sera were previously assayed by TSSApep-II/V/VI RDT[22].

**Prediction of glycosylation sites within gTSSA-I recombinant.** In order to determine the presence of O and N glycosylation sites on the TSSA-I epitope, the amino acid sequence was submitted to NetOGlyc 4.0 (www.cbs.dtu.dk/services/NetOGlyc/) and NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/) online servers. Prediction included coverage of the SUMO component of gTSSA-I.

***L. tarentolae*** **production of recombinant antigens gTSSA-I and gTSSA-II/V/VI.** The TSSA-specific sequences were cloned into separate expression plasmids pLEXSY_I-blecherry3 with an upstream His tag and SUMO fusion partner to aid solubility of the resulting recombinant proteins, hereafter called gTSSA-I or gTSSA-II/V/VI, expressed in the *L. tarentolae* system (Jena Biosciences, Germany). Figure 1a,b depict the sequences of these recombinant antigens, with N-terminal histidine tag (blue), SUMO sequence (green) and TSSA-I sequence (red).

**a** gTSSA-I

```
          10         20         30         40         50         60         70
HHHHHHGSDS EVNQEAKPEV KPEVKPETHI NLKVSDGSSE IFFKIKKTTP LRRLMEAFAK RQGKEMDSLR
          80         90        100        110        120        130
FLYDGIRIQA DQTPEDLDME DNDIIEAHRE QISSGHMANG GSTSSTPPGK DKKTAAGGTP SPSGASSG
                                                 ●●●●  ●  ●  ● ●
```

**b** gTSSA-II/V/VI

```
          10         20         30         40         50         60         70
HHHHHHGSDS EVNQEAKPEV KPEVKPETHI NLKVSDGSSE IFFKIKKTTP LRRLMEAFAK RQGKEMDSLR
          80         90        100        110        120        130
FLYDGIRIQA DQTPEDLDME DNDIIEAHRE QISSGHMANG GSTSSTPPSG TENKPATGEA PSQPGASSG
                                                      *
```

**c**

| TSSA residue | | | |
|---|---|---|---|
| **Native** | **gTSSA-I** | **Amino acid** | **Score** |
| 29 | 112 | Ser | 0.98 |
| 30 | 113 | Thr | 0.95 |
| 31 | 114 | Ser | 0.95 |
| 32 | 115 | Ser | 0.99 |
| 33 | 116 | Thr | 0.97 |
| 41 | 124 | Thr | 0.93 |
| 46 | 129 | Thr | 0.90 |
| 48 | 131 | Ser | 0.81 |
| 50 | 133 | Ser | 0.82 |
| 53 | 136 | Ser | - |
| 54 | 137 | Ser | - |

**d**

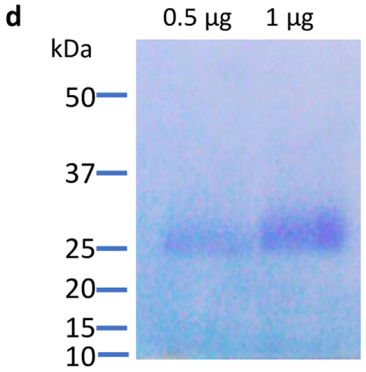


**Figure 1.** *T. cruzi* TSSA recombinant proteins produced in *L. tarentolae* expression system. (**a**) gTSSA-I protein sequence (based on GenBank GU059925) and (**b**) gTSSA-II/V/VI protein sequence (based on GenBank GU075675): histidine tag (blue), SUMO sequence (green), linker sequences (black) and TSSA sequences (red). Dots: Residues predicted to be O-glycosylated. Starred: Ser residue present in native TSSA-II/V/VI but absent in native TSSA-I. Underlined: lineage-specific polymorphic residues. (**c**) Predicted likelihood scores for O-glycosylation of TSSA-I sequence range between 0 (least)—1 (highest); scores below 0.5 not listed. (**d**) Coomassie blue stained gel of purified gTSSA-I.

Production of recombinant protein, based on the methodology of Rooney et al.[29] was carried out in 1 L baffled Erlenmeyer flasks in BHI medium (supplemented with antibiotics and hemin) and the medium was harvested when the $OD_{600}$ reached 4 (approx. 70 h post inoculation, $10^8$ cells/ml). All media components were from Jena Bioscience. Clarified medium was concentrated 20- fold on a Pellicon XL 50 Ultrafiltration cassette (10 kDa MWCO) and diluted four times in binding buffer (20 mM Phosphate, 500 mM NaCl, 10 mM Imidazole) before addition to an equilibrated His Trap (GE Healthcare) FPLC column. Bound proteins were eluted using an increasing Imidazole gradient (20 mM Phosphate, 500 mM NaCl, 500 mM Imidazole). Peak protein containing fractions, as determined by $A_{280}$ nm measurement, were combined, desalted and concentrated by centrifugation in Amicon Ultra-15 device (5 kDa MWCO). Purified recombinant gTSSA-I was run on Coomassie blue-stained pre-cast 4–12% BisTris gradient SDS-PAGE gel (Novex) using the MOPS running system, along with Precision Plus Protein Unstained Standards (BioRad). The final protein was stored at 1 mg/ml in solution in PBS, 15% glycerol at – 20 °C.

**Glycoproteomics.** For glycoproteomics 20 µg of the glycoprotein with the gTSSA-I epitope were denaturated in 10 µL 8 M guanidine hydrochloride solution and reduced with 1 µL of 10 mM DTT (Thermo Fisher Scientific) in 50 mM ammonium bicarbonate buffer (pH 8.4) for 30 min at 56 °C. 1 µL of 55 mM iodoacetamide (Thermo Fisher Scientific) was added and incubated at room temperature for 30 min in the dark. Finally, the digest was diluted with 30 µl of 50 mM ammonium bicarbonate buffer prior to adding 1 µL of trypsin (1:20 w/w enzyme:protein ratio).

The online ESI-LC–MS data were recorded in MSE mode for 60 min using a Waters Synapt G2 mass spectrometer (Waters, Milford, MA). Separations were achieved on C18 Acquity UPLC M-Class column (HSS T3 1.8 µm 75 µm × 150 mm) equilibrated at 40 °C. The mobile phases were A: 0.1% (v/v) formic acid (Biosolve Chemicals) in LC–MS grade water (Greyhound) and B: 0.1% (v/v) formic acid (Biosolve Chemicals) in acetonitrile (Greyhound). Lockmass was set to m/z: 785.80 Glu-1-Fibrinopeptide B (200 nmol/µl, Waters). The Synapt G2-S mass range was operated between 50–2000 m/z.

Initial peptide mapping was carried out using BiopharmaLynx 1.3.3 (Waters, Milford, MA) to locate the epitope region of the gTSSA-I construct in the chromatogram. Interpretations of glycopeptide MS data were

performed manually by previously reported methods based on amino acid and sugar masses together with the known fragmentation of glycopeptides[30], with the assistance of MassLynxV4.1. (Waters, Milford, MA).

**Assessing serological efficacy of the recombinant antigen gTSSA-I.** Separate wells of a 96-well flat bottomed ELISA plates (735–0465: Immulon 4HBX, VWR) were coated with 50 µl/well of 1 µg/ml *L. tarentolae*-expressed gTSSA-I, and with 100 µl/well of 2 µg/ml lysate of *T. cruzi* TcII strains IINF/PY/00/Chaco23 or MHOM/BR/00/Y in coating buffer (15 mM $Na_2CO_3$, 34 mM $NaHCO_3$, pH 9.6). Lysates were prepared as described previousy[19]. After overnight incubation at 4 °C, plates were washed three times with PBS/0.05% (v/v) Tween 20 (P7949: Sigma Aldrich, UK) (PBST), then blocked with 200 µl of PBS/2% skimmed milk powder (PBSM) at 37 °C for 2 h. Following three washes, 100 µl of 1:200 dilutions of serum in PBSM with 0.05% Tween 20 (PBSMT) was applied and incubated at 37 °C for 1 h; after six further washes, 100 µl of donkey anti-human IgG (H + L)-HRP (709–035–149: Jackson ImmunoResearch, USA) diluted 1:2000 (Peruvian and Bogotá sera) or 1:4000 (Medellín, Ecuadorean, Venezuelan, Bolivian sera) in PBSMT was added and incubated at 37 °C for 1 h. Following six washes, wells were developed with 100 µl/well of 50 mM phosphate/citrate buffer (pH 5.0) containing 2 mM o-phenylenediamine HCl (P1526: Sigma Aldrich) and 0.005% (vol/vol) $H_2O_2$ (216763: Sigma Aldrich); the plates were incubated in the dark at room temperature for approximately 10 min. Reactions were stopped by 50 µl/well of 2 M $H_2SO_4$, and absorbance values read at a wavelength of 490 nm. For Gambian malaria samples, sera were used at 1:100 and anti-human IgG 1:2000.

**Excluding cross reaction with the SUMO component of recombinant antigens.** A subset of 34 Colombian (Medellín) samples were assayed for serological cross reactivity with the SUMO component of the recombinant antigens. This was done by parallel performance of ELISAs, as described above, with the recombinant antigen gTSSA-II/V/VI coated in separate wells from the gTSSA-I.

**Assessing the contribution of glycosylation and secondary structure to antigenicity of gTSSA-I.** Contribution of glycosylation to antigenicity was assessed using an assay based on the protocol of Woodward et al.[31], and employed subsequently[32,33], which described the oxidative cleavage of carbohydrate vicinal –OH groups and subsequent reduction of generated aldehyde groups to prevent non-specific antibody binding. After the blocking and washing steps of the ELISA, described above, all wells were rinsed in periodate buffer (50 mM sodium acetate buffer, pH 4.5), and the wells that had been coated with gTSSA-I received 5 mM freshly-made sodium (meta)periodate (71859: Sigma Aldrich) in periodate buffer; the remaining wells received periodate buffer only. Plates were incubated in the dark at room temperature for 1 h, followed by rinsing of all wells with periodate buffer. The periodate-treated wells were then reduced with freshly-made 50 mM sodium borohydride (71320: Sigma Aldrich) in PBS for 30 min; the remaining wells received PBS only. Following this step, wells were washed three times with PBST before addition of sera and subsequent processing, as described above.

To investigate the contribution of secondary structure to the antigenicity of gTSSA-I, an aliquot of gTSSA-I in coating buffer was heated > 95 °C for up to 10 min, prior to coating onto the plate and performance of the serological efficacy was assessed as described above.

**Statistical analysis.** Replica ELISA plates were run in duplicate simultaneously. Cut-off values were determined by first subtracting the background absorbance values (i.e., mean of wells with coating buffer only, no antigen) from the mean reading for each sample; samples that were then greater than three standard deviations above the mean of seronegative non-endemic controls were considered positive. *P* values were determined by performing two-sample T test either unpaired (gTSSA-I against gTSSA-II/V/VI) or paired (unmodified gTSSA-I against oxidised or denatured) with GraphPad Prism (GraphPad Software, San Diego, USA).

## Results

**Synthetic peptides were not recognised by TcI endemic chagasic sera.** All Colombian (Medellín and Bogotá), Ecuadorean, Venezuelan, and northern Peruvian sera samples (n = 146) were from regions considered to be endemic for TcI principally[11], and were predominantly from chronic cases of Chagas disease in rural locations[1,34]. All sera were seropositive with *T. cruzi* lysate; (TcII and TcI lysate antigens do not discriminative between lineage infections). Apart from Colombia (Medellín) and Peruvian samples, all had been previously assayed with synthetic peptide TSSApep-I, and no reaction had been identified[19]. Here, there was no TSSApep-I recognition by the Colombian sera (Medellín) and no indication of the presence of infection with any of the other synthetic peptides.

**Glycosylation prediction.** Figure 1c shows the bioinformatic analysis predicting the likelihood score for O-linked glycosylation of the TSSA-I sequence used in gTSSA-I. No N-linked glycosylation was predicted by NetNGlyc 1.0, nor was any glycosylation predicted on the SUMO component of the recombinant proteins.

**Recombinant antigens produced in *L. tarentolae*: gTSSA-I and gTSSA-II/V/VI.** Recombinant proteins were produced by the *L. tarentolae* LEXSY system and purified by NiNTA from the culture media. The predicted mass is 15 kDa but a broad smear between 25 and 35 kDa is typical for a glycosylated protein (Fig. 1d, and supplementary Figure S1).

| Source | gTSSA-I reactive (%) |
|---|---|
| Colombia (Medellín) | 47/55 (85.5%) |
| Colombia (Bogotá) | 7/31 (22%) |
| Ecuador | 6/14 (42.9%) |
| Venezuela | 2/4 (50%) |
| Peru | 12/42 (28.6%) |

**Table 1.** Recognition of gTSSA-I by chagasic sera from northern South America.

**Mass spectrometry analysis proves glycosylation of recombinant gTSSA-I.** Confirmation of glycosylation was demonstrated by mass spectrometry based glycoproteomics. An O-glycosylated glycopeptide was identified, consisting of the sequence TAAGGTPSPSGASSG substituted with a single N-acetylhexosamine (HexNAc) residue (Supplementary Figure S2). Such O-glycosylation with a single HexNAc residue is consistent with previous reports of *L. tarentolae* recombinant glycoprotein glycosylation, confirming the prediction of the NetOGlyc 4.0 programme[28]. As predicted by NetNGlyc 1.0, no N-linked glycosylation was found.

**Recognition of gTSSA-I.** Overall (74/146; 50.7%) of chagasic sera from TcI endemic regions of northern South America were seropositive with gTSSA-I (Table 1).

**Absence of SUMO cross-reactivity between gTSSA-I and gTSSA-II/V/VI.** A subset of Colombian (Medellín) sera positive for gTSSA-I (n = 34) were assayed by ELISA against gTSSA-II/V/VI, to assess levels of potential cross-reactivity to the SUMO component of gTSSA-I. No recognition of gTSSA-II/V/VI was observed with these sera (Fig. 2a). Grouping these data, there was a significant difference in the absorbance values between these two recombinant antigens ($P < 0.0001$) (Fig. 2b). Thus, there was no cross reactivity between these two recombinant antigens attributable to antibody recognition of the SUMO component. Bolivian sera shown previously to be reactive with synthetic peptide TSSApep-II/V/VI (n = 5) reacted with gTSSA-II/V/VI; two sera also recognising gTSSA-I (Fig. 2c), suggesting TcII/V/VI and TcI co-infection.

**gTSSA-I antigenicity is principally dependent on glycosylation, not structure.** A subset of Colombian (Medellín) samples that recognised gTSSA-I (n = 12) were assayed against periodate-treated, heat denatured and unmodified gTSSA-I on the same ELISA plates. Recognition of gTSSA-I decreased substantially after periodate treatment, compared to the heat denatured antigen, for all samples except sample 2 (Fig. 3a). The absorbance values were significantly lower ($P = 0.0002$) after periodate treatment, with only a single sample retaining the same level of recognition as with the unmodified antigen. After heat denaturation the absorbance values of all 12 samples were reduced to a much lesser extent, albeit with a significant difference ($P < 0.0001$) (Fig. 3a).

**gTSSA-I recognition by some Gambian malaria sera is dependent on structure.** Unexpectedly, recognition of gTSSA-I was observed with 13/24 Gambian malaria sera samples. Eight of these reactive samples were assayed against periodate-treated (oxidised) and heat denatured gTSSA-I. Oxidation had little effect on recognition of gTSSA-I, with no significant difference between absorbance values ($P = 0.5732$) and recognition of gTSSA-I was retained by all 8 samples. However, this was abolished by heat denaturation of this antigen ($P = 0.0089$). One sample remained above the cut-off, though the absorbance value had been greatly reduced (Fig. 3b,c). Any recognition of gTSSA-I by non-endemic healthy controls (NEHC) was also abrogated by heat-denaturation.

## Discussion

Lineage-specific serology for *T. cruzi* infections provides a powerful tool for understanding the epidemiology and ecology of Chagas disease. Here, we have investigated lineage-specific epitopes to determine the importance of glycosylation in antigen recognition, particularly in relation to TcI.

The *L. tarentolae* expression system has been used previously to produce *Trypanosoma brucei gambiense* surface antigens, as a low cost alternative to harvesting diagnostic antigens from *T. b. gambiense* grown *in vitro*[29]. Expression in *L. tarentolae* has also been applied in attempts to improve diagnostic antigens for the trypanosomatids *Leishmania braziliensis*[35] and *Leishmania donovani*[36].

Here, we applied *L. tarentolae* expression to produce recombinant *T. cruzi* gTSSA-I, and to determine whether consequent glycosylation or more *bona fide* structural conformation of the protein conferred serological recognition upon the antigen.

All Colombian, Venezuelan, and Ecuadorean sera that were positive here with gTSSA-I were previously seronegative with the synthetic peptide TSSApep-I. The Medellín samples showed by far the highest proportion that recognised gTSSA-I; 85.5%. Reasons for this are unclear, but may be related to lower levels of anti-*T. cruzi* IgG in the other sera; in some localities there are low IgG antibody levels in *T. cruzi* infections[37]. Furthermore, the gTSSA-I positive samples were not seropositive with gTSSA-II/V/VI, demonstrating that there was no serological cross reaction with the SUMO component of the recombinant antigens. Thus, we have demonstrated

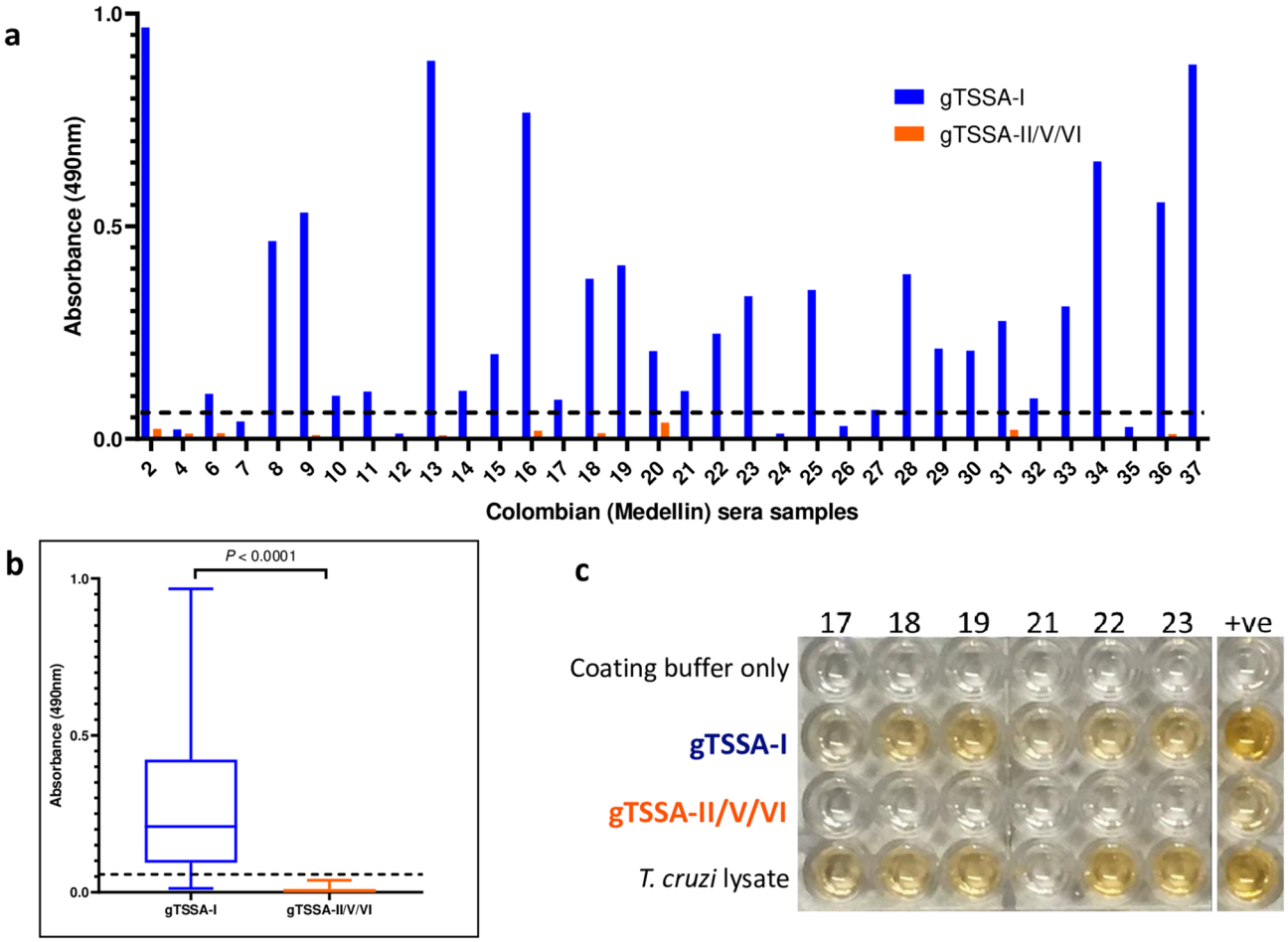


**Figure 2.** Antigenicity of gTSSA-I is due to TSSA-I sequence. (**a**) Absorbance values with Colombian (Medellín) sera recognising gTSSA-I (blue bars) and gTSSA-II/V/VI (orange bars). (**b**) Overall absorbance values for Colombian (Medellín) sera against gTSSA-I (blue) and gTSSA-II/V/VI (orange); gTSSA-I is recognised, whereas gTSSA-II/V/VI is not ($P < 0.0001$). (**c**) ELISA plate illustrating the recognition of gTSSA-I but not of gTSSA-II/V/VI; all samples were seropositive with lysate, and coating buffer controls were negative. Sample numbers correspond with (**a**). Positive control: serum from a Bolivian patient previously shown to be reactive with synthetic peptide TSSApep-II/V/VI and also seropositive with gTSSA-I, indicating co-infection (see text).

clear, robust TcI lineage-specific serology with sera originating from countries where TcI has been identified by genotyping as the principal cause of Chagas disease.

In comparison with synthetic peptides or production of recombinants in the bacterium *E. coli,* heterologous expression in the eukaryote *L. tarentolae* enables glycosylation of the trypanosomatid proteins and adoption of a more natural conformation. O- and N-linked glycosylation of recombinant proteins in *L. tarentolae* has been described, and the wild type pattern in *T. cruzi* also shown to be O-linked (GlcNAc)[28,38]. Assays with periodate-treated antigen showed that the protein glycosylation (predicted by the online algorithms and demonstrated by glycoproteomics), and not the protein conformation, was important for serological recognition of gTSSA-I. Thus, expression in *L. tarentolae* revealed antigenic properties of TSSA-I that are not evident in synthetic peptides or *E. coli*-expressed recombinant proteins.

The specificity of gTSSA-I was questioned, due to some cross-reactivity with non-endemic (Gambian) malaria control sera. The sera from malaria were included due to breadth of immune response associated with antigenic variation, and recent research interest in whether antibodies to *Plasmodium* may recognise glycosylated antigens[39]. Two of these controls were also seropositive with the lateral flow diagnostic test specifically to detect exposure to *T. b. gambiense* infection (data not shown), suggesting an explanation for their cross reactivity. However, heating gTSSA-I abolished recognition by all Gambian malaria sera, demonstrating that this was due to analogous protein structure of gTSSA-I, not glycosylation. Since this is contrary to gTSSA-I recognition by chagasic sera, heating of gTSSA-I prior to performance of diagnostic ELISA or to incorporation into RDTs provides TcI-specific diagnosis via recognition of glycosylation antigenicity.

We have previously demonstrated a link between serological recognition of TSSApep-II/V/VI and severity of chagasic cardiac symptoms[19,22]. Availability of gTSSA-I serology enables parallel investigation of clinical status associated with TcI infection, and serological detection of sporadic TcII/V/VI and TcI co-infections, which occur in some Bolivian and Brazilian endemic foci[40,41]. As with application of TSSA-II/V/VI serology to sylvatic

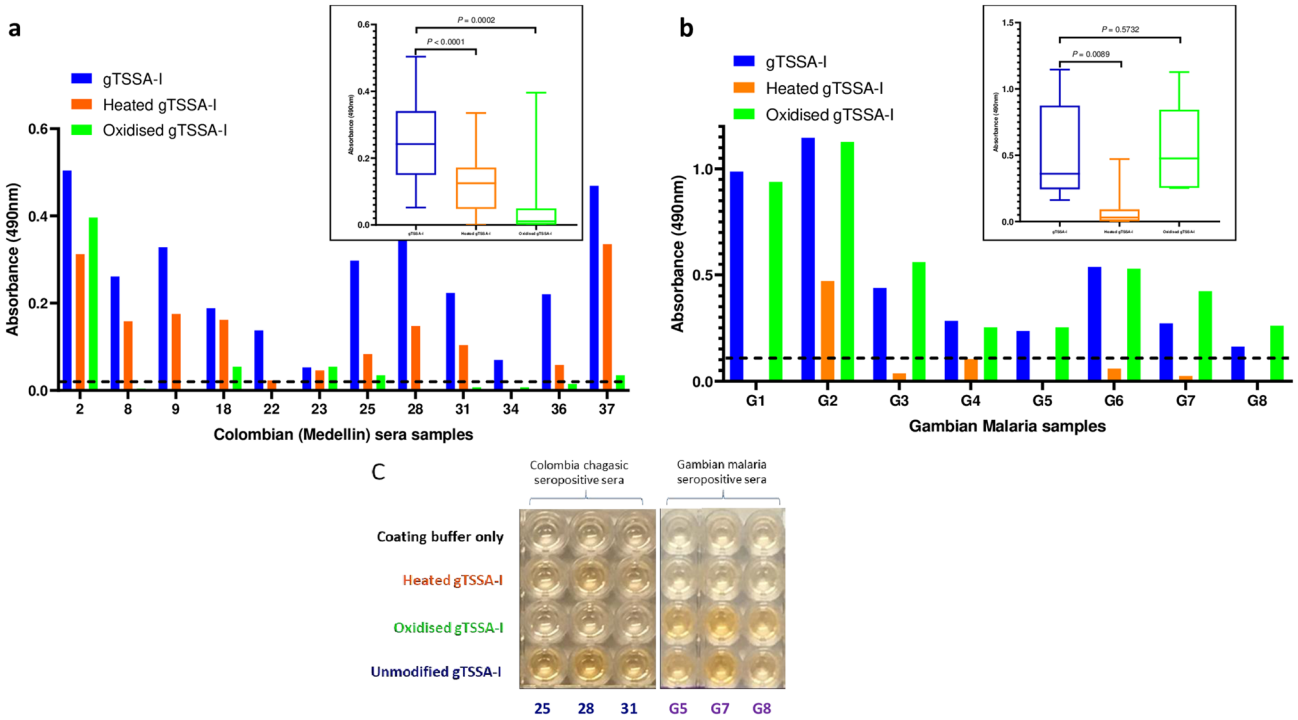


**Figure 3.** gTSSA-I antigenicity is dependent on glycosylation, whereas recognition by non-endemic malaria sera is dependent on structure. (**a**) Absorbance values of Colombian Medellín sera recognising unmodified gTSSA-I (blue bars), heat denatured gTSSA-I (orange bars) and oxidised gTSSA-I (green bars); oxidation decreases absorbance values substantially more than heat denaturing for all samples except 2. (**b**) Absorbance values of Gambian malaria (G) sera recognising unmodified gTSSA-I (blue bars), heat denatured gTSSA-I (orange bars) and oxidised gTSSA-I (green bars); heat denaturing decreases absorbance values substantially, whereas oxidation does not. For each of the data sets represented in (**a**) and (**b**) individual samples are presented on the main graph and composite absorbance values in the inset box-and-whisker plots. (**c**) ELISA plate illustrating the effects of modifications: coating with of unmodified gTSSA-I (fourth row); heat denatured gTSSA-I (second row), with slight signal reduction for Colombian sera but ablation for Gambian malaria sera; oxidised gTSSA-I (third row), with considerable reduction for Colombian sera, yet no reduction with Gambian malaria sera; coating buffer only (no antigen) control (first row). Sample numbers correspond with (**a**) and (**b**).

mammals[20,21,23,42,43], gTSSA-I can be deployed for resolution of TcI domestic and sylvatic transmission cycles, and for discovery of reservoir hosts. Thus, expression of *L. tarentolae* recombinant antigens representing epitopes specific to each of the *T. cruzi* lineages, may facilitate comprehensive epidemiological investigations. Our results encourage *L. tarentolae* expression to improve efficacy of candidate diagnostic antigens.

Proteins have been preferentially pursued for decades as highly sensitive and specific diagnostic antigens, and as vaccine candidates. Perhaps most importantly, as recognised in recent research on malaria[39] and bacteria[44] we have indicated here that glycosylation cannot be ignored in the search for improved diagnostics or efficacious vaccines.

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## Author contributions

Produced the recombinant antigens: B.R., C.M.S. Provided sera: O.T.C., R.H.G., K.T., C.D. Performed glycoproteomics: A.K., S.M.H. Performed ELISAs: N.M., T.B., V.O.R., M.P., J.T. Analysed the data: N.M., B.R., T.B., J.B-S., M.A.M. Wrote the manuscript: N.M., T.B., B.R., M.A.M. Supervised the project: M.A.M.

## Competing interests

The authors declare no competing interests.

## Additional information

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# CHAPTER 4 ASSESSING ANTIBODY DECLINE AFTER CHEMOTHERAPY OF EARLY CHRONIC CHAGAS DISEASE PATIENTS

## 4.1 Key points and implications

- Unless successfully treated, *T. cruzi* usually persists as a life-long infection and treatment options are limited. Currently there are no distinctive parasitological or serological biomarkers of cure and seroconversion from positive to negative may take many years, hindering patient care and management.
- In a pilot study a decrease was shown in IgG1 titre against *T. cruzi* lysate by ELISA in three out of seven Bolivian migrants living in Spain one-year post treatment. There was no antibody decline for IgG2 and IgG4, and one patient showed a decline in IgG3.
- For the main study described here, 71 patient pre- and post-treatment samples from the Argentine Chaco were assayed for IgG and IgG1 antibody titres against TSSApep-II/V/VI and *T. cruzi* lysate. All except one of the 71 post-patient samples remained positive by conventional serology, whilst kDNA-PCR was negative. The cohort was split into two groups, Group A: completed treatment (n=58) and Group B: reported interruption of treatment (n=13).
- The 71 Argentine patient samples were screened first with the Chagas Sero K-SeT RDT to detect recognition of TSSApep-II/V/VI; of these 91.5 % were positive, confirming the predominance of these lineages in this area.
- IgG and IgG1 of Argentine patients showed significant decline post chemotherapy against both lysate and TSSApep-II/V/VI. IgG1 decline was more discriminative than IgG, the Chagas Sero K-SeT was found to be the least discriminative.
- Incomplete treatment was associated with high IgG1 post-treatment titres against lysate, as were IgG post-treatment titres to TSSApep-II/V/VI.
- Due to restricted sensitivity, IgG1 should not be used as a diagnostic marker but has promise, with further development, as a biomarker of cure.

## 4.2 Candidate's contributions

- Planned and performed all pre-and post-treatment ELISAs and RDTs in the main study.
- Generated and analysed data, including statistical analysis with two-tailed paired t-tests and un-paired two-sample t-tests.

- Prepared draft (text and figures) of manuscript and Corresponding Author for /submission to journal *Parasites & Vectors*.

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| **Thesis Title** | Development of rapid diagnostic tests for Trypanosoma cruzi lineage-specific serology, comparative epidemiology and for monitoring efficacy of chemotherapy. | | |
| **Primary Supervisor** | Michael Miles | | |

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Parasites & Vectors

RESEARCH

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# Assessing antibody decline after chemotherapy of early chronic Chagas disease patients

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## Abstract

**Background:** Chagas disease remains a significant public health problem in Latin America. There are only two chemotherapy drugs, nifurtimox and benznidazole, and both may have severe side effects. After complete chemotherapy of acute cases, seropositive diagnosis may revert to negative. However, there are no definitive parasitological or serological biomarkers of cure.

**Methods:** Following a pilot study with seven Bolivian migrants to Spain, we tested 71 serum samples from chronic patients (mean age 12.6 years) inhabiting the Argentine Chaco region. Benznidazole chemotherapy (5–8 mg/kg day, twice daily for 60 days) was administered during 2011–2016. Subsequently, pre-and post-chemotherapy serum samples were analysed in pairs by IgG1 and IgG ELISA using two different antigens and Chagas Sero K-SeT rapid diagnostic tests (RDT). Molecular diagnosis by kDNA-PCR was applied to post-treatment samples.

**Results:** Pilot data demonstrated IgG1 antibody decline in three of seven patients from Bolivia 1 year post-treatment. All Argentine patients in 2017 (averaging 5 years post-treatment), except one, were positive by conventional serology. All were kDNA-PCR-negative. Most (91.5%) pre-treatment samples were positive by the Chagas Sero K-SeT RDT, confirming the predominance of TcII/V/VI. IgG1 and IgG of Argentine patients showed significant decline in antibody titres post-chemotherapy, with either lysate (IgG, $P=0.0001$, IgG1, $P=0.0001$) or TcII/V/VI peptide antigen (IgG, $P=0.0001$, IgG1, $P=0.0001$). IgG1 decline was more discriminative than IgG. Antibody decline after treatment was also detected by the RDT. Incomplete treatment was associated with high IgG1 post-treatment titres against lysate ($P=0.013$), as were IgG post-treatment titres to TcII/V/VI peptide ($P=0.0001$). High pre-treatment IgG1 with lysate was associated with Qom ethnicity ($P=0.045$). No associations were found between gender, age, body mass index and pre- or post-treatment antibody titres.

**Conclusions:** We show that following chemotherapy of early chronic Chagas disease, significant decline in IgG1 antibody suggests cure, whereas sustained or increased IgG1 is a potential indicator of treatment failure. Due to restricted sensitivity, IgG1 should not be used as a diagnostic marker but has promise, with further development, as a biomarker of cure.

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**Keywords:** *Trypanosoma cruzi*, Chagas disease, Serology, ELISA, Rapid diagnostic test, IgG, IgG1, Pre-treatment, Post-treatment

## Background

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, remains a major cause of disability in the Americas, particularly in the Gran Chaco region of Argentina, Bolivia and Paraguay. *Trypanosoma cruzi* is primarily transmitted via infected faeces of the triatomine bug vector, during a blood meal, when the parasite can enter the host through mucosal membranes and abraded skin. Transmission may also be congenital, by blood or organ donation, and orally via triatomine contamination of food or drink [1]. The initial acute phase of Chagas disease is often asymptomatic or without specific symptoms, although fatalities may occur [2]. The subsequent chronic phase may develop years later, in about 30% of individuals, principally with cardiomyopathy, and/or megasyndromes of the oesophagus and colon [3]. Infection can be cleared by a full course of chemotherapy with benznidazole (or nifurtimox). However, both drugs require prolonged treatment (30–60 days), and can be interrupted by severe adverse effects, particularly in adults. Delivery of chemotherapy has gained renewed impetus in the last 10 years, and treatment is now more accessible to rural communities [4–7] and urban centres [8]. However, the potential for improving long-term prognosis and for controlling transmission is usually lost due to the lack of early diagnosis and treatment, and delay in delivering insecticide control of infested dwellings, respectively [9].

Serological techniques to identify anti-*T. cruzi* immunoglobulin G (IgG), which are used principally in the chronic phase when parasites are sequestered in the tissues and rare in the circulating blood [10], include the enzyme-linked immunosorbent assay (ELISA), indirect haemagglutination (IHA), indirect immunofluorescence (IIF) and several commercial rapid diagnostic tests (RDTs), among the most commonly employed [10–12]. However, tests vary in practicality, sensitivity and specificity, and can be discordant between patients from different geographical locations [13]. During the chronic phase, other diagnostic techniques can be used, including molecular methods, for example kDNA-PCR, which amplifies sequences in the *T. cruzi* kinetoplast, a dense network structure of repetitive mitochondrial DNA, but these methods may lack sensitivity due to the paucity of circulating parasites. Therefore, serological identification of *T. cruzi*-specific IgG antibodies is considered the standard. However, the World Health Organization recommends at least two tests using different methods and/or detecting antibodies to different antigens and potentially a third test if the results are conflicting, in order to make a definitive diagnosis [14, 15]. Many biomarkers have been assayed as criteria of cure; however, reversion of conventional serology from positive to negative is considered the best and most reliable indicator of successful parasitological cure [14]. Nevertheless, except in treatment of initial acute cases or in the chronic phase during early childhood, the decline of conventional antibody (IgG) titres may take many years [16, 17], and patients therefore remain without confirmation of treatment outcome. Not having a definitive answer soon after chemotherapy is a fundamental impediment that can complicate patient management, and patients may be unwilling to start prolonged drug treatment if there is a risk of adverse side effects, with uncertain improvement of clinical prognosis, such as prevention of cardiomyopathy [18, 19]. Furthermore, with increased national and international migration, long-term patient follow-up is proving difficult and impractical. Thus, an early biomarker of cure is urgently needed [20].

*Trypanosoma cruzi* is composed of six genetic lineages or discrete typing units (DTUs), TcI–VI [21], with a possible seventh, TcBat [22]. TcI, TcII, TcV and TcVI are the most common in human infections, whilst TcIII and TcIV are principally associated with sylvatic cycles. It has long been proposed that the differing lineages may contribute to the varying clinical forms of Chagas disease throughout South America [23].

Various *T. cruzi* antigens or antigenic fractions that elicit a serological response have been evaluated for post-treatment biomarkers [24–26], with relative success [24]. The MultiCruzi assay, a serological assay incorporating 15 *T. cruzi*-specific antigens, when used with an interpretation formula, has been proposed for use as a predictive tool to assess parasitological cure in infants and children [27]. In paediatric cases post-chemotherapy, antibody titres to the trypomastigote small surface antigen (TSSA) shared by TcII, TcV and TcVI (TSSA-II/V/VI) decreased significantly faster than those against crude parasite homogenates [28]. Serology with a synthetic peptide TSSApep-II/V/VI epitope also revealed an association between seropositivity and severity of chagasic cardiomyopathy [29], and ELISAs and RDTs with protein G detection had the capacity to resolve host, ecological and epidemiological associations in the Argentine Chaco [30].

IgG is the most common class of immunoglobulin in human serum, the major antibody of the secondary

immune response and is split into four subclasses, IgG1, IgG2, IgG3 and IgG4. IgG1 is at the highest levels in adult sera, with a relative abundance of 60% [31]. In comparison, although children are born with a relatively high level of IgG1 from the mother, this quickly drops to low levels due to non-sustained antigenic stimulation. From 6 months of age, the level of IgG1 increases, and by 5 years of age the level of IgG1 is 75% of that found in adult sera [32].

During the acute stage of infection, IgG is found at relatively low levels, and IgM is the most abundant antibody; however, as the infection changes from acute to chronic, there is a switch to IgG [33]. Of the four subclasses of IgG, IgG1, IgG2 and IgG3 are found at high titres during *T. cruzi* infection, with IgG1 being the most abundant, whereas IgG4 is at relatively low levels [34, 35]. Increased anti-*T. cruzi* IgG1 titres have also been associated with increased severity of Chagas disease cardiomyopathy [33]. Here we address whether IgG1 may be an early biomarker of cure after treatment of chronic Chagas disease. Following a pilot study, we assess IgG and IgG1 antibody decline in treated early chronic Chagas disease patients living in the Argentine Chaco where domestic transmission was interrupted, using separately whole cell lysate and TSSApep-II/V/VI antigens, and we show that IgG1 is more discriminative as a biomarker for assessing cure than IgG, irrespective of antigen.

## Methods

### Pilot serological study

For a pilot cohort of chronic patients ($n=7$, all of Bolivian origin) presenting in Barcelona, Spain, serum samples at 0, 60 and 365 days (post-treatment) were assayed for anti-*T. cruzi* IgG and IgG1 levels by ELISA against *T. cruzi* lysate (described below). The data from these preliminary assays informed and encouraged the wider investigation in the main study.

## Main study

### Sample collection, serological surveillance and treatment in Argentina

Field work took place in the rural area of Pampa del Indio, Chaco Province, in northern Argentina [36]. In this municipality, intense domestic transmission of *T. cruzi* occurred and was suppressed by sustained actions against *Triatoma infestans,* the main vector, as part of an ongoing intervention programme launched in 2007, which virtually eliminated domestic infestation by the second year post-interventions [37–40]. During 2010–2016, we scaled up delivery of serodiagnosis and chemotherapy of seropositive people in the rural area, divided for operational reasons (areas I–IV) and achieved approximately 50% of serodiagnosis coverage of nearly 9000 inhabitants (Additional file 1: Figure S1). Patients included members of the Qom and Creole ethnic groups. The seroepidemiology and long-term impact of sustained vector control on domestic transmission are described elsewhere [40–43].

All serum samples were preserved at −20 °C until assayed for *T. cruzi* infection by conventional serology. In Argentina, the WHO guidelines for conventional serology are followed: the use of two serological tests, either ELISA, or IHA of IIF, detecting different parasite antigens or whole parasites [14, 15]. Here, duplicate ELISAs with non-recombinant (Chagastest, Wiener) and recombinant antigens (ELISA Rec v3.0, Wiener) were used, according to the manufacturer's instructions. A serum sample was considered seropositive if reactive in two different assays [36]. Two serologically discordant human samples were sent to the National Institute of Parasitology "Dr. Mario Fatala Chabén" (ANLIS-Malbrán, Buenos Aires, Argentina) for final serodiagnosis, where they were tested by IHA, ELISA and IIF.

Benznidazole (5–8 mg/kg day) was administered twice daily for 60 days to all seropositive individuals, except in 2012 when nifurtimox was used (8–10 mg/kg day) (Additional file 1: Figure S1). Chemotherapy rounds were launched between 2011 and 2016. For the cohort treated in 2011, blood samples by venipuncture were taken by local physicians at day 0, 20–30, 60 and 180 post-treatment. Conventional serological tests were performed, and molecular diagnosis was applied by qPCR and kDNA-PCR to determine the infection status of the patients in the cohort treated in 2011 [36]. For this cohort, pre-treatment samples were collected between November 2010 and January 2011. The subsequent post-treatment samples were collected, as described above, when patients were resampled in 2017 in house-to-house visits.

### Molecular assays

For DNA extraction, samples were mixed immediately after collection with an equal volume of 6 M guanidine hydrochloride and 0.2 M EDTA in pH 8.0 buffer. Guanidine/EDTA blood samples were heated in a boiling water bath for 15 min. Total DNA was purified using a DNeasy Blood & Tissue Kit (Qiagen, USA) according to manufacturer's instructions, slightly modified to exclude proteinase K and buffer AL [44] and eluted in 200 µl of distilled water. A total of 24 out of 34 pre-treatment Argentinean samples were positive by kDNA-PCR [36].

### Chagas Sero K-SeT RDT

As previously described [29, 30], the Chagas Sero K-Set is a novel RDT manufactured by Coris BioConcept using TSSApep-II/V/VI (GTENKPATGEAPSQPG) as the

antigen and colloidal gold-conjugated protein G detection of bound IgG. Each test was assessed visually and independently by two individuals after 15 min of incubation. A test was considered valid if the control line was present, determined as positive if there was a signal of any intensity at the test (antigen) line, and negative if there was no signal at the test line. The intensity of signal at the test band was assessed visually as strong, weak or absent.

### ELISA

Immulon 4HBX 96-well flat-bottom ELISA plates (735–0465, VWR, UK) were divided into 16 sections of 3 columns × 2 rows of wells, to allow simultaneous assay of IgG1 and IgG paired pre- and post-treatment samples, as further described below. Wells were coated either with 100 µl/well of 1 × coating buffer (15 mM $Na_2CO_3$, 34 mM $NaHCO_3$, pH 9.6) as a no-antigen control or with TSSApep-II/V/VI synthetic peptide (5 µg/ml) or lysate of *T. cruzi* TcI strain ISAN/US/00/Florida (2 µg/ml, prepared as described in [45]) diluted in 1× coating buffer. Following overnight incubation at 4 °C, plates were washed three times with PBS/0.05% Tween (PBST), then blocked with 200 µl/well of PBS/2% skimmed milk powder at 37 °C for 2 h. After three washes, 100 µl/well of serum at 1:200 dilution in PBST/2% milk was applied, such that for each paired sample per plate section, the upper row contained pre-treatment serum, and the lower row post-treatment serum. Following incubation at 37 °C for 1 h and six washes in PBST, 100 µl/well of horseradish peroxidase-labelled anti-human IgG (709–035-149, Jackson ImmunoResearch, USA) or anti-human IgG1 (ab99774, Abcam, UK), diluted 1:5000 in PBST/2% milk, were added to the second and third columns of each section, respectively. The first column of each section received PBST/2% milk only. After 1 h of incubation and six PBST washes, 100 µl/well of substrate solution (50 mM phosphate/citrate buffer, pH 5.0) containing 2 mM *o*-phenylenediamine HCl (P1526, Sigma-Aldrich) and 0.009% $H_2O_2$ (216,763, Sigma-Aldrich) was added to the entire plate, which was then incubated in the dark. Reactions were stopped with 50 µl/well of 2 M $H_2SO_4$, and absorbance values were measured at 490 nm. Duplicate (replica) plates were performed simultaneously, and mean results obtained. Cut-off values were calculated by subtracting the plate background absorbance values from each of the samples, including the negative controls; samples that were higher than the mean of the negative controls plus three standard deviations were considered positive. Only Chagas Sero K-Set RDT-reactive samples were assayed with TSSApep-II/V/VI ELISA; other lineage-specific RDTs are not yet available [30, 46].

### Data analysis

Two-tailed paired *t*-tests (pre- and post-treatment samples), unpaired two-sample *t*-tests or one-way ANOVA were used to determine statistical significance (GraphPad Prism, 8.4.3, San Diego, CA, USA). Values of $P<0.05$ were considered statistically significant.

A serum sample was considered to decline significantly (herein called "clear decline") if absorbance dropped by 50% (ELISAs) or exhibited a prominent decrease in RDT test line intensity (Additional file 2: Figure S2). For a given assay, a reactive serum pre-treatment and non-reactive post-treatment was considered seronegativisation. Re-examined patients ($n=71$) were grouped according to whether they completed the 60 days of chemotherapy. Thirteen of the 71 patients reporting interruption of therapy [36] were designated as group B (age range, 2–20 years), and those with completed treatment [36] or self-reporting completion as group A (age range, 6–19 years), including the patient with therapeutic failure [36]. We compared the percentage of patients showing clear decline of antibodies or seronegativisation between groups by means of Fisher's exact tests. Group B patients were excluded from IgG and IgG1 response pre-/post-treatment, but group A and group B were compared in the univariate analysis. We merged our database with that previously published [36], updating the data for corresponding patients, to investigate the association of antibody titres with individual patient data (i.e., age, body mass index [BMI], ethnic group). The relationship between antibody decline (for each serological test) and age at treatment, treatment groups and time elapsed since treatment (in years) was tested by multiple logistic regressions implemented in R using "lme4" and "sjPlot" packages [47–49]. Continuous variables were standardised.

## Results

### Pilot study shows trend of IgG1 decline in paired pre- and post-treatment sera

To assess the trend of IgG subclass titres post-chemotherapy, sequential serum samples from seven adult Bolivian patients living in Barcelona were assayed by ELISA against *T. cruzi* lysate at 0, 60 and 365 days post-treatment. IgG1 antibody titre declined in three (42.3%) participants 365 days post-treatment (Fig. 1), whilst there was no antibody decline for IgG2 or IgG4; one participant showed IgG3 antibody decline 365 days post-treatment (data not shown).

## Main study

### Conventional serology remains positive after chemotherapy

Conventional serological assays were used with all 71 samples collected in 2017; all except one remained consistently positive. People re-examined were on average

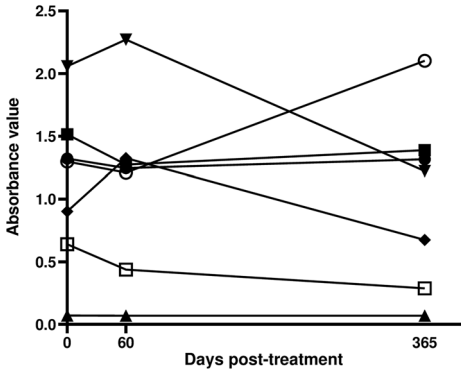


**Fig. 1** Pilot study supports IgG1 decline post-treatment: ELISA absorbance values are shown for individual participants at 0, 60 and 365 days post-treatment. Each line represents an individual patient. Three of seven show a decline in IgG1

12.6 years of age when treated; 47.9% were female, 52.1% were Qom descendants, and on average 5.0 years had elapsed since treatment. Around a third (34%) of the study people had moved from their original household to a new residence within the municipality when revisited in 2017.

### Molecular assays

kDNA-PCR was carried out with post-treatment samples collected in 2017 to indicate presence of parasitaemia. The predicted 330-bp amplicons were produced with pre-treatment samples but not produced with post-treatment samples (data not shown). All of the 71 post-treatment samples were negative.

### TSSApep-II/V/VI seropositivity by Chagas Sero K-SeT RDT

All 71 pre-treatment samples were initially screened with this RDT to detect recognition of TSSApep-II/V/VI. Fifty-four of the 58 pre-treatment samples from group A and 11 of the 13 pre-treatment samples from group B were positive by this RDT (Table 1) giving an overall seropositivity of 91.5%.

### Comparison between treatment groups

We found a significantly higher percentage of patients showing a clear decline of antibody titres after treatment in group A than in group B when assayed against TSSApep-II/V/VI ELISA with IgG (Fisher's exact test, $P=0.04$; OR = 0.04; 95% CI = 1.07–23.33). We found no significant between-group differences in the percentage of positive patients before treatment and in those who became seronegative after treatment (Fisher's exact test, $P \geq 0.1$ in all cases) (Table 1).

**Table 1** Changes in anti-*T. cruzi* seroreactivity according to assay and treatment group

| Antigen | Number positive/examined (%) by detection assay | | | | |
|---|---|---|---|---|---|
| | Protein G: Chagas Sero K-SeT | IgG1 ELISA | | IgG ELISA | |
| | TSSApep-II/V/VI | Lysate | TSSApep-II/V/VI[a] | Lysate | TSSApep-II/V/VI[a] |
| Group A ($n=58$) | | | | | |
| Positive (pre-treatment) | 54/58 (93.1%) | 46/58 (79.3%)[f] | 22/53[b] (41.5%)[f] | 57/58 (98.3%)[g] | 41/53[b] (77.4%)[g] |
| Clear decline | 25/54 (46.3%) | 35/46 (76.1%) | 22/22 (100%) | 21/57 (36.8%) | 34/41 (82.9%)[e] |
| Seronegativisation | 11/54 (20.4%) | 19/46 (41.3%)[h] | 15/22 (68.2%)[i] | 6/57 (10.5%)[h] | 18/41 (43.9%)[i] |
| Remained seronegative[c] | 4/58 (6.9%) | 12/58 (20.7%) | 30/53[d] (56.6%) | 1/58 (1.7%) | 12/53[d] (22.6%) |
| Group B ($n=13$) | | | | | |
| Positive (pre-treatment) | 11/13 (84.6%) | 11/13 (84.6%) | 7/10[b] (70.0%) | 13/13 (100%) | 10/10[b] (100%) |
| Clear decline | 5/11 (45.5%) | 6/11 (54.5%) | 6/7 (85.7%) | 6/13 (46.2%) | 5/10 (50.0%)[e] |
| Seronegativisation | 1/11 (9.1%) | 2/11 (18.2%) | 5/7 (71.4%)[j] | 1/13 (7.7%) | 2/10 (20.0%)[j] |
| Remained seronegative[c] | 2/13 (15.4%) | 2/13 (15.4%) | 3/10 (30.0%) | 0/13 (0%) | 0/10 (0%) |

Group A: completed treatment; group B: reported interruption of treatment

[a] Only assayed for samples positive by TSSApep-II/V/VI Chagas Sero K-SeT RDT

[b] Two RDT-positive samples (one each from group A and group B) were not assayed by TSSApep-II/V/VI-ELISA

[c] Remained seronegative = negative pre- and post-treatment

[d] One sample seroconverted (changed from negative to positive) in this assay

[e–j] Statistically significant differences between superscript pairs are discussed in the text

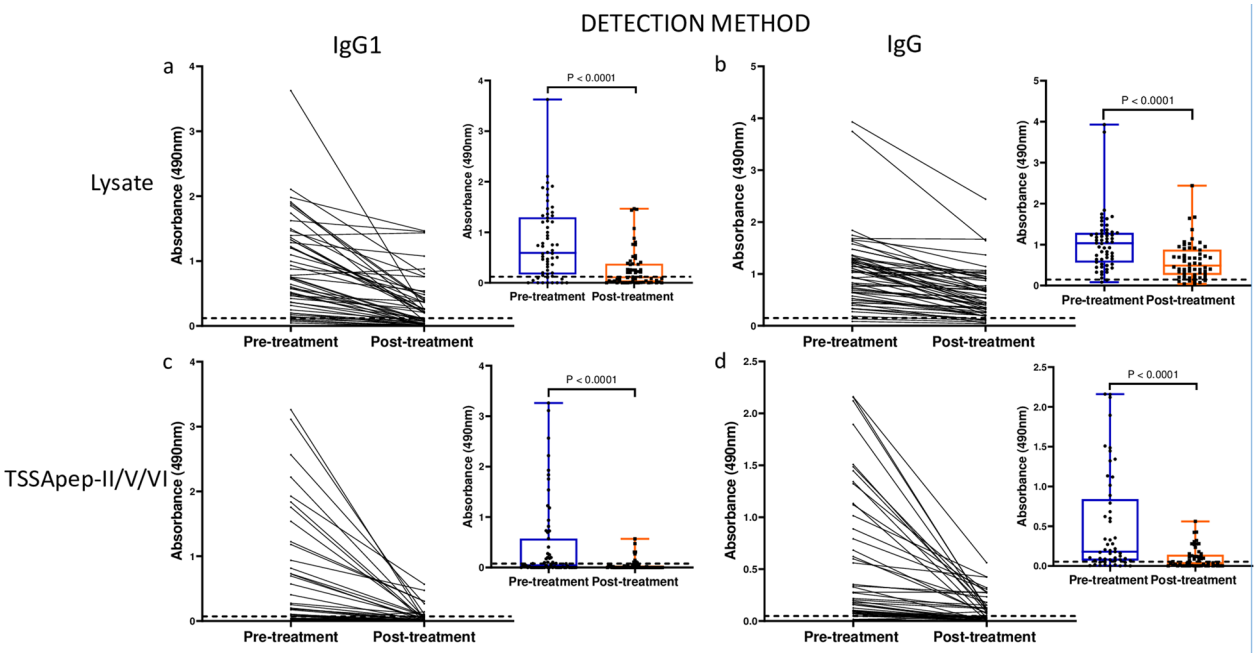


**Fig. 2** IgG1 decline is more discriminative than IgG in paired pre- and post-treatment sera for patients that completed treatment (group A). In each panel (**a**–**d**), ELISA absorbance values are shown connected for individual sample pairs, and in the insets as composite box and whisker plots, blue (pre-treatment) and red (post-treatment). **a** IgG1 with lysate ($P < 0.0001$ for pre- versus post-treatment); **b** IgG with lysate ($P < 0.0001$); **c** IgG1 with TSSApep-II/V/VI ($P < 0.0001$); **d** IgG with TSSApep-II/V/V ($P < 0.0001$). The dashed line represents each assay cut-off value

### Chagas Sero K-SeT RDT is the least discriminative of the TSSApep-II/V/VI assays

Using the Chagas Sero K-SeT RDT in patients from group A, 25/54 (46.3%) of samples showed a clear decrease of band intensity post-treatment, in comparison to 22/22 (100%) or 34/41 (82.9%) by TSSApep-II/V/VI ELISA with IgG1 or IgG, respectively (Table 1). A similar trend was observed in patients from group B.

### ELISA with IgG1 is more discriminative than IgG in assessing seronegativisation and decline in antibody levels

The sensitivities with pre-treatment samples were significantly greater against lysate than TSSApep-II/V/VI with both IgG1 and IgG (Fisher's exact test, $P < 0.0001$; OR = 5.40; 95% CI = 2.31–11.91 for IgG1 and $P = 0.001$; OR = 16.68; 95% CI = 2.76–181.6 for IgG) (Table 1). However, the post-treatment decline in IgG1 was more discriminative than for IgG, regardless of antigen (Fig. 2). In group A patients, a clear decline in IgG1 was observed in 35/46 (76.1%) and 22/22 (100%) samples against lysate and TSSApep-II/V/VI, respectively, compared to 21/57 (36.8%) and 34/41 (82.9%) for IgG (Table 1). Overall, antibody titres also showed a greater decline with TSSApep-II/V/VI compared to lysate for IgG1 (paired *t*-test, $P < 0.0001$, $t_{(62)} = 4.18$ and $P < 0.0001$, $t_{(70)} = 7.35$, respectively; Fig. 2). Similarly, in group B patients, ELISA with IgG1 was more discriminative than with IgG (Table 1). The seronegativisation percentage was significantly higher with IgG1 regardless of the antigen employed (Fisher's exact test, $P < 0.0001$; OR = 5.98; 95% CI = 2.26–17.70 for lysate and $P = 0.057$; OR = 2.74; 95% CI = 0.92–7.64 for TSSApep-II/V/VI) in Group A patients and for the TSSApep-II/V/VI in Group B patients (Fisher's exact test, $P = 0.052$; OR = 10.00; 95% CI = 1.12–69.44) (Table 1).

In multiple logistic regression analysis, the occurrence of antibody decline (both IgG and IgG1) in ELISAs with lysate antigen decreased significantly with increasing age at treatment, but not with treatment group or time elapsed since treatment (Additional file 3: Table S1). Using TSSApep-II/V/VI as antigen, the occurrence of IgG antibody decline was significantly associated negatively with age at treatment and positively with treatment group. Seronegativisation was significantly negatively associated with both age at treatment and time elapsed since treatment (Additional file 4: Table S2). IgG1 antibodies assayed with TSSApep-II/V/VI were not included in this analysis, because virtually all samples declined.

### Univariate associations with serology

Univariate associations between IgG and IgG1 levels and lysate or TSSApep-II/V/VI ELISA are shown in Table 2.

The Qom population had a significantly higher pre-treatment IgG1 titre against lysate. In contrast, the Creole population had a significantly higher pre-treatment IgG antibody titre against TSSApep-II/V/VI. In addition, there was a statistically significant association between patients not completing treatment and higher pre-treatment IgG titre against TSSApep-II/V/VI ($P=0.049$, $t_{(61)}=2.01$), higher post-treatment IgG titre against TSSApep-II/V/VI ($P=0.0001$, $t_{(61)}=4.32$) or higher 5-year post-treatment IgG1 titre against lysate ($P=0.013$, $t_{(69)}=2.56$), although titres were not significantly higher against TSSApep-II/V/VI. Furthermore, there was a significant association between a positive pre-treatment kDNA-PCR and higher post-treatment IgG antibodies against lysate (Table 2, $P_{(32)}=0.019$, $t=2.46$). No associations were found for the Chagas Sero K-Set RDT results pre- and post-treatment.

## Discussion

In Chagas disease, current methods to establish chemotherapeutic parasite clearance are technically demanding and inconclusive. Post-chemotherapy reversion of conventional serology from positive to negative may take decades to confirm parasitological cure. Failure to determine cure complicates patient follow-up, because physicians are unable to inform patients on their long-term prognosis and risk of developing chagasic cardiomyopathy or intestinal pathology. Furthermore, follow-up of patients may be difficult if they migrate to other regions or move to new households, and they may be unwilling to accept prolonged chemotherapy with potential adverse effects and no definitive outcome. Thus, there is a great need for identifying a biomarker for a rapid point-of-care test of cure. Molecular assays may be considered the most useful for assessing treatment response in the short term. However, there are no commercial molecular assay kits available, and typically only a fraction of seropositive chronic individuals are PCR-positive before treatment; moreover, a negative PCR cannot establish absence of infection, although a positive PCR proves treatment failure [50].

The role of the IgG subclasses as an early diagnostic tool, indicator of parasite clearance or predictor of disease prognosis has been assessed in other protozoan infections including malaria [51–54] and toxoplasmosis [55, 56]. In visceral leishmaniasis, the level of IgG1 response has been shown to be a potential therapeutic marker, principally in India; patients considered to be cured had significantly lower levels of anti-*Leishmania* IgG1 compared to those with treatment failure or relapse [57, 58], possibly due the lack of sustained antigenic stimulus associated with successful chemotherapy.

In a previous report of anti-*T. cruzi* IgG1 levels following accidental infection with *T. cruzi*, the anti-*T. cruzi* IgG1, IgG3 and IgG4 returned to pre-infection levels 55 days post-chemotherapy and 80 days after infection, whereas the IgG2 titres remained high at 300 days after infection [59]. Anti-*T. cruzi* IgG1 serology has been shown to be highly sensitive and specific for screening blood donors when conventional serological methods (ELISA and IIF) previously gave inconclusive results [60]. Similarly, anti-epimastigote IgG1 has been able to distinguish between *T. cruzi*-infected and non-infected individuals [61]. Most recently, the Human Chagas-Flow ATE-IgG1 has been able to differentiate between TcI, TcVI and TcII lineages with high accuracy [62].

Our pilot study of treated Bolivian patients residing in Barcelona initially detected a possible trend for a decrease in IgG1 titre compared to IgG and the other IgG subclasses. In the Argentine cohort of the main study, almost all patients remained seropositive post-treatment as determined by conventional serology, namely two commercial ELISAs that detect IgG with non-recombinant and recombinant antigens, showing that this could not determine whether treatment was successful. We assessed IgG and IgG1 antibody decline with ELISA against lysate and TSSApep-II/V/VI antigens pre- and post-treatment of early chronic Chagas disease patients. To our knowledge, there are few reports of TSSApep-II/V/VI as an antigen for assay with paired samples from chronic patients. Of pre-treatment samples screened by Chagas Sero K-SeT RDT for TSSApep-V/V/VI infection, 91.5% were positive, confirming that these are the predominant infecting lineages in the region, as previously reported [30, 63]. Among the patients who completed chemotherapy, a proportion of Chagas Sero K-SeT RDT positives (43.6%) showed IgG decline post-treatment, by visual assessment of band intensity. In contrast, IgG (82.9%) and IgG1 (100%) showed substantial decline by TSSApep-II/V/VI ELISA and even seronegativisation (Fig. 2). We propose an algorithm for incorporating IgG1 and IgG serological assays to infer the treatment outcome after chemotherapy when 5 years have elapsed (Fig. 3). In the ELISAs, IgG/IgG1 are detected with specific conjugates, whereas in the RDTs, antibodies are detected with protein G. Developing IgG1 RDTs could provide a useful tool for monitoring chemotherapy. Moreover, whereas the ELISA uses serum samples at a dilution of 1:200, RDTs use undiluted samples at the point of application, which may also explain the high RDT sensitivity with pre-treatment samples and the lower capacity to detect decline in antibody response. Modification of

**Table 2** Univariate associations with IgG1 and IgG serology

| ELISA antigen | Category | Categories | *N* | IgG Pre-treatment | | IgG Post-treatment | | IgG1 Pre-treatment | | IgG1 Post-treatment | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean absorbance value | *t*-test *P*-value | Mean absorbance value | *t*-test *P*-value | Mean absorbance value | *t*-test *P*-value | Mean absorbance value | *t*-test *P*-value |
| Lysate | Ethnic group | Creole | 30 | 1.120 | 0.355 | 0.650 | 0.364 | 0.599 | 0.045* | 0.244 | 0.529 |
| | | Qom | 28 | 0.950 | | 0.541 | | 0.980 | | 0.307 | |
| | Completed treatment | Yes | 58 | 1.038 | 0.540 | 0.597 | 0.209 | 0.783 | 0.079 | 0.274 | 0.013* |
| | | No | 13 | 1.159 | | 0.778 | | 1.180 | | 0.589 | |
| | Pre-treatment kDNA-PCR result | Positive | 24 | 1.270 | 0.092 | 0.676 | 0.019* | 0.910 | 0.156 | 0.121 | 0.281 |
| | | Negative | 10 | 0.854 | | 0.315 | | 0.557 | | 0.267 | |
| TSSApep-II/V/VI | Ethnic group | Creole | 26 | 0.700 | 0.030* | 0.111 | 0.584 | 0.593 | 0.256 | 0.033 | 0.682 |
| | | Qom | 27 | 0.321 | | 0.091 | | 0.327 | | 0.046 | |
| | Completed treatment | Yes | 53 | 0.507 | 0.049* | 0.101 | < 0.0001*** | 0.478 | 0.660 | 0.038 | 0.261 |
| | | No | 13 | 0.964 | | 0.478 | | 0.336 | | 0.081 | |
| | Pre-treatment kDNA-PCR result | Positive | 21 | 0.738 | 0.640 | 0.105 | 0.996 | 0.591 | 0.194 | 0.0233 | 0.429 |
| | | Negative | 9 | 0.592 | | 0.105 | | 0.172 | | 0.012 | |

Unpaired *t*-test *P* values are shown

*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$

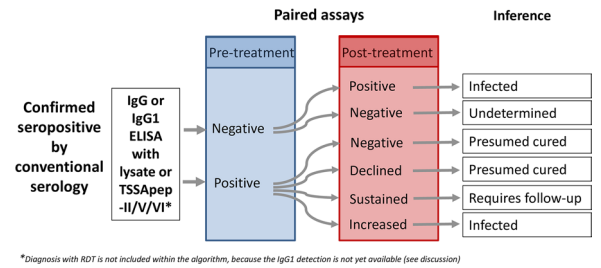


**Fig. 3** Suggested algorithm for chemotherapy follow-up after an average of 5 years post-treatment. Proposed pathway for incorporating IgG and IgG1 serology to infer chemotherapy outcome

sample volume, together with specific IgG1 detection, could allow RDT assessment of antibody decline following chemotherapy (Fig. 3). Both antibody decline and seronegativisation were also observed in patients reporting an incomplete course of chemotherapy. Although the number of days of pill intake was missing for six out of 13 patients (group B), the remaining seven received an average of 20 days of treatment (range = 6–31) of the 60 days prescribed, thus on average less than half the treatment course. Shorter chemotherapy courses are now being trialed to avoid adverse reactions while sustaining parasiticidal effects [64, 65]. Similarly, all patients, including the 13 with incomplete treatment, had a negative kDNA-PCR post-treatment, suggesting that treatment may be effective with reduced length of chemotherapy. However, kDNA-PCR may have low sensitivity, and may not yield the correct diagnosis if there is a low number of circulating parasites.

We considered whether treatment completion, ethnic group, pre-treatment kDNA-PCR result, gender, age and BMI were univariate factors associated with IgG1 or IgG levels. Significant associations were found between post-treatment high IgG1 and failure to complete treatment. High IgG1 has been associated with greater deterioration of cardiac function in Chagas disease patients [35]. Interestingly, a significant association was found between lower levels of IgG post-treatment and a negative pre-treatment kDNA-PCR. We speculate that there may be higher parasitaemia in patients with a positive pre-treatment kDNA-PCR, resulting in slower antibody decline. No associations were found between pre- or post-treatment antibody titres and gender, age or BMI.

The Qom ethnic group had significantly higher IgG1 titre pre-treatment against lysate. Qom households receive a lower level of formal education compared to Creoles [66]. Furthermore, the Qom do not use screens or apply insecticides as regularly, and their households had a higher prevalence of domestic infestation with *T. infestans* [67] and of *T. cruzi*-infected dogs and cats [66, 68], increasing the likelihood of repeat infection by *T. cruzi* and therefore increased production of antibodies [69]. However, we do not have an explanation for the higher pre-treatment titres among the Creole community with the less discriminative IgG assay.

Limitations to this study could be addressed with a larger study cohort, more representative (broader) age distribution and additional intervening time points. Employment of qPCR may have revealed a few cases with very low parasitemia, not detected by kDNA-PCR [36]. We had insufficient pre-treatment ECG data to use in our analysis of IgG1 levels, although this would be an interesting aspect of future research. Including a cardiological and clinical evaluation may shed light on the current status of the study patients.

## Conclusion

Here, we show that IgG1 decline is more discriminative than IgG. A larger proportion of post-treatment samples showed anti-*T. cruzi* IgG1 decline in comparison to IgG, regardless of the antigen employed. Overall, post-treatment samples had significantly lower IgG1. Although IgG1 has restricted sensitivity and should not be used as a diagnostic assay, with further development it clearly has potential as a biomarker of cure. Our results emphasise the importance of early diagnosis and treatment of affected populations in endemic areas.

### Abbreviations

BMI: Body mass index; Bz: Benznidazole; Nf: Nifurtimox; TSSA: Trypomastigote small surface antigen; TSSApep: Lineage-specific TSSA peptide; RDT: Rapid diagnostic test.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-021-05040-6.

**Additional file 1: Figure S1.** Timeline of field work and main activities undertaken in Pampa del Indio, Chaco, Argentina.

**Additional file 2: Figure S2.** Decline in RDT test line intensity. Examples of notable decline in Chagas Sero K-SeT test line intensity observed between pre- and post-treatment samples. a. From strong to moderate, b. From moderate to weak and c. From weak to negative. C: Control line T: Test line.

**Additional file 3: Table S1.** Multiple logistic regression analysis of antibody decline as a function of selected predictors.

**Additional file 4: Table S2.** Multiple logistic regression analysis of seronegativisation as a function of selected predictors.

**Additional file 5: Table S3.** Database.

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**Authors' contributions**
MVC, HF, MAM and REG conceived the study and the analysis. MVC, GFE, NPM and AA conducted field work. PMS, IM, REG and MVC provided sera. NM, MVC, PMS and TB generated the data. NM, MVC, PMS, TB and MAM analysed the data. PM and QG developed and produced reagents. NM, MVC, TB and MAM wrote the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
The data sets generated during and/or analysed during the current study are available as an additional file. (Additional file 5: Table S3).

## Declarations

**Ethics approval and consent to participate**
Bolivian samples: the study protocol for collection of samples was approved by the Ethical Review Board of Vall d'Hebron Hospital (Barcelona, Spain), and all procedures were carried out in accordance with ethical standards laid out in the Helsinki Declaration as revised in 2000. All patients provided written informed consent, and samples were anonymised. Argentine samples: the collection, serological surveillance and treatment protocol were approved and supervised by the Dr. Carlos Barclay "Comité de Ética en Investigación Clínica" (Ethics Committee in Clinical Research), Buenos Aires, Argentina (IRB No. 00001678; Protocol No. TW-01-004), and all clinical investigations were carried out in accordance with principles of the Declaration of Helsinki. All individuals (or parents/guardians) provided written informed consent to participate in the serological surveillance and treatment. When information on the study was given to indigenous populations, an indigenous healthcare worker or community member was provided as translator [36]. The London School of Hygiene and Tropical Medicine (UK) Ethics Committee approved further work and secondary analysis of these samples.

**Consent for publication**
Not applicable.

**Competing interests**
P.M. and Q.G. are employees of Coris BioConcept with no shares in the company.

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## Publisher's Note

# 4. DISCUSSION

## 4.1 General discussion and key outputs

Every year there are an estimated 30,000- 40,000 new cases and approximately 10,000 deaths caused by Chagas disease (5). It is still a major public health concern in Latin America and is rapidly becoming a global health threat due to increased migration. Research priorities for Chagas disease are focused on improved testing and treatment of at-risk populations and case follow-up (112). Throughout my PhD work, I have contributed to these research priorities by accomplishing the following aims: 1) Establish the *L. tarentolae* expression system at LSHTM for the production of *T. cruzi* lineage-specific recombinant proteins; 2) Utilise lineage-specific serological assays (ELISA and RDT) to clarify transmission pathways and mammalian hosts in the Chaco region of Northern Argentina; 3) Evaluate the antigenicity of recombinant gTSSA-I produced in a *L. tarentolae* expression system; 4) Assess IgG and IgG1 as potential biomarkers for the development of a test to monitor the outcome of chemotherapy.

### 4.1.1 Establishment of the *L. tarentolae* system at LSHTM, with successful transfection and production of TSSA proteins.

As detailed throughout this thesis work, the TSSA peptides allow lineage specific serology. However, they do not have 100 % sensitivity. When incorporated into an RDT, TSSApep-II/V/VI is more sensitive than in ELISA format, but it is still only predicted to have a sensitivity of 73.7 % (Murphy et al 2019 (113)). Furthermore, the TSSA peptides specific to lineages TcIII and TcIV have seldom been recognised by Chagasic sera. Therefore, there is a need for better and more sensitive lineage-specific serological markers. I established the *L. tarentolae* expression system at LSHTM to produce glycosylated TSSA antigens, with the aim of increased recognition by Chagasic sera compared to their synthetic counterparts. To date, I have successfully produced all the TSSA antigens (TSSA-I (T), TSSA-II/V/VI, TSSA-III, TSSA-IV and TSSA-V/VI) using the *L. tarentolae* expression system, demonstrated by confirmatory PCR and/or Ble-cherry fluorescence of transfected cells; and have shown increased recognition compared to the synthetic linear peptide for TSSA-II/V/VI and TSSA-V/VI. Unfortunately, due to time constraints, I was unable to see further develop this project. Further work is needed to assess the antigenicity of these recombinant proteins, determine if the antigenicity is due to glycosylation and/or to the conformation. It will also be important to

determine that there is no cross reaction between lineages or with other parasitic diseases before incorporating them onto RDTs.

### 4.1.2 First application of lineage-specific TSSA peptide ELISA and Chagas Sero K-SeT RDT, in the Argentine Chaco, and identification of epidemiological associations with TSSA-II/V/VI.

I utilised lineage-specific serological assays (ELISAs and RDT), to clarify transmission pathways and mammalian hosts in the Gran Chaco, Argentina. During two trips to Argentina (Chapter 2 and Appendix 2), I have shown that lineage-specific serology can identify *T. cruzi* lineages without the need for parasite isolation and genotyping. The incorporation of TSSApep-II/V/VI into the low-cost Chagas Sero K-SeT RDT offers a quicker alternative, delivering results in 15 minutes rather than several hours, and also has greater sensitivity compared to the TSSApep ELISA. Additionally, this RDT uses protein G as its detection molecule making it applicable to several non-human mammalian species, such as dogs and opossums, without the need for specific secondary antibodies. Assays performed as part of my research visit found that in humans the prevalence of TSSApep-II/V/VI seropositivity was 69.5 %, similar to what had previously been shown in Bolivian patients (104), and identified associations between TSSA-II/V/VI infection and locality, with increasing and decreasing age within the Qom and Creole populations, respectively. In dogs, TSSApep-II/V/VI sero-prevalence was linked to being born before the insecticide spraying campaign, indicating an increased risk of infection before the campaign and cumulative risk with age; and Qom households, who are predicted to have a higher prevalence of *T. cruzi* infection (113, 114).

To note, this is the lineage-specific infection pattern in human blood samples collected between 2014 and 2017. Appendix 2 details the infection pattern of blood samples collected solely between 2016- 2017 from Pampa del Indio, Avia Teria and Juan José Costelli. Interestingly, there appears to be a decrease in the prevalence of TSSA-II/V/VI by ELISA in humans, from 34.2 % to 10.8 %. For dog samples the reactivity to TSSApep-II/V/VI remained similar at 38.8 % compared to 46.2 %. Further work is needed to unravel fully the magnitude of this change and how this may impact patient care and control strategies in the future.

### 4.1.3 First direct genotyping of *T. cruzi* from triatomine bugs using the Lewis (2009) protocol.

I performed the first application of the Lewis ( 2009 (115)) protocol to direct genotype *T. cruzi* lineages from triatomine rectal ampules (Appendix 2), demonstrating the first identification of TcI in *T. infestans* collected from Avia Teria, and a higher percentage of mixed infections than had previously been found in the Chaco (116). The opossum *Didephis abliventris* is the probable main source of TcI infection in sylvatic transmission cycles (85), and in the Chaco province *D. albiventris* has exclusively been found to be infected with TcI (117-120). Mice are also known to be infected with TcI (121). TcI infection has also been shown in *T. sordida* but this bug is linked to sylvatic transmission cycles (116).

The cause of this increased frequency of TcI in domestic and high-risk domestic ecotypes in both rural and peri-urban environments are multifactorial, and may or may not be associated with the lower TSSApep-II/V/VI serology described above. One factor is changes in the local environment. The Gran Chaco has one of the highest deforestation rates in the world, every month it is estimated that 133 square miles are lost (122). Twenty-five per cent of the Argentine Gran Chaco has been cleared for farmland for livestock and soya bean production (122), leading to the displacement of local wildlife into neighbouring communities, causing an overlap of sylvatic TcI and domestic TcII/V/VI transmission cycles, particularly along the rural-urban gradient. This may also explain why such a similar distribution of lineages was seen in both peri-urban and rural environments. Post-intervention TcI infection was only found in high-risk domiciles, *T. cruzi* infiltration is most likely introduced from the sylvatic cycles (116).

A second factor may be increased migration, particularly from Paraguay, Peru and Bolivia; between 2018-20, 36 % of residency permits were given to people originating from Venezuela (123), where TcI is the secondary cause of Chagas disease (81, 85). Furthermore, 60 % of individuals migrating to Argentina were between the ages of 19 and 65, an age group that is associated with an increased risk of infection (124).

A limitation of this study is that I was unable to assay the collected samples for TcI serology and no human serum samples were collected from the houses where bugs infected with TcI were identified. A further limitation was that the Chagas Sero K-SeT RDT was not available on my second visit to Argentina; it was found to be more sensitive than the ELISA for TSSSA-

II/V/VI, and with around 70 % of human samples positive TSSA-II/V/VI (included herein as **Murphy et al., 2019** & **2021**), this may have given a more accurate picture of the TcII/V/VI prevalence. Furthermore, the use of direct sampling of infecting lineage from *T. infestans* meant that there was not always enough DNA to distinguish between the hybrid lineages and TcII (TcII/V/VI).

Overall, this highlights the dynamic nature of the parasite's transmission and the need for continuous and real-time monitoring of the prevalence of *T. cruzi* lineages throughout the community. Although a useful tool, this would be too complex and time consuming by standard DNA isolation and genotyping but may be plausible with a highly sensitive and specific rapid diagnostic test. Furthermore, it also indicates the importance of monitoring the *T. cruzi* lineages in domestic bugs, as this is a good early predictor for human infection.

### 4.1.4 First demonstration of *L. tarentolae*-produced antigen for *T. cruzi* serology and crucial role of glycosylation for antigenicity.

I evaluated the glycosylated form of TSSA-I peptide for its applicability to TcI-specific serology (included herein as **Murphy et al 2020**). Before the commencement of my PhD, there was no robust serological marker for the identification of TcI, despite it having a unique TSSA sequence; synthetic peptide or recombinant protein produced by *E. coli* was not recognised reliably by chagasic sera. This was thought to be either due to lack of antigenicity of the TSSA-I sequence, the role of TSSA-I has not been define; or due to the linear structure/ lack of post-translational modifications of the peptide. The *Leishmania tarentolae* expression system, is a allows for the production of recombinant proteins, with the addition of post-translational modifications, making them more naturalistic. Here, I showed that a TSSA-I recombinant protein produced in *L. tarentolae* has increased recognition by TcI endemic sera, in particular Colombian chagasic sera, and that this was mediated by the glycosylation rather than the protein conformation, the converse to what was observed with cross-reacting malaria sera (125). To the best of my knowledge, this is the only known serological marker for TcI, it can be incorporated onto an RDT for resolving TcI transmission cycles and investigating the link between *T. cruzi* lineage infection and clinical prognosis.

### 4.1.5 Following pilot in Bolivian patients, IgG and IgG1 levels showed significant decline post-chemotherapy, in a cohort of Argentine patients, with IgG1 decline being more discriminative than IgG.

I assessed the decline of IgG and IgG1 as a potential biomarker for cure after chemotherapy (included herein as **Murphy et al 2021**). A research priority is the development of a rapid point-of-care test of cure, conventional serology detecting anti-IgG antibodies can take years to revert to negative, impacting drug adherence, follow-up and care. The use of IgG subclasses as an indicator of parasite clearance, early diagnostic tool or predicator of disease, has been demonstrated in other parasitic diseases, including malaria (126-129) and visceral leishmaniasiss (130). In *T. cruzi* infection IgG1 titres decrease more rapidly after treatment in the acute phase (131), can identify infected individuals with high sensitivity (62, 132) and in studies on FACS, could distinguish between the lineage infections (133). In this thesis work, after pilot data demonstrated that IgG1 declined in three out of seven Bolivian patients residing in Barcelona one-year post-treatment, I assessed the potential of IgG and IgG1 as a biomarker of cure in 60 chronic patients from Argentina. After treatment, most of the patients remained seropositive when assayed by conventional serology, whereas all post-treatment K-DNA-PCR samples were negative. However, this could not indicate treatment success due to the limitations in PCR with isolating the parasites during the chronic phase. I found IgG1 to be more discriminative than IgG but lacking sensitivity, indicating that it should not be used as a diagnostic assay but has potential with further development as a biomarker of cure.

## 4.2 Future directions

Throughout this thesis work I have made several advances in *T. cruzi* lineage-specific serological. I have used the Chagas Sero K-SeT RDT to identify epidemiological associations with infecting lineage, assessed IgG1 as a biomarker for cure, shown how a glycosylated version of TcI has increased antigenic, and used the *L. tarentolae* expression system to produce recombinant TSSA proteins, increasing their antigenicity. However, this has not been without its limitations and constraints, which have highlighted some exciting prospects for future work.

### 4.2.1 Further investigations into the prevalence of *T. cruzi* lineages in the Gran Chaco, Argentina, with particular focus on emerging TcI.

In Avia Terai, the notable drop in seroprevalence of TSSA-II/V/VI in humans was of particular interest. A key future objective here is to assay the samples from both collections against gTSSA-I to see if TcI is crossing from sylvatic to domestic transmission cycles in this region. The gTSSA-I antigen could also aid greatly in investigating the link between infecting lineage and clinical presentations, it has long been proposed that the different *T. cruzi* lineages may contribute to the disparate clinical presentations (79). Assaying chagasic populations from different localities with matched clinical data against the TSSA antigens would give us a better understanding and has the potential to gain further insight into disease progression which could aid with patient management and treatment options.

A further aim would be for the expansion of serological techniques to incorporate a larger number of mammalian species by using an alternative detection molecule to Protein G. Whilst, detection by Protein G can be used for humans and a wide range of mammals (canines and primates), it does not work for all, for example felids. Two alternatives are Protein A and Protein A/G. Protein A is thought to have better binding capacity for rabbit, pig and cat immunoglobulins, and it has been shown to be a good detection molecule for armadillo IgG (134, 135). Whilst Protein A/G is a recombinant fusion protein that includes the IgG binding sites of Protein A and G. The efficacy of these detection molecules should be compared to protein G and their species-specific secondary antibodies across a large range of mammalian species and once established, an RDT incorporating the proteins could be developed. The inclusion of a greater range of mammalian species, in lineage-specific serology, without the need for species specific secondary antibodies would help to elucidate better *T. cruzi* transmission pathways and identify reservoir species.

As discussed above, in Avia Terai there was an increase of TcI and mixed infections identified in captured *T. infestans*, greater than had previously been found. Understanding the possible route of infection could help improve future control strategies. Schwabl et al 2020 (136), have shown how a genome wide sequence typing (GWST) tool, which uses a multiplex PCR amplifying TcI 203 targets simultaneously, can identify parasite diversity at a local and cross-country scale, distinguishing parasites infecting sympatric vectors, and clearly distinguishing TcI, TcIII, TcIV and TcV + TcVI, as well as TcI with inter-strains. They describe how the target selection model can be modified to a specific goal, I suggest designing a panel of targets to investigate further the diversity of the *T. cruzi* lineages infecting *T. infestans* infesting households in the Argentine Chaco, and comparing them to those isolated from surrounding sylvatic cycles, this may identify inter-lineage diversity, genetic proximity, and potentially map the route of TcI introduction.

### 4.2.2 Assessment of glycosylated TSSA recombinant proteins, and incorporation onto RDTs.

During this thesis work, pandemic-responsive constraints and consequent effects, such as having to request fresh batches of *L. tarentolae* from the commercial supplier and the expiry of key culture supplements, impacted experimental progress. Therefore, future work would be to evaluate the recombinant TSSA proteins further, by assaying with a broader range of samples from different localities in Latin America, establishing the roles of glycosylation and conformation in antigenicity, as well as against sera from non-endemic healthy controls and from other infections such as *Leishmania* and *Plasmodium* to assess specificity, with potential incorporation into RDTs. This will be especially interesting for TSSA antigens specific to TcIII and TcIV, as there are relatively low sensitive ELISAs for these lineages and the TSSA synthetic peptides have had limited recognition. The antigenicity of glycosylated versions of these antigens should be assessed against a large range of mammalian sera, either experimentally infected or sera collected from sylvatic animals in endemic areas, with these lineages, before being incorporated onto RTDs.

### 4.2.3 Maximising the potential of the *Leishmania tarentolae* expression system.

Now established at LSHTM, the *L. tarentolae* expression system can be used to express diagnosis antigens of other infectious diseases, to assess for improved antigenicity, for example Visceral Leishmaniasis (VL). Current serological diagnostic assays for VL are unable to differentiate between past and active infection and lack specificity. Two main diagnostic

serological antigens are rK39, a recombinant protein consisting of a 46-amino acid region followed by 6.5 non-identical repeats of 39-amino acids of a kinesin-like protein, and rK28 a recombinant protein comprised of kinesin repeats from Sudanese *L. donovani* and sequences of the hydrophilic acylated surface proteins (HASPB) from an Ethiopian *L. donovani* (137). Recombinant proteins rk39 and Lbk39 expressed through the *L. tarentolae* system have been assessed for diagnostic potential for *L. infantum* (138) and *L. braziliensis* (139), but not for rK28. However, Simonson et al 2020, has shown through via heat denaturing that antigenicity of the kinesin component is dependent on protein conformation, rather the linear epitope (137), nonetheless it would be interesting to see if glycosylation improves antigenicity. One major limitation of rk28 is its cross reactivity with other infectious diseases (Malaria, tuberculosis and schistosomiasis), Malaria shares a similar HASPB epitope (137), I have previously shown that recognition of gTSSA-I by Gambian Malaria sera can be removed by heat-denaturing the protein, it would be of value to see if disrupting the protein confirmation of the rK28 through heat-denaturing also removes recognition by malaria sera.

The *L. tarentolae* expression system has been used for the production of possible vaccine targets in pathogenic parasitic species *Leishmania* (140, 141) and *Toxoplasma gondii* (142). An interesting topic of research would be. use the *L. tarentolae* system to produce proteins for other organisms such as *Plasmodium*, where it has been suggested that glycosylation may improve protection seen from vaccine targets circumsporozoite protein (CSP) and thrombospondin-related adhesion protein (TRAP) (143). CSP and TRAP are *Plasmodium* sporozoite surface proteins, essential for motility and live-stage infection, both are targets for *Plasmodium* vaccine development. TRAP has been combined with multiple T cell epitopes from *Plasmodium* to develop the viral vector protein ME-TRAP and αTSR region of CPS is incorporated into RTS,S. Interestingly, it is suggested that this αTSR region may not be properly folded in the RTS.S vaccine (144). Vaccines combining TRAP and CPS have been explored with limited success (144), which may be due to them not having the correct conformation structure and post-translational modifications. A glycosylated recombinant TRAP-CPS protein that joined the I and TSR domains of TRAP to the αTRS domain in CSP with the repeated region in CPS found within RTS,S. expressed, through a mammalian system, has shown protection against infection challenge in vaccinated mice (144). An interesting, research objective would to express the TRAP-CPS proteins through the *L. tarentolae* system, as well as other pre erythrocyte malaria vaccine targets, LSA-I (recombinant live stage Antigen

1) and EXP1 (Exported protein 1), to see if the addition of post-translation modifications (glycosylation and protein confirmation) increases their vaccine efficiency, by inoculating mice, measuring antibody titres at multiple time points by post vaccination and by introducing a *Plasmodium* infection challenge.

### 4.2.4 Enhancement of *T. cruzi* serological diagnostics by the development of a point-of cure test and identification of new serological biomarkers.

A point-of-care test for treatment outcome is also needed; I showed that IgG1 does significantly decline post-chemotherapy, so assessing the other IgG subclasses for decline as well as the identification of other biomarkers should be a key research priority. Two other potential biomarkers that warrant further investigation are SAPA (shed acute phase antigen), a portion of the trans-sialidase expressed by infectious forms of *T. cruzi*, and TcD, an antigenic epitope expressed by trypomastigotes, both have shown significant decline after treatment in mice, compared to those not treated or treated with lower dosages (145). Demonstrated on a multiplex assay, this serological tool also offers a great advantage in identifying novel biomarkers for diagnosis and treatment outcome, with the ability to assess multiple antigens simultaneously, it offers a rapid alternative to ELISAs and should therefore be utilised (145-148).

No current serological diagnostic tool has 100 % sensitivity, which additionally can vary greatly depending on the geographic origin of the population. Therefore, there is still a great need to identify a novel universal and lineage-specific serological marker, as well as a test for cure. Romer et al (2023) recently showed greater variations within the TSSA sequences: one particular amino acid change, N40K, increased recognition by a pool of Mexican sera; they also identified two key antigenic epitopes of sequence one, which is not included in the current TSSA peptides, so it would be interesting to see what the inclusion of this epitope region has on antigenicity and incorporating the variants of TSSA sequences through the creation of fusion proteins containing variations of the sequence (92). Another candidate antigen that should be further explored is Tc24. Tc24 is a trypomastigote excretory-secretory protein, with very little genetic variation between lineages originating from different geographic localities in South America (149) and is conserved within TcI strains in Mexico (150), making it a strong candidate antigen for serological diagnosis, especially in Mexico where current tests can have very low reactivity.

## 4.3 SUMMARY/ CONCLUSION

In summary, *T. cruzi* is an extremely complex parasite, it is highly genetically diverse split into six distinct lineages (TcI- TcVI) and sequesters in host cells hampering the ability to isolate parasites to identify associations between the lineages, and its reservoir hosts, transmission cycles and clinical manifestations. Even so, it has long been proposed that the disparity in clinical manifestations witnessed in different geographical areas is due to the different lineages.

Here I have shown that lineage-specific serological assays can establish the prevalence of *T. cruzi* lineages in communities, and discover epidemiological associations. A rapid diagnostic test (Chagas Sero K-SeT) incorporating the TSSApep-II/V/VI can be used instead of ELISA. I demonstrate that a glycosylated version of TcI specific antigen increases recognition by chagasic sera, compared to its linear synthetic counterpart, and glycosylated recombinant proteins for the other TSSA lineages can be produced through the *L. tarentolae* expression system. I identified IgG1 is a potential biomarker for cure.

I have demonstrated how fundamental lineage-specific serology may further our knowledge of this complicated parasite. The production of improved lineage-specific RDTS, able to identify infection at each stage of disease is more important now than ever. As demonstrated here, transmission cycles of *T. cruzi* are constantly changing and will continue to do so. Changes in the local environments caused by human intervention or environmental factors will bring with it new challenges, early identification of changes in transmission cycle, and discovery of novel hosts is vital to improve control interventions and strategies. In addition, with the increased internal and international migration of people from endemic areas, sensitive and timely diagnosis is important to control transmission and for patient management.

Furthermore, improved lineage-specific serology, especially with a biomarker for TcI, allows for the discovery of associations between *T. cruzi* lineage and clinical manifestations, which could be used to predict disease progression, personalise treatment plans and improve disease prognosis.

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# APPENDIXES

## Appendix 1: Table 3. Publicly available published *T. cruzi* genomes and assembly statistics methods.

TryTrip and NCIB database were used to search for published and accessible T. cruzi genomes. last accessed and downloaded 29/04/2024. If there was a duplicate of the same isolate genome the largest genome was chosen for inclusion. BUSCO comparison against Euglenzoa genome, BUSCO version: 5.4.7, performed on Linux command line using these commands: busco -i ~/Tc_Genomes -o big_tc_busco -m genome -c 40 -l euglenozoa_odb10 (151).

# Appendix 2: Identification of infecting *T. cruzi* lineages in Humans and Triatomine bugs from the Argentine Chaco.

In this section, I discuss the data obtained during my second research visit to Argentina, in which I identified the infecting lineages of *T. cruzi* from humans, canines and *T. infestans* using both serological and molecular techniques to further our understanding of domestic and sylvatic transmission cycles in the Argentine Chaco.

Three study sites within 150 km were used for the collection of serum samples in the Argentine Chaco, Pampa del Indio, Juan José Costelli and Avia Terai. Avia Terai is a multi-principality situated in the Gran Chaco region, it is 700 $km^2$ and consists of approximately 13,000 people in 2016 (152). The area has been divided into three environments, urban, semi-urban and rural (153). *T. infestans* is the main vector of *T. cruzi* in these areas, and although attempts at controlling the vector have been made through control programs with mass spraying campaigns, they have had limited success.

## 2.1 Materials and Methods

### *2.1.1 Ethics statement for collection and use of human samples*

Ethics approval for the collection and use of human samples for secondary analysis as described in Chapters 2, 3 and 4.

### *2.1.2 Sources of sera for lineage-specific diagnosis*

Serum samples from seropositive patients and *T. cruzi* infected samples were from archives stored at University of Buenos Aires and the London School of Hygiene and Tropical Medicine, as described in Chapters 2, 3 and 4.

### *2.1.3 Lineage-specific TSSApeptide ELISA for serological diagnosis of infecting T. cruzi lineage*

To identify the infecting *T. cruzi* lineage the TSSApeptide ELISA was performed on human and animal serum samples from the Gran Chaco, Argentina, as described in Chapter 2.

### *2.1.4 Sources of T. cruzi DNA for molecular diagnosis of infecting lineage*

*T. cruzi* DNA isolated from *T. infestans* rectal samples collected from Avia Terai, Argentina (Table A1).

**Table A1.** Panel of DNA isolated from *Triatoma infestans* rectal samples for identification of infecting *T. cruzi* lineage

| Isolate | Collection site | Environment |
|---|---|---|
| AT Pi 15o1042 | domicile | Peri-urban |
| AT Pi 15o991 | domicile | Peri-urban |
| AT Pi 15o994 | domicile | Peri-urban |
| AT Ri 15d2401 | domicile | Rural |
| AT Ri 15d2402 | domicile | Rural |
| AT Ri 15d3069 | High risk* | Rural |
| AT Ri 15o3287 | Domicile | Rural |
| 29 | High risk* | Rural |
| 513 | High risk* | Rural |
| 498 | domicile | Rural |
| 502 | domicile | Rural |
| 126 | High risk* | Peri-urban |
| 711 | domicile | Peri-urban |
| 712 | domicile | Peri-urban |

*****Areas with a high probability of *T. cruzi* bug infection (granaries, mud ovens, kitchens, storerooms and kennels) due to presence of mammals (dogs, cats and rodents) as defined in Alverdo et al (2021) (153).

*2.1.5 Direct genotyping of T. cruzi lineage from triatomines rectal ampules*

To identify if a triatomine bug was infected with *T. cruzi* and by which lineage, DNA was isolated from the triatomine bug rectal ampules. Rectal ampules were preserved in 50 µL sterile water, boiled for 10 minutes and the DNA extracted using DNAzol® (Invitrogen, Carlsbad, CA, USA) as described in Alvedro et al (2021) (153). To assess the infecting *T. cruzi* lineages, the Lewis et al (2009) protocol was used (115), which incorporates two PCR-RFLP (Restriction Fragment Length Polymorphism) reactions based on a SNPs in the HSP60 and GPI loci and a third PCR product size polymorphism of the LSU rDNA loci, the PCR banding pattern of the three considered together was used to identify lineage, according to Table A2.

**Table A2.** PCR banding patterns to identify *T. cruzi* lineage (Lewis 2009 method).

| | | *T. cruzi* lineage | | | | | |
|---|---|---|---|---|---|---|---|
| | | TcI | TcII | TcIII | TcIV | TcV | TcVI |
| Gene target | HSP60 | ~450 bp | ~450 bp | 314 bp<br>~130 bp | ~450 bp | ~450 bp<br>314 bp<br>~130 bp | ~450 bp<br>314 bp<br>~130 bp |
| | GPI | 817 bp<br>447 bp | 490 bp<br>447 bp<br>253 bp | 817 bp<br>447 bp | 490 bp<br>447 bp<br>253 bp | 817 bp<br>490 bp<br>447 bp<br>253 bp | 817 bp<br>490 bp<br>447 bp<br>253 bp |
| | LSU rRNA | 110 bp | 125 bp | 110 bp | 120 bp | 110 bp | 125 bp |

## 2.3 Results and Discussion

*2.3.1 Lineage-specific serological and molecular assays to clarify transmission pathways in the Gran Chaco, Argentina: Lower prevalence of T. cruzi lineage TcII/V/VI than previously found*

Forty-one human serum samples and 26 from dog were assayed against the linear synthetic TSSA peptides. One dog sample was subsequently removed from analysis due to suspected non-specific binding. The prevalence of TSSApep-II/V/VI was found to be 10.8 % and 46.2 % in humans and dogs respectively, results for each of the TSSA lineage specific peptides are listed in Table A3.

**Table A3.** Human and dog samples assayed by lineage specific TSSA-pep ELISA.

| | Antigen | Positives/ total (%) |
|---|---|---|
| Humans | TSSA-II/V/VI | 4/41 (10.8%) |
| | TSSA-III | 0 / 41 (0 %) |
| | TSSA-IV | 0 / 41 (0 %) |
| | TSSA-V/V | 0 / 41 (0 %) |
| Dogs | TSSA-II/V/VI | 12/ 26 (46.2 %) |
| | TSSA-III | 0 / 36 (0 %) |
| | TSSA-IV | 0 / 36 (0 %) |
| | TSSA-V/VI | 1 / 36 (2.9 %) |

*2.3.2 Identification of infecting T. cruzi lineage by direct genotyping of DNA isolated from Triatoma infestans.*

The Lewis (2009) PCR-RFLP protocol identified the infecting lineage in 50 % (7/ 14) of DNA isolates from triatomine rectal ampules, five were partial identification, unable to differentiate between TcII, TcV or TcV, one showed a mixed infection and the remaining one showed TcI infection (Figure A1), banding patterns and full results are shown in Table A4. Previous lineage identification had been performed using Burgos et al (2010) protocol (94).

**Table A4.** Banding patterns for Lewis protocol (2009) identification of *T. cruzi* lineage in triatomine rectal samples.

| **Rectal sample** | **Previous lineage identification** | **HSP60 / *Eco*RV** | **GPI / *Hha*I** | **S24 gene (bp)** | **Lewis method identification** | **Consensus** |
|---|---|---|---|---|---|---|
| AT Pi 15o1042 | TcV or TcV & TcVI/II | | | | | |
| AT Pi 15o991 | Tc VI or TcII | 3 bands | 4 bands | 125 | Tc VI | TcII/V/VI |
| AT Pi 15o994 | TcV or TcV, VI/II | | | | | |
| AT Ri 15d2401 | TcVI | | | | | |
| AT Ri 15d2402 | Tc VI or TcII | | | 125 | Tc VI or II | TcII/V/VI |
| AT Ri 15d3069 | TcI & TcV | 1 band | | 110 | TcI | TcI |
| AT Ri 15o3287 | TcI & TcV | | | 125 | TcII or VI | TcI & TcV/VI |
| 29 | Tc I | | | | | |
| 513 | TcII or TcVI | | | 125 | TcII or VI | TcII/V/VI |
| 498 | TcV or TcVI & V | | | | | |
| 502 | TcII or TcV or TcVI | | | | | |
| 126 | TcI & TcV or TcII or TcVI | | | | | |
| 711 | not done | 3 bands | 4 bands | 125 | TcVI | TcII/V/VI |
| 712 | not done | 3 bands | Smeared | 125 | TcVI | TcII/V/VI |

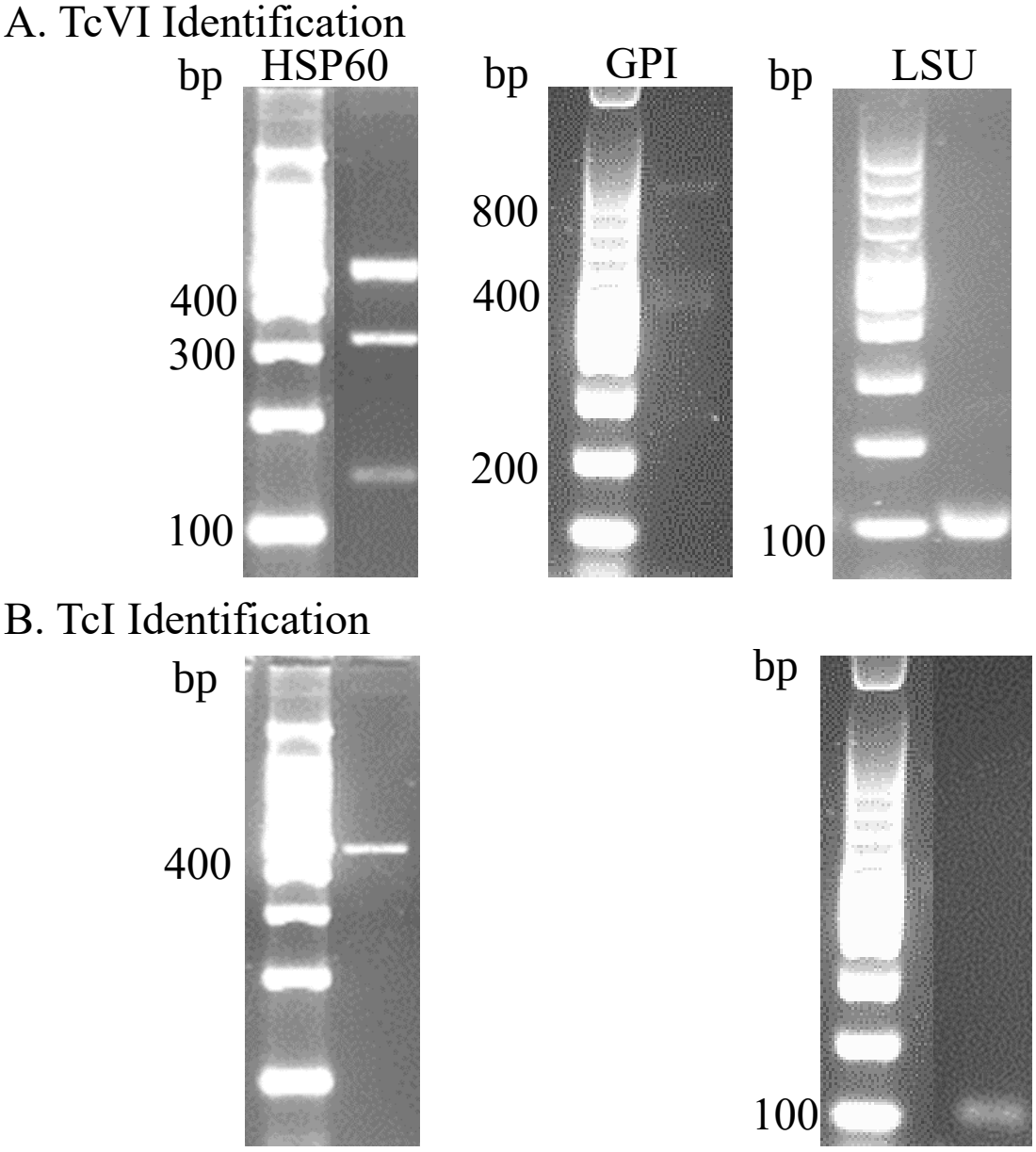


Figure A1. Lewis protocol Identification of *T. cruzi* infecting lineage of triatomine bugs. Using the Lewis protocol *T. cruzi* infecting lineage can be identified by using the banding patterns of three PCRs. A. Demonstrates a typical banding pattern for TcVI, HSP60 3 bands, GPI 4 bands, and LSU 1 band at 125 bp. In contrast B shows the banding pattern for TcI, HSP60 1 band and LSU 1 band at 110 bp, the GPI PCR did not work for this sample.

*2.3.3 T. cruzi genotyping from triatomine rectal samples*

To the best of my knowledge, this was the first time the Lewis (2009) protocol was used for direct PCR genotyping of *T. cruzi* infection from DNA extracted from triatomine bugs' rectal ampules, although other methods have been reported (Table A5).

**Table A5.** Published methodologies of direct PCR genotyping of *T. cruzi* from Triatomine faecal samples.

| Publication | Collection Location | Method/genomic targets | Result/Lineage |
|---|---|---|---|
| Marcet et al 2006 (154) | Northwestern Argentina | Panel of PCR-based genomic markers<br>Intergenic region of spliced-leader DNA (SL-IR)<br>24Sα rRNA PCR<br>18Sα RNA PCR<br>A10 sequence | 2/28 TcI<br>24/28 TcIIe<br>1/28 TCII<br>1/28 mixed TCI +TCII<br>(TC11 culture) |
| Schijman et al 2006 (155) | Northwestern Argentina | kDNASat-DNA; SL-DNA | No amplification |
| | | D7 Domain of the 24Sα rRNA | 2 250bp |
| Zulantay et al 2007 (156) | Chile | hyper variable region of kDNA PCR | Increased sensitivity of by XD-PCR compared to XD with microscopy |
| Mejia-Jaramillo et al 2009 (157) | Colombia | SL-LSSP; kDNA | SL-LSSP-PCR identification the same with both faeces and culture, whilst kDNA different depending on the source |
| Maffey et al 2012 (116) | Argentine Chaco | Rectal ampule<br>Burgos et al protocol (76) | Identification by rectal ampules 52% of *T. infestans* and 100 % *T. sordida* |

| | | | |
|---|---|---|---|
| Padillia et al 2017 (158) | Peru | DNA extracted from insect intestinal contents: kDNA; SL-IR; 24Sα rRNA; 18Sα RNA | 95/ 113 TcI<br>5/113 TcII<br>4/113 TcIII<br>4/113 TcIV |
| Schwabl et al 2020 (136) | Colombia, Venezuela & Ecuador | Genome-wide locus sequence typing (GLST), a multiplex PCR amplifying a 203-target panel for TcI | Identification of 780 SNPs, markers able separate TcI, TcIII, TcIV and TcV + TcVI and multiclonal infections within TcI |

# Appendix 3: Other publications

**Murphy, N**. (2022) Identification of Biomarkers of cure in Chagas disease. Bug Bitten. https://blogs.biomedcentral.com/bugbitten/2022/01/18/identification-of-biomarkers-of-cure-in-chagas-disease/

McClean MCW, Bhattacharyya T, Mertens P, **Murphy N**, Gilleman Q, et al. (2020) A lineage-specific rapid diagnostic test (Chagas Sero K-SeT) identifies Brazilian *Trypanosoma cruzi* II/V/VI reservoir hosts among diverse mammalian orders. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0227828

Bhattacharyya T, **Murphy N**. & Michael M. (2019) *Trypanosoma cruzi* lineage specific serology: new rapid tests for resolving clinical and associations. *Future Science*. https://doi.org/10.2144/fsoa-2019-0103

Bhattacharyya T, Messenger LA, Bern C, Mertens P, Gilleman Q, Zeippen N, Bremer Hinckel BC, **Murphy N**, Gilman RH, Miles MA; Chagas Working Group in Bolivia and Peru (2018). Severity of Chagasic cardiomyopathy is associated with response to a novel rapid diagnostic test for *Trypanosoma cruzi* TcII/V/VI. *Clin Infect Dis*. doi: 10.1093/cid/ciy121

Bhattacharyya T, Marlais T, Hinckel B, Mateus D, Le H, **Murphy N**, Yeo M, Mertens P, Miles MA (2018). Comparative Genomics and the Improved Diagnosis of Trypanosomiasis and Visceral Leishmaniasis. In Whitehouse & Rapley (eds.) *Genomics and clinical Diagnostics*, Royal Society of Chemistry, UK.

# Research poster presentations/ conferences

Lewis MD, Khan AA, Langston HC, **Murphy N**, Phelan J, et al Hybridisation of *Trypanosoma cruzi* insect stages in axenic culture. ICOPA, Copenhagen 2022

**Murphy N**, Macchiaverna NP, Cardinal MV, Tapan Bhattacharyya T, et al Lineage-specific rapid diagnostic tests can resolve Trypanosoma cruzi TcII/V/VI ecological and epidemiological associations in the Argentine Chaco. British Society of Parasitology Spring Meeting, Poster Presentation, University of Manchester. 2019

**Murphy N**, Macchiaverna NP, Cardinal MV, Tapan Bhattacharyya T, et al Lineage-specific rapid diagnostic tests can resolve Trypanosoma cruzi TcII/V/VI ecological and epidemiological associations in the Argentine Chaco. RSTMH Research in Progress, Poster Presentation, London. 2018