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Distinct proteomic profiles are associated with *Trypanosoma cruzi* I strains displaying high/low infectivity towards mammalian cells *in vitro*

Tesis previa a la obtención del título de Magister en Biología de Enfermedades Infecciosas

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Certifico que la Tesis de Maestría en Biología de Enfermedades Infecciosas de la Sra. Camila Cilveti Rodríguez Riglos ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Jaime Costales, PhD

Director de la Tesis

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Distinct proteomic profiles are associated with *Trypanosoma cruzi* I strains displaying high/low infectivity towards mammalian cells *in vitro*

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Abstract

The causative agent of Chagas disease, *Trypanosoma cruzi*, has a digenetic life cycle, comprising life stages in invertebrate and mammalian hosts. In mammalian hosts, the parasites multiply intracellularly; therefore, the process of cellular invasion is required for life cycle completion. *T. cruzi* is a genetically diverse species and six different genetic lineages are currently recognized (TCI-TCVI), TCI being the most widespread and genetically variable. TcI strains present significant differences in their ability to invade mammalian cells *in vitro*, which may be rooted both in genetic differences as well as in protein expression levels. We report the identification of sets over and underexpressed of 2-DE spots statiscally associated with high and low infectivity strains. Proteins associated with high infectivity strains identified through mass spectrometry showed a major presence of proteins related to the cytoskeleton and motility of the parasite, which suggests that the more infective strains could be more motile than the less infective ones. Additionally, high infective strain proteins associated with antioxidant activity were also present, which may be related to more aggressive invasive patterns. Ultimately, identification of specific proteins and pathways associated to the ability of trypomastigotes to infect mammalian cells will open up the possibility of functional confirmation through overexpression and knockout experiments and may unveil potential drug and vaccine targets.

Introduction

Chagas Disease (CD) is a zoonotic disease caused by the protozoan parasite *Trypanosoma cruzi* [1]. It is estimated that it currently affects 10 million people, while around 30 million are currently at risk of infection [2]. CD is endemic to Latin America; however, human migration has allowed the disease to spread to non-endemic regions such as the US, Canada, Japan and Australia, among other countries[3].

CD is transmitted primarily by the action of hematophagous vectors (Reduviidae: Triatominae), although infection through blood transfusions, as well as oral and congenital transmission also occur [4]. The initial two months after the onset of CD are known as the acute phase, which are followed by the chronic phase (which is lifelong in the absence of treatment) and may be symptomatic (up to 20% of infected individuals can develop irreversible chronic heart disease) [5]. Although a small percentage of individuals (specially children) die during the acute phase, slowly developing debilitating chronic damages, including chronic chagasic cardiomyopathy, megaesophagous and megacolon, constitute the severest clinical manifestations of Chagas disease, leading to disability and death among those infected [6]. Currently, there is no vaccine available for Chagas disease and the available treatment is not completely satisfactory due to incomplete efficacy (especially in the chronic phase) [5].

T. cruzi is genetically variable, and currently six genetically different lineages or discrete typing units (DTUs) are recognized, named from TcI to TcVI [7]. Furthermore, an additional genotype known as Tcbat, which primarily infects bats has been described [8].

These lineages have a different but overlapping geographical distribution in the Americas [9]. There are no proven associations between DTUs and clinical manifestations of CD [10].

TcI is the most widely distributed DTU and it is considered to be the main cause of chagasic heart disease in the northern Amazon region [11, 12]. TcI possesses high intra DTU genetic diversity, which is associated with many different human infections, mainly with chagasic heart disease but in a region where megasyndromes are rare [13, 14]
The cellular invasion of *T. cruzi* represents an advantage in the persistence of the disease in the human hosts [15]. Despite the immune response of the host, the parasite manages to endure because of its ability to infect many different cell types [15].

The parasitic stage in vertebrates has an obligatory intracellular component. Trypomastigotes invade mammalian host cells and differentiate into the replicative form known as amastigote and proliferate [16]. Cellular invasion begins with the parasite attachment to the cell membrane, which leads to different signaling processes that mediate the internalization of the parasites through a parasitophorous vacuole [17]. Once in the cytoplasm of the host cell, it replicates and differentiates back into the invasive trypomastigote, which are released into the bloodstream to continue with the life cycle [16, 18].

Trypomastigotes can infect a wide variety of nucleated non-phagocytic mammalian cells [17]. The initial interaction between the parasite and the host cell plasma membrane is mediated by parasite surface-membrane proteins [19] such as the

mucins, glycoproteins which can interact with mammalian cells through their sugar residues [17]. Another group of proteins belonging to the *trans*-sialidase Family (gp82, gp90, gp33/50 and gp30, among others) are involved in the initiation of the cellular invasion by *T. cruzi* [19]. In this case, proteins like gp82 and gp30 can trigger a Ca^{2+} response in the host cell that leads to parasite internalization [17]. Meanwhile, proteins like gp90 and gp33/50 negatively regulate the Ca^{2+} release and prevent cellular invasion resulting in poorly invasive strains [19]. On the other hand, strains overexpressing gp82 have been shown to be more infective to mammalian cells *in vitro*, while expression of gp90 is correlated to low infectivity [20].

In this study, we employed two-dimensional electrophoresis (2-DE) to characterize the proteomic profiles of seven TcI strains displaying differential infectivity of cultured mammalian cells. We report the identification of sets over and underexpressed of 2-DE spots statiscally associated with high and low infectivity strains. The identification of the proteins represented in such spots through mass spectrometry is currently underway, and will allow for gene ontology and pathway analysis through genomic resources for trypanosomatids. Ultimately, identification of specific proteins and pathways associated to the ability of trypomastigotes to infect mammalian cells will open up the possibility of functional confirmation through overexpression and knockout experiments and may unveil potential drug and vaccine targets.

Materials and Methods

1. Quantitative analysis of the *in vitro* infectivity of seven TcI-lineage *Trypanosoma cruzi* strains

Cell line and parasite maintenance

LLcMK$_2$ monkey kidney cells, were maintained with 10 mL of Dulbecco Modified Eagle Medium (DMEM) (Hyclone, Waltham, MA, USA) with 1 % penicilin/streptomycin (Hyclone, Waltham, MA, USA), 10% of fetal bovine serum (FBS) (DMEM10) (Hyclone, Whaltam, MA, USA) and were kept in a 37ºC, 5% CO_2 atmosphere [21].

Selected strains were available as epimastigote forms. Epimastigote cultures were maintained in Liver Infusion Tryptose (LIT), supplemented with 1% of gentamicin (GIBCO Invitrogen, Carlsbard, CA, USA), 1% of penicilin/streptomycin (SIGMA, St Louis, MO, USA) and 10 % of FBS (Hyclone, Whaltam, MA, USA) at 28ºC. Epimastigote cultures were seeded and allowed to reach the stationary phase in order to induce metacyclogenesis. The cultures were then seeded in LLcMK$_2$ confluent monolayers to let the metacyclic trypomastigotes infect the cells. The metacylcic cultures were obtained by complement lysis selection as described previously by Nogueira *et al*., 1975 [22]. Horse Serum (Gibco, Thermo Fischer Scientific, New Zealand) was used for 2 weeks in order to obtain a trypomastigotes-only culture.

Tissue culture derived trypomastigotes were generated by serial infection passages in LLcMK$_2$ in order to establish the culture of each strain of the study. DMEM with 1 %

135 penicilin/streptomycin, 12% of fetal bovine serum (FBS) was used (DMEM2) (Hyclone,
136 Whaltam, MA, USA).
137
138 Seven TcI parasite strains were selected for study, as follows: Brazil, Human cl3, Ecua
139 7, BJ 401, TRC 2377, Sylvio X10 and TSW 2339 (Table 1).
140
141 **Table 1. TcI strains included in the study**

Strain	DTU	Origin	Host	Reference
Brazil	TC I	Brasil	*Homo sapiens*	Zingales, B., et al. 2009[23]
Human cl3	TC I	El Salvador	*Homo sapiens*	Costales, J., et al. 2015[24]
Ecua7	TC I	El Oro, Ecuador	*Homo sapiens*	Garzon, E., et al. 2002[25]
BJ401	TC I	Portoviejo, Ecuador	*R. ecuadoriensis*	Ocaña, S., et al. 2010[26]
TRC 2377	TC I	San Vicente, Ecuador	*R. ecuadoriensis*	Ocaña, S., et al. 2010 [26]
Sylvio X10	TC I	Brasil	*Homo sapiens*	Zingales, B., et al. 2009[23]
TSW 2339	TC I	Montecristi, Ecuador	*R. ecuadoriensis*	Ocaña, S., et al. 2010[26]

142
143 **Infectivity of *Trypanosoma cruzi* I isolates**
144 Three round sterile coverslips of 12 mm (Fisherbrand) were placed in each well in a 6-
145 well tissue culture plate. A total of 1,5 x 10^5 cells were suspended in 2 ml DMEM10, as
146 previously described, seeded in for each well of the plate and incubated for 48 hours at
147 37ºC, 5% CO_2. Subsequently, the plate was infected with 1,5 X 10^7 parasites per well for 2
148 hours (MOI 100). Then, each well was washed three times with PBS 1X, and filled with 2
149 ml DMEM2. The infection was allowed to proceed for 48 hours, the coverslips removed,
150 washed on phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and stained
151 with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (D9542 SIGMA-ALDRICH).
152
153 Three hundred cells per coverslip were counted at 40 X using a fluorescence scope
154 (Olympus BX51) equipped with an Olympus DP72 Camera. Every cell that presented one

155 or more parasites in their cytoplasm was counted as an infected cell, otherwise when
156 only a cell nucleus was observed it was counted as a non-infected cell. Each sample had
157 three independent technical repetitions. These results were analyzed by a one-way
158 ANOVA and a Tukey post-hoc test was also performed in order to group the samples into
159 different categories.

160 **2. Two dimensional gel electrophoresis (2-DE)**

161 **2.1 Sample Preparation**

162 $1x10^9$ tissue culture-derived trypomastigotes were collected and pelleted for 20
163 minutes at 1500 g at room temperature. The pellet was washed with 50 ml PBS 1X and
164 resuspended in 15 ml PBS. The parasites were pelleted once again and resuspended in
165 1ml of PBS 1X and transferred to a 1,5 ml microcentrifuge tube. The microcentrifuge
166 tubes were then centrifuged at 1500 g for 20 min at room temperature; the weight of the
167 pellet was recorded and the sample stored at −80ºC for up to three months.
168

169 **2.2 Cellular lysis and soluble protein extraction**

170 The samples prepared in 2.1 were thawed and 300 μl of solubility buffer (7M Urea,
171 2M Thio Urea, 4% chaps, 0,5% Triton, 40 mM Tris, 0,25% SDS and traces of bromophenol
172 blue) per 300 mg of pellet was added [27].
173

174 In order to rupture the parasite cells, samples were immersed in liquid nitrogen for 5
175 seconds and then thawed at RT. This process was repeated 4 times after which bensonaze
176 nuclease was added and incubated with the samples for 10 min at RT to remove nucleic
177 acids residues from the sample. The tubes were then centrifuged at 13 000g for 20 min at

178 4ºC. The supernatant was recovered and the pH was adjusted to 8.0 with NaOH.
179 Subsequently, four volumes of ice-cold acetone were added to precipitate proteins and to
180 remove organic-soluble contaminants (small ionic molecules like nucleotides, metabolites
181 and phospholipids) [27]. The samples were stored overnight at −20ºC, and then
182 centrifuged at 13 500g for 45 min at 4ºC. The supernatant was discarded and the pellet
183 was washed with 500 µl of cold acetone and centrifuged once again under at the same
184 conditions. This process was repeated 3 times. Finally, the supernatant was discarded and
185 the pellet was allowed to dry for 1 minute and resuspended in solubilization buffer (7M
186 Urea, 2M Thio Urea, 4% chaps, 0,5% Triton, 40 mM Tris, 0,25% SDS and traces of
187 bromophenol blue).
188

189 **2.3 Quality and quantity sample verification**

190 Integrity of the protein samples was verified by elecrophoresis in 10 % SDS-
191 polyacrylamide mini-gels stained with Commassie blue. Protein concentration in the
192 samples was determined by the Bradford method [28]. Linear regression analysis was
193 performed by measuring the absorbance of known concentrations of BSA and the
194 unknown sample at 550 nm in a spectrophotometer (ELX800 Universal Microplate Reader
195 BIO-TEK Instruments, INC).
196

197 **2.4 Isoelectric focusing**

198 The first dimension separation of the protein samples was performed in an Ettan
199 IPGphor instrument (GE Healthcare) using 3-10 pH non-linear gradient IPG-strips. Strips
200 were rehydrated for 16 hours with 200 mg of the sample and covered with 3 mL of

DryStrip Cover Fluid (GE) to avoid sample evaporation. The proteins were focused sequentially as follows: 60 V for 2 hours, 1000 V for 3 hours (gradient), 8000V for 4 hours (gradient), 8000V constant for 7 hours and then held in 30 V.

2.5 SDS-PAGE

Strips were reduced with 20 mL of equilibrium buffer (6M Urea, 100 mM Tris pH 8.8, 30% glycerol, 2% SDS, and 1Lt of dH_2O) and 200mg of dithiothreitol (DTT) for 10 min, after which the strips were alkylated with 20 mL of equilibrium buffer and 400 mg of iodoacetamide for another 10 min to avoid the re-oxidation of proteins. Subsequently, the strips were washed three times with ddH_2O and placed on an electrophoresis chamber SE600 RUBY Complete (GE Healthcare) and electrophoresed on 12%, 18 cm, polyacrylamide gels at constant 50 V overnight. Subsequently, the gels were fixed as follows: 20 min submerged in 5 mL of F1 fixation solution (50% Absolut Ethanol and 50% Acetic Acid), 10 min submerged in 5 mL of F2 fixation solution (50% Absolut Ethanol and 50% dH_2O), washed during 5 min with dH_2O and repeated 3 times. The gels were stained with InstantBlue Protein Stain (Sigma-Aldrich) for 24 hours with agitation. Finally, the gels were distained with dH_2O 5X, replacing water every 2 hours, and subsequently maintaining the gel in dH_2O overnight with gentle agitation. High-resolution digital images TIFF format of each gel in were obtained with an Epson Perfection V850 Pro Scan.

2.6 Proteomic profile analysis

Gel images were analyzed with Progenesis SameSpot 3.1 Software (Nonlinear Dynamics), which is designed to assure the objective analysis of the differential

224 expression of the proteins in 2-DE gel and its reproducibility. The software allows to
225 subtract background, detect and quantify spots and match them across different gels
226 [29]. The Software also gives spots volumes (by combining the pixel intensity of the spot
227 and the area of the spot measured in mm^2). The volume is normalized to a standard,
228 which was a Brazil gel with no background. Subsequently, the spots were chosen and
229 screened according to their individual ANOVA p-value and their differential expression
230 between the statistically determined groups (high infectivity vs. low infectivity).
231
232 The selected spots were then excised from the gel and submitted to Mass
233 Spectrometry analysis. Twenty-two spots of interest were analyzed. Additionally, three
234 control spots where included. Control spots consisted in the same spot from three
235 individual gels from Brazil, Ecua 7 and TSW2339 strains.
236

237 **3. Mass Spectrometry Analysis**

238 The samples were analyzed using a nano-flow HPLC (Ultimate 3000 RSLC, Thermo
239 Fisher Scientific) coupled to a mass spectrometer equipped with a nanoelectrospray
240 source (Q-Exactive Plus, Thermo Fisher Scientific). Peptides were separated on a capillary
241 column (reverse phase C18, NanoViper, Dionex) following a gradient of 0-40% B in 33 min
242 (60 min run) (A = 0.1% formic acid, B = 0.1 % formic acid in 80% acetonitrile) at a flow rate
243 of 300 nl / min. The spectra were recorded in the Xcalibur software (Thermo Fisher
244 Scientific). These devices are regularly checked (cleaning, fluids, calibration). The spectral
245 data were analyzed using ProteomeDiscoverer v1.4 and mascot v2.6 software (Mascot
246 Significance Threshold 0.01 filters, $\geqslant$ 2 single peptides). The database was
247 RefProteome_TRYCR-all 2018_01 (*Trypanosoma cruzi*, 19,243 sequences; 9,671,180

residues) (source UniProtKB) with the fixed modification Carbamidomethyl (C) and the following variable modification: Oxidation (M).

4. Data Analysis

Protein IDs (UniProtKB accession number) for the most abundant match for each spot were entered in the UniProtKB database to find the corresponding TryTripDB gene code for each protein, as well as gene ontology (GO) classification for molecular function. Subsequently, the integrated database trypanosomatid genomic analysis (TryTripDB) was used to identify the biological processes to which the differentially expressed proteins were associated to through gene ontology (GO) analysis [30]. Finally, the lists of GO terms generated in TryTripDB were summarized and visualized employing the REVIGO (reduce + visualize gene ontology) server, in order to identify patterns among the complex lists of GO terms generated in the analysis [31].

Results

1. Quantitative analysis of the in vitro infectivity of seven TcI-lineage Trypanosoma cruzi strains

LLcMK$_2$ cells were infected with each studied strain, and the number of infected cells was determined as described under the materials and methods section. The number of cells infected by each strain was analyzed by one-way ANOVA (Table S1), and trypomastigotes from the studied strains displayed statistically significant differences ($p < 0,001$) in terms of their infectivity towards mammalian cells. A Tukey post-hoc test clustered the strains in two groups, which we termed “high infectivity” strains (Brazil,

Human cl3 and Ecua 7), and “low Infectivity” strains (BJ 401, TRC 2377, Sylvio X10 and TSW 2339) (Fig 1).

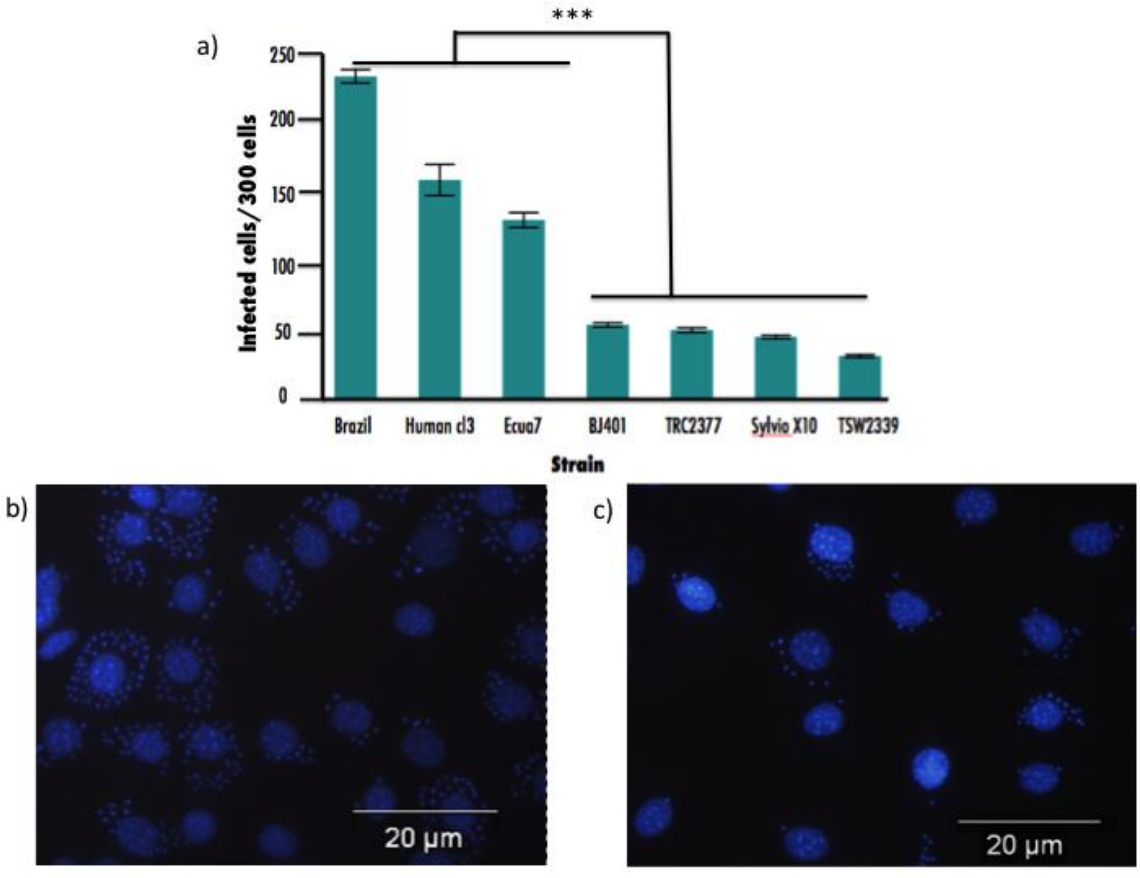

Fig 1. Infectivity rate for the strains included in the study. LLcMK$_2$ cells were infected for two hours using tissue culture derived trypomastigotes from each of the studied strains at an MOI of 100. Infection was allowed to proceed for 48 hours (to allow for parasite replication and facilitate differentiating infected from uninfected cells) and the DNA from the cells and parasites was stained with DAPI. Three hundred fields were counted at 40X on a fluorescence microscope to quantify the number of cells infected by each strain. The experiment was repeated independently three times.
a) Number of infected cells for each of the strains included in the study; **b)** and **c)** Representative fluorescence micrographs (40X) of LLcMK$_2$ cultured cells infected with a high infectivity strain (Brazil) and a low infectivity strain (TSW 2339) respectively; *** $p < 0{,}001$ black bars indicate the groups to which strains are assigned to according to the Tukey-test.

2. Two dimensional gel electrophoresis (2-DE)

Four gels were generated for each of the studied strains. The digital images corresponding to the gels were aligned with the Progenesis SameSpot 3.1 Software. All gel images were aligned to that of a gel corresponding to the Brazil strain, which had no background noise. The employed software identifies spots, which appear consistently in the independent replicate experiments, and compares their intensities through an ANOVA test, in order to identify differentially expressed spots. Forty spots with statistically different intensities between high and low infectivity strains were identified (Fig 2). ANOVA p-values, fold-change and normalized average volume for each spot are summarized in Table 2.

Differentially expressed spots were visually inspected in each gel image. The following cases were encountered: 1) spots present in high infectivity strains and absent in low infectivity ones, 2) spots present in present in both groups, which were over expressed in high infectivity strains and 3) spots present only in low infectivity strains and absent in high infectivity strains. Representative examples of each of these cases are shown in Fig. 3, panels a-c.

Based on the visual inspection, 22 differentially expressed spots were chosen for mass spectrometry analysis. Low quality spots (see Fig 3, panel d for an example) were excluded from downstream analysis.

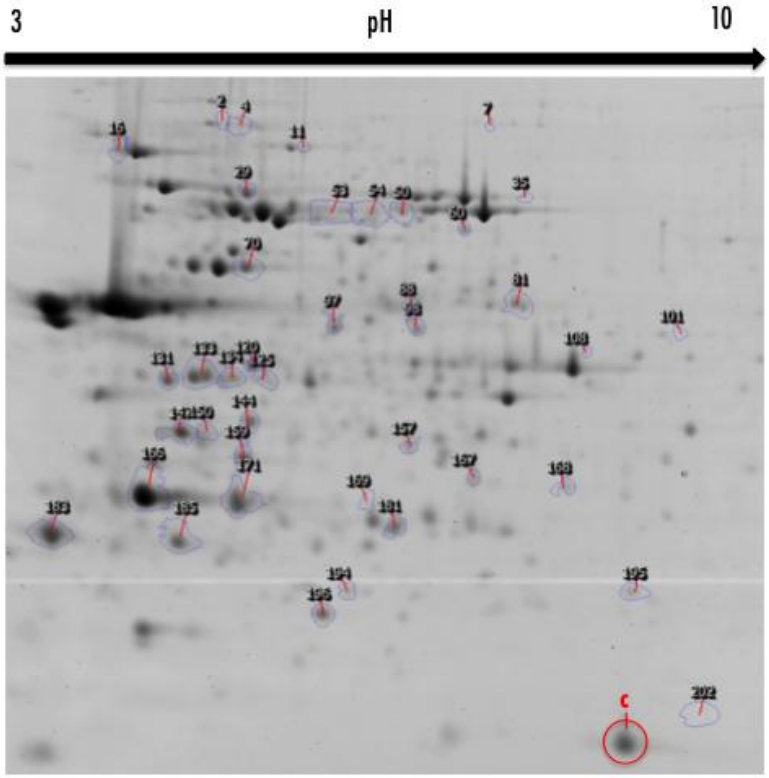

Fig 2. Differentially expressed spots identified by the 2D electrophoresis analysis. A representative gel (corresponding to the Brazil strain) is shown, displaying the observed 2D electrophoresis pattern. A total of 147 spots were consistently observed in the gels, 40 of which showed statistical differences in intensity between the high infectivity and low-infectivity strains as identified by SameSpot Software analysis. Spot labelled as C corresponds to the control spot excised from different gels to test the consistency of the protein identification through mass spectrometry.

Table 2. Data for differentially expressed spots identified in the study.

Spot ID #	p-value (ANOVA)	Fold -change	Average Normalised Volumes		Selected for Mass Spectrometry
			High infectivity	Low Infectivity	
2	0,00	2.6	3768543	9642765	
147	0,01	2.3	65350000	27990000	+
134	0,01	1.8	19520000	11080000	
171	0,09	1.8	109900000	60090000	+
185	0,09	1.9	76950000	41030000	+
4	0,12	2.2	10450000	22550000	+
194	0,16	1.8	9688342	5512649	+
144	0,33	1.7	24640000	14150000	+
60	0,71	1.9	6585488	3404248	+
7	0.001	3.1	1091505	3366528	+
97	0.002	1.6	8879261	5694202	+
125	0.002	1.4	11980000	8388125	
11	0.003	2.1	2262029	4729070	+
131	0.005	1.8	33480000	18670000	+
54	0.005	1.6	25120000	41000000	
183	0.006	1.6	137100000	88040000	+
159	0.008	1.5	24140000	16590000	+
16	0.008	1.7	20710000	34430000	
150	0.010	1.5	15350000	10510000	
202	0.010	3.1	6122510	18870000	
81	0.011	1.4	26640000	38530000	
88	0.013	1.5	10400000	6992105	
196	0.014	2.0	29680000	14850000	+
98	0.016	1.5	14370000	9560042	+
169	0.018	1.6	7792838	12110000	
133	0.018	1.4	64310000	47010000	+
120	0.020	1.9	6597920	3474884	
195	0.020	1.7	16440000	9705530	+
70	0.023	1.8	31680000	57940000	+
157	0.028	1.4	7157488	4979133	+
108	0.028	4.3	1133720	4882848	
101	0.032	9.3	1439719	13350000	
29	0.037	1.3	18690000	14690000	
166	0.038	1.4	309800000	225900000	+
53	0.039	1.4	42820000	60070000	
181	0.042	1.3	23150000	17220000	+
167	0.042	1.3	5063439	4046781	
50	0.043	1.4	14750000	20980000	+
35	0.045	2.4	1559189	3783124	
168	0.045	1.5	5537490	8493638	

[+] Spot chosen for Mass Spectometry Analysis

Data for the 40 spots displaying statistical differences in intensity between high and low infectivity strains as identified by SameSpot Software analysis is shown. Based on the visual inspection of the spots on the gel images (see example on Figure 3d), 22 significant spots were selected for mass spectrometry analysis.

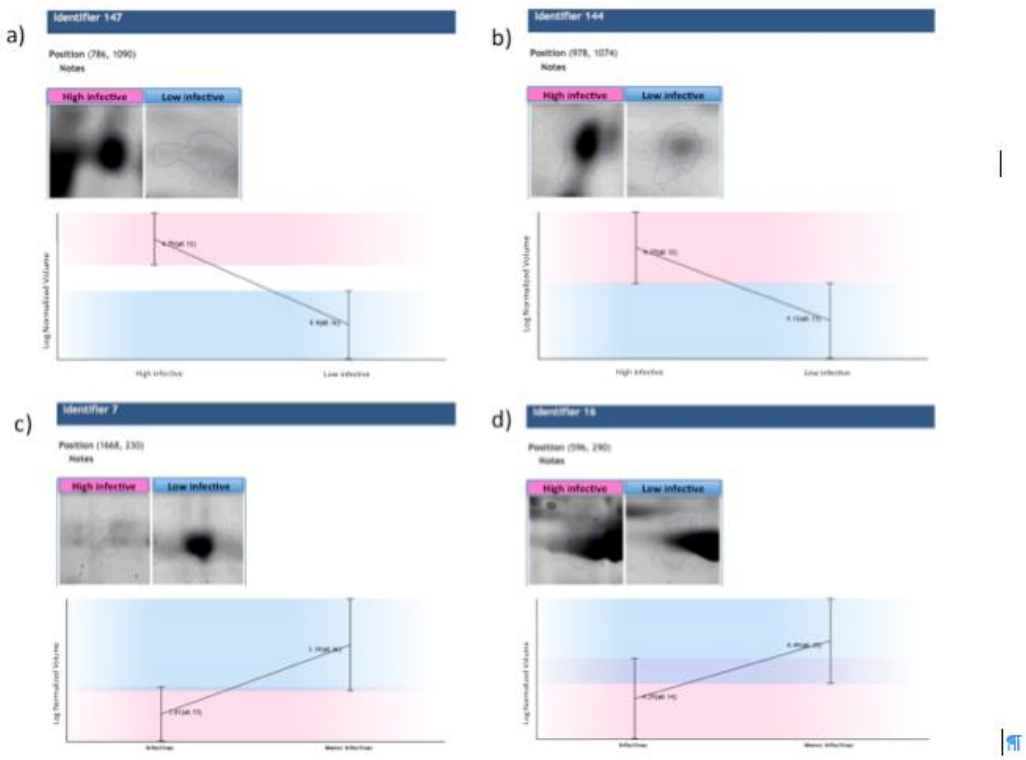

Fig 3. Representative examples of the types of spots chosen for subsequent mass spectrometry analysis. Visual examination of each spot chosen by SameSpot Software was performed in order to filter out low quality spots. a-c show examples of good quality spots: a) A spot present in high infectivity strains and absent in low infectivity ones; b) Over expressed spot in high infectivity strains c) Spot present in low infectivity strains and absent high infectivity strains. d) Example of low quality spot excluded from the analysis.

3. Mass Spectrometry Data Analysis

The mass spectrometry analysis allowed for protein identifications for each of the spots submitted for analysis. Although more than one protein can be present in each spot (Table S2, included as digital file), the proteins with higher number of peptide spectrum matches are likely those more abundant, and thus were employed for downstream analysis. Importantly, three independent control spots from gels corresponding to different parasite strains (Brazil, Ecua 7 and TSW2339) all yielded the same protein ID: the putative enolase, accession # QD4Z98 from the *T. cruzi* CL Brenner genome, providing confidence in the mass spectrometry results.

348

349 Molecular functions for the most abundant protein for each spot were obtained in
350 the UniProtKB Data Base. Proteins associated with low infectivity strains include
351 Lipophosphoglycan biosynthetic protein, Elongation factor 2, Chaperonin HSP60
352 (mitochondrial) and Heat shock protein 70 (HSP70) (Table 3). Besides from elongation
353 factor 2, all other proteins overexpressed by low infectivity strains are associated with
354 ATP binding as their molecular function (Fig 4a).

355

356 **Table 3. Proteins overexpressed in low infectivity strains and their characteristics**

Protein ID	Name	MW [kDa]	Coverage	Molecular Function
Q4DW89	Lipophosphoglycan biosynthetic protein	87.0	32.15	ATP binding
				unfolded protein binding
Q4D3T1	Elongation factor 2	94.1	33.33	GTPase activity
				GTP binding
				translation elongation factor activity
Q4DYP5	Chaperonin HSP60, mitochondrial	59,13	81,4	ATP binding
Q4DTM8	Heat shock protein 70 (HSP70)	73,25	38,26	ATP binding

357

358

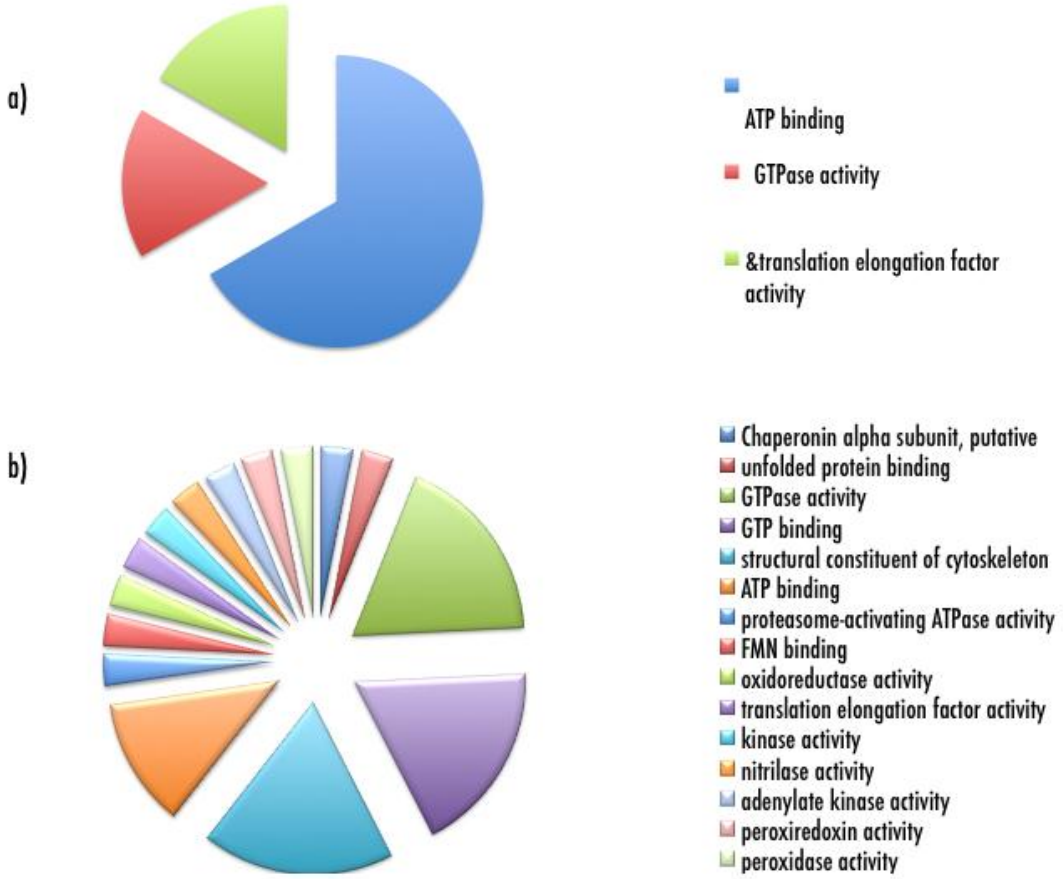

Fig 4. Molecular functions of differentially expressed proteins. Molecular functions for the most abundant protein for each spot were obtained from UniProtKB Data Base. **a)** Proteins overexpressed in the low infectivity strains **b)** Proteins overexpressed in the high infectivity strains

On the other hand, proteins associated with high infectivity strains (Table 4), include the paraflagellar rod protein, the tubulin beta chain, and the proteasome regulatory ATPase subunit 5. The main molecular functions of the high infectivity strain proteins are the GTPase activity, GTP binding and structural constituent of cytoskeleton, followed by the ATP binding molecular function (Fig 4).

372 **Table 4. Proteins overexpressed in high infectivity strains and their characteristics**

Protein ID	Name	MW [kDa]	Coverage	Molecular funtion
Q4DIF6	69 kDa paraflagellar rod protein, putative	69,51	33,83	calmodulin binding
Q4D0F4	Chaperonin alpha subunit, putative	59,35	27,97	Chaperonin alpha subunit, putative
				unfolded protein binding
Q4DQP2	Tubulin beta chain	49,66	28,73	GTPase activity
				GTP binding
				structural constituent of cytoskeleton
Q4DVH1	Proteasome regulatory ATPase subunit 5, putative	49,02	43,76	ATP binding
				proteasome-activating ATPase activity
Q4DTM8	Heat shock protein 70 (HSP70), putative	73,25	27,18	ATP binding
Q4E3A4	60S acidic ribosomal protein P0	34,93	44,58	
Q4E4V7	Prostaglandin F2alpha synthase	42,21	30,08	FMN binding
				oxidoreductase activity
Q4DQP2	Tubulin beta chain	49,66	35,97	GTPase activity
				GTP binding
				structural constituent of cytoskeleton
Q4DQP2	Tubulin beta chain	49,66	51,81	GTPase activity
				GTP binding
				structural constituent of cytoskeleton
Q4D3T1	Elongation factor 2, putative	94,12	27,66	GTPase activity
				GTP binding
				translation elongation factor activity
Q4DTN2	Activated protein kinase C receptor, putative	35,00	55,97	kinase activity
Q4DQP2	Tubulin beta chain	49,66	36,43	GTPase activity
				GTP binding
				structural constituent of cytoskeleton
Q4CLA1	Tubulin alpha chain	49,76	38,36	GTPase activity
				GTP binding
				structural constituent of cytoskeleton
Q4D9K8	Nitrilase, putative	30,84	51,45	nitrilase activity
Q4CU95	Heat shock 70 kDa protein, putative	40,83	50,14	ATP binding
Q4E1S6	Adenylate kinase, putative	29,37	42,69	adenylate kinase activity
				ATP binding
Q4CX87	Tryparedoxin peroxidase, putative	25,49	66,37	peroxidase activity
				peroxiredoxin activity

373

374

375 In order to attain a global understanding of the biological processes in which the
376 proteins of interest are involved, the gene ontology (GO) enrichment analysis tools built
377 within TryTripDB (the integrated database trypanosomatid genomic analysis) were
378 employed.
379 Preliminary analysis employing the lists of proteins overexpressed in high and low
380 infectivity strains separately identified enriched GO terms; however, in most cases only
381 one of the proteins from the list was related to each GO term. Therefore, in order to
382 maximize the probability of identifying biologically relevant GO functions and metabolic
383 pathways associated to the differentially expressed proteins identified by proteomics, the
384 entire list of proteins of interest (proteins overexpressed in both high and low infectivity
385 strains) was analyzed.
386 When GO term enrichment for molecular function was employed, multiple (up to four)
387 proteins were found to be related to individual GO terms. The list of GO terms obtained
388 for both “CL Brenner Esmeraldo-like” and “CL Brenner Esmeraldo non-like” components
389 of the *T. cruzi* genome were merged, and are displayed in Table 5.
390

391

392 **Table 5**. GO term analysis from the Trytrip Database

GO ID	GO Term	Genes in the background with this term	Genes in result with this term	Percent of background genes in result	Fold-enrichment	Odds ratio	P-value
GO:0019201	nucleotide kinase activity	7	1	14.3	111.62	155.87	8.93e-3
GO:0004017	adenylate kinase activity	6	1	16.7	130.22	187.08	7.66e-3
GO:0051920	peroxiredoxin activity	4	1	25.0	141.47	215.05	7.05e-3
GO:0051087	chaperone binding	3	1	33.3	260.44	468.0	3.84e-3
GO:0016209	antioxidant activity	21	1	4.8	26.95	32.14	3.65e-2
GO:0036094	small molecule binding	731	4	0.5	3.1	5.22	2.74e-2
GO:1901265	nucleoside phosphate binding	724	4	0.6	3.13	5.28	2.65e-2
GO:0000166	nucleotide binding	724	4	0.6	3.13	5.28	2.65e-2
GO:0003924	GTPase activity	45	2	4.4	25.15	34.7	2.60e-3
GO:0043168	anion binding	710	4	0.6	3.19	5.4	2.48e-2
GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	11	1	9.1	51.44	64.41	1.93e-2
GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	15	1	6.7	52.09	66.69	1.91e-2
GO:0017076	purine nucleotide binding	644	4	0.6	3.51	6.06	1.77e-2
GO:0032553	ribonucleotide binding	642	4	0.6	3.53	6.08	1.75e-2
GO:0097367	carbohydrate derivative binding	642	4	0.6	3.53	6.08	1.75e-2
GO:0032555	purine ribonucleotide binding	633	4	0.6	3.58	6.18	1.66e-2
GO:0032550	purine ribonucleoside binding	632	4	0.6	3.58	6.2	1.65e-2
GO:0001883	purine nucleoside binding	632	4	0.6	3.58	6.2	1.65e-2
GO:0001882	nucleoside binding	632	4	0.6	3.58	6.2	1.65e-2
GO:0032549	ribonucleoside binding	632	4	0.6	3.58	6.2	1.65e-2
GO:0035639	purine ribonucleoside triphosphate binding	631	4	0.6	3.59	6.21	1.64e-2
GO:0019205	nucleobase-containing compound kinase activity	12	1	8.3	65.11	84.93	1.53e-2
GO:0016776	phosphotransferase activity, phosphate group as acceptor	12	1	8.3	65.11	84.93	1.53e-2
GO:0019001	guanyl nucleotide binding	95	2	2.1	11.91	15.86	1.12e-2
GO:0005525	GTP binding	95	2	2.1	11.91	15.86	1.12e-2
GO:0032561	guanyl ribonucleotide binding	95	2	2.1	11.91	15.86	1.12e-2
GO:0005516	calmodulin binding	6	1	16.7	94.31	128.97	1.06e-2
GO:0010181	FMN binding	8	1	12.5	97.67	133.57	1.02e-2

393

394 Finally, the merged list of GO terms was analyzed employing REVIGO (reduce +
395 visualize gene ontology), a resource which allows to summarize and visualize GO terms in
396 different graphic formats, removing redundant GO terms [31], and therefore facilitating
397 the identification of patterns among complex lists of GO terms. A scatterplot of the
398 REVIGO analysis for our data is shown in Fig 5.

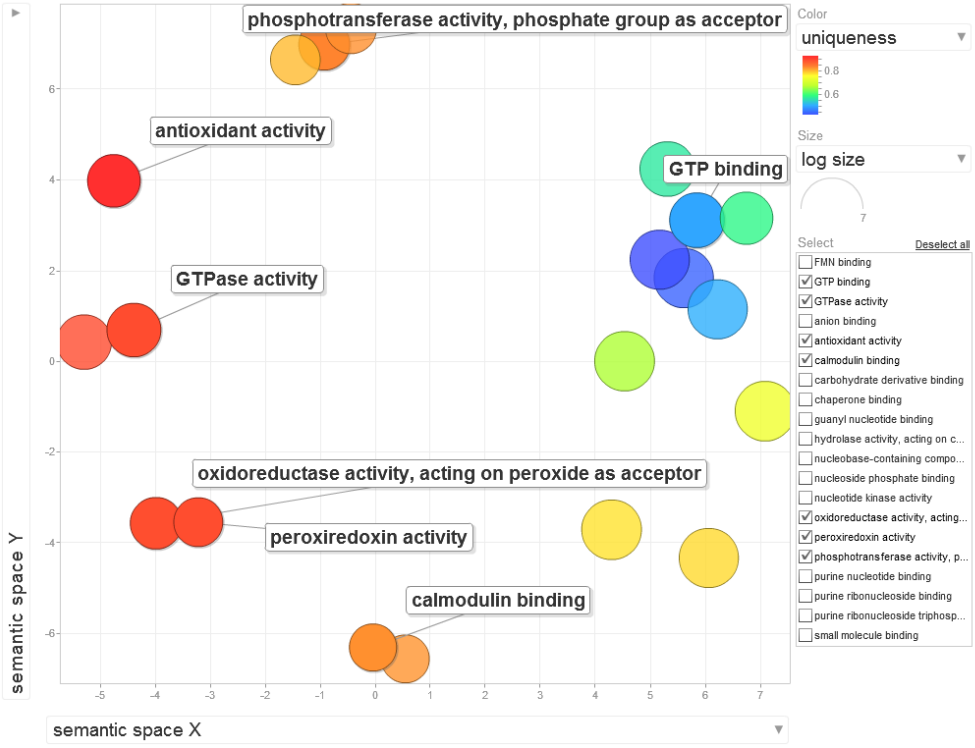

Fig 5. REVIGO scatterplot showing the cluster similarities of GO terms associated to the proteins identified by the proteomic analysis. The colors represent the uniqueness (a measure of how different a given GO term is from others in the GO term database). It is desirable to preserve "unique" GO terms when summarizing the results, while other terms can be either dispensed or collapsed into more general GO terms.

Discussion

T. cruzi is an obligate intracellular parasite which must successfully invade mammalian cells in order to complete its life cycle [32]. Understanding its cell invasion process is crucial to the efforts related to prevent Chagas disease: blocking entry to mammalian host cells through vaccination or chemotherapeutic strategies would be highly beneficial for the control and prevention of this parasitosis, for which an effective vaccine and satisfactory treatment are not currently available [10].

The cellular biology involved in the invasion of mammalian host cells by *T. cruzi* has been extensively studied over the last three decades [16, 33-35] and the sum of this

information depicts elegant mechanisms of manipulation of host cell function by the parasite [36]. The current model of cell invasion indicates that the parasite takes advantage of the wound repair machinery of the cell that begins with a Ca^{2+} trigger signal to promote lysosomal exocytosis by actin reorganization [37]. Once the lysosomes are in the entry site a release of the lysosomal contents happens, liberating the acid sphingomyelinase (ASM), which catalyzes the breakdown of sphingomyelin facilitating plasma membrane invagination. This membrane invagination facilitates parasite`s entry into the cytoplasm of the host cell [15, 36].

However, *T. cruzi* is a highly variable species where at least six genetic lineages or discrete typing units (DTUs), named TcI to TcVI, are currently recognized [23, 38]. Although no proven association exists to date between the *T. cruzi* DTUs and the different clinical manifestations of Chagas disease [10], the DTUs display different geographic distribution patterns and host preferences, as well as tissue distribution and physiopathology [39]. TcI constitutes the most widespread DTU, spanning the entire distribution range of the parasite (from the South of the United States to Northern Chile and Argentina) and is involved in both sylvatic and domestic transmission cycles [39].

Additionally, TcI displays the highest intra-DTU genetic variability among the *T. cruzi* DTUs [13]. Due to the low prevalence of TcI among chronic patients in the Southern Cone countries [39] this DTU has been erroneously disregarded as non-pathogenic in the past [40]. However, TcI constitutes the most important DTU north of the Amazon [10, 39] and it is well associated with chronic chagasic cardiomyopathy, the most devastating chronic clinical manifestation of Chagas disease [14, 24].

Besides a handful of studies, which have shown that different DTUs could have differences in tissue tropism and geographical distribution, the infectivity of a given strain does not seem to be determined only by the DTU to which it belongs [41-43]. We explored the *in vitro* infectivity of several TcI strains, including standard laboratory strains, an isolate from a chronic chagasic patient from El Salvador and several Ecuadorian isolates [26, 44]. Interestingly, wide variability was displayed by the studied strains in terms of the ability of tissue culture-derived trypomastigotes to infect tissue culture cells: while some strains infected 80% of cells in culture, other infected 11%. These results clearly support the view that the strain infectivity is not directly determined by the DTUs. Additionally, our statistical analysis clearly grouped the strains in two classes: three strains displaying higher infectivity, and the remaining four strains displaying lower infectivity.

Proteomic profiles obtained by 2D electrophoresis analysis have been shown to be correlated to the *T. cruzi* DTUs [29]. Although the differential proteomic profiles may be determined by broad genetic differences among parasite strains (i.e. DTUs), they are the result of gene expression and thus they hold the potential to allow for identification of the protein factors that determine the infective capacity of a given strain. We employed 2D electrophoresis to obtain proteomic profiles for tissue culture-derived trypomastigotes from each of the studied strains and compared these between strains displaying high and low infectivity. The methodology we standardized allowed for characterization of proteomic profiles comprised of 147 spots, where 40 spots were found to be differentially displayed by high and low infectivity strains. Among these, spots

464 that were present in one of the classes and absent in the other, and spots which were
465 present in both, but at different intensities were identified. The majority of differentially
466 expressed spots were expressed in high infectivity strains (30) while only a few (10) were
467 expressed in low infectivity strains.
468
469 The spots showing the best quality in the proteomic profiles (22) were selected for
470 identification via Mass Spectrometry analysis. When three replicates of the same spot
471 were submitted to mass spec analysis, the results consistently identified the same
472 protein: enolase, showing the reliability of the obtained IDs.
473
474 Interestingly, tubulin α and specially β were both found to be among the most
475 clearly overexpressed proteins associated with the highly infective strains. These proteins
476 are components of microtubules, including the subpellicular microtubules, which
477 constitute the parasite cytoskeleton, as well as the microtubules present in the
478 cytopharinx and flagellar pocket [45]. Additionally, microtubules constitute the
479 paraflagellar rod and the flagellum, which are involved in motility [45]. Among the GO
480 functions associated with the overexpressed proteins, GTP binding, GTPAse activity,
481 calmodulin binding and phosphotransferase activity are associated with tubulin and
482 microtubule dynamics. Interestingly, parasite motility and, specifically the flagellum, have
483 been associated with the capacity of the parasite to invade cells [46]. Our results suggest
484 that more infective strains may be more motile or have a more intense flagellar function,
485 which could correlate with their greater capacity to infect non-phagocytic mammalian
486 culture cells. Our results complement and extend previous reports of tubulin expression

being overexpressed among TcI strains displaying more aggressive infection patterns in phagocytic cells and mice [47].

The fact that β-tubulin was associated with different spots in our gels could be explained by the occurrence of several variant of this protein in the parasite, due to the expression of two different length mRNAs [48], as well as numerous post-translational modifications, including glutamylation, acetylation and detyrosination [49].

GO functions related to antioxidant activity, peroxiredoxin and oxidoreductase activity are associated with antioxidative functions of the parasite. Importantly, tryparedoxin, one of the major antioxidative proteins in the parasite, and a protein we found to be overexpressed in the more infective strains, has been studied as a possible vaccine candidate for *T. cruzi* [50] as well as for its trypanosomatid relative Leishmania [51, 52]. Furthermore, the capacity of TcI strains to respond to oxidative stress has also been reported to be related to TCI strains displaying more aggressive infection patterns in mice as well as the capacity of the parasite to survive phagocytosis by macrophages [53]. The role that the antioxidant network of *T. cruzi* could play during invasion of non-phagocytic cells is less intuitive, and will require further study.

Additionally, it is important to mention that at least five transialidase-family members (identified in the Tritryp database with codes Tc00.1047053508853.20, Tc00.1047053506455.30, Tc00.1047053506751.50, Tc00.1047053511603.90 and Tc00.1047053506409.170) are overexpressed among the highly infective strains and it is important to mention them although they were not considered the major components of

any of the spots in our analysis. Transialidases have also been evaluated as vaccine candidates for *T. cruzi* [50, 54, 55].

Despite great success in Chagas disease control and considerable reduction of its prevalence in Latin America [56], Chagas remains the most important parasitic disease in the Americas [10]. Human migration has caused it to reach non endemic countries such as the US, Canada, Europe and Japan [3]. Our results show that protein expression patterns correlate with infectivity of *T. cruzi* strains, and open up new venues to explore the intricacies of the host cell invasion process, i.e. a more detailed knowledge about the role of parasite motility and antioxidant activity in the invasion of non-phagocytic cells. A better understanding of the cell invasion process by *T. cruzi* increases the probability of identifying new potential targets to block host cell invasion through chemotherapy or vaccination.

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694

Supplementary Information

Table S1. Anova analysis of the infected cells from the strains examined

ANOVA

Infected

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	172642.032	1	172642.032	163.087	.000
Within Groups	42343.611	40	1058.590		
Total	214985.643	41			

Table S2. Mass spectrometry results from all the spots analyzed. The supplementary table from the mass spectroemtry results is available as a digital file.

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Reference style	PLOS uses "Vancouver" style, as outlined in the ICMJE sample references. See reference formatting examples and additional instructions below.
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	Genes, mutations, genotypes, and alleles	Write in italics. Use the recommended name by consulting the appropria genetic nomenclature database (e.g., HUGO for human genes). It is sometimes advisable to indicate the synonyms for the gene the first time appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MY
	Allergens	The systematic allergen nomenclature of the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee should be used for manuscripts that include the description or use of allergenic proteins. For manuscripts describing new allergens, the systematic name of the allergen should be approved by the WHO/IUIS Allergen Nomenclature Sub-Committee prior manuscript publication. Examples of the systematic allergen nomenclatu can be found at the WHO/IUIS Allergen Nomenclature site.

Submission Guidelines

Related information for authors

- Submission system
- Journal scope and publication criteria
- Getting started guide
- Guidelines for revisions
- Publication fees
- Chinese translation of PLOS policies: PLOS 编辑与出版规定

Style and Format

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Other elements	• Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately. • Tables are inserted immediately after the first paragraph in which they are cited. • Supporting information files are uploaded separately.

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			(SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-randomized, controlled trial
Short title	100 characters	State the topic of the study	Cigarette smoke exposure and innate immunity SODIS and childhood diarrhoea

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

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Authorship requirements

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Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled “current address.” At a minimum, the address must include the author’s current institution, city, and country.

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If a manuscript is submitted on behalf of a consortium or group, include the consortium or group name in the author list, and provide the full list of consortium or group members in the Acknowledgments section. The consortium or group name should be listed in the manuscript file only, and not included in the online submission form. Please be aware that as of October 2016, the National Library of Medicine's (NLM) policy has changed and PubMed will only index individuals and the names of consortia or group authors listed in the author byline itself. Individual consortium or group author members need to be listed in the author byline in order to be indexed, and if included in the byline, must qualify for authorship according to our criteria.

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Acknowledgments

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Source	Format
	example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.
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Online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available from: arXiv:1403.3301v1. Cited 17 March 2014.
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html Cited 17 March 2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

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Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). Download additional translations of the form from the Downloads and Translations page. More information about patient privacy, anonymity, and informed consent can be found in theInternational Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

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- Microarray experiments: MIAME
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- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: “Caucasian” should be changed to “white” or “of [Western] European descent” (as appropriate); “cancer victims” should be changed to “patients with cancer.”

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- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's "Fig 1"

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

Animal research

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

Manuscripts reporting animal research must state in the Methods section:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s).
- Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why. Provide any relevant regulations under which the study is exempt from the requirement for approval.
- Relevant details of steps taken to ameliorate animal suffering.

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Protocol Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Authors should always state the organism(s) studied in the Abstract. Where the study may be confused as pertaining to clinical research, authors should also state the animal model in the title.

To maximize reproducibility and potential for re-use of data, we encourage authors to follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for all

submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information.

Non-human primates

Manuscripts describing research involving non-human primates must report details of husbandry and animal welfare in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research* (PDF), including:

- Information about housing, feeding, and environmental enrichment.
- Steps taken to minimize suffering, including use of anesthesia and method of sacrifice, if appropriate.

Random source animals

Manuscripts describing studies that use random source (e.g. Class B dealer-sourced in the USA), shelter, or stray animals will be subject to additional scrutiny and may be rejected if sufficient ethical and scientific justification for the study design is lacking.

Unacceptable euthanasia methods and anesthetic agents

Manuscripts reporting use of a euthanasia method(s) classified as unacceptable by the American Veterinary Medical Association or use of an anesthesia method(s) that is widely prohibited (e.g., chloral hydrate, ether, chloroform) must include at the time of initial submission, scientific justification for use in the specific study design, as well as confirmation of approval for specific use from their animal research ethics committee. These manuscripts may be subject to additional ethics considerations prior to publication.

Humane endpoints

Manuscripts reporting studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, must comprehensively report details of study design, rationale for the approach, and methodology, including consideration of humane endpoints. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality.

Definition of a humane endpoint

A humane endpoint is a predefined experimental endpoint at which animals are euthanized when they display early markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress. Humane endpoints are used as an alternative to allowing such conditions to continue or progress to death following the experimental intervention ("death as an endpoint"), or only euthanizing animals at the end of an experiment. Before a study begins, researchers define the practical observations or measurements that will be used during the study to recognize a humane endpoint, based on anticipated clinical, physiological, and behavioral signs. Please see the NC3Rs guidelines for more information. Additional discussion of humane endpoints can be found in this article: Nuno H. Franco, Margarida Correia-Neves, I. Anna S. Olsson (2012) How "Humane" Is Your Endpoint? — Refining the Science-Driven Approach for Termination of Animal Studies of Chronic Infection. PLoS Pathog 8(1): e1002399 doi.org/10.1371/journal.ppat.1002399.

Full details of humane endpoints use must be reported for a study to be reproducible and for the results to be accurately interpreted.

For studies in which death of an animal is an outcome or a planned experimental endpoint, authors should include the following information in the Methods section of the manuscript:

- The specific criteria (i.e. humane endpoints) used to determine when animals should be euthanized.
- The duration of the experiment.
- The numbers of animals used, euthanized, and found dead (if any); the cause of death for all animals.
- How frequently animal health and behavior were monitored.
- All animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions.

If humane endpoints were not used, the manuscript should report:

- A scientific justification for the study design, including the reasons why humane endpoints could not be used, and discussion of alternatives that were considered.
- Whether the institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design.

Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use. Read the policy.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.

- **Ethics.** *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported.

Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information

Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants' experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.

For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.

See our reporting guidelines for human subjects research.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

For *de novo* (new) cell lines, including those given to the researchers as a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

Blots and gels

Manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with our policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast.
- All blots and gels that support results reported in the manuscript should be provided.
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files.
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given.

- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples.
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made.

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such asAntibodypedia or CiteAb.

Small and macromolecule crystal data

Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

Small molecule single crystal data

Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

Macromolecular structures

Authors reporting novel macromolecular structures must have deposited their data prior to submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a recognized validation functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, or databases as the primary focus of the manuscript if they meet the following criteria:

Utility

The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

Validation

Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

Availability

If the manuscript's primary purpose is the description of new software or a new software package, this software must be open source, deposited in an appropriate archive, and conform to the Open Source Definition. If the manuscript mainly describes a databse, this database must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. In these cases, authors should provide a direct link to the deposited software or the database hosting site from within the paper.

Software submissions

Manuscripts whose primary purpose is the description of new software must provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

Read the PLOS policy on sharing materials and software.

New taxon names

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be

legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni *Fisher* ***sp. nov.*** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called "Nomenclatural Acts":

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Solanum aspersum S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the **Methods** section, include a sub-section called "Nomenclature" using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Hymenogaster huthii. Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]

You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.
In the **Methods** section, include a sub-section called "Nomenclature" using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):
The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].
All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.
Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.

DECLARACIÓN Y AUTORIZACIÓN

Yo, CAMILA CILVETI RODRÍGUEZ RIGLOS**,** con CC.# 1713168878, autorA del trabajo de graduación intitulado "DISTINCT PROTEOMIC PROFILES ARE ASSOCIATED WITH *TRYPANOSOMA CRUZI* I STRAINS DISPLAYING HIGH/LOW INFECTIVITY TOWARDS MAMMALIAN CELL *IN VITRO*" previa la obtención del grado académico de magíster en Biología de Enfermedades Infecciosas, en la Facultad de Ciencias Exactas y Naturales.

1.- Declaro tener pleno conocimiento de la obligación que tiene la Pontificia Universidad Católica del Ecuador, de conformidad con el artículo 144 de la Ley Orgánica de Educación Superior, de entregar a la SENESCYT en formato digital una copia del referido trabajo de graduación para que sea integrado al Sistema Nacional de Información de la Educación Superior del Ecuador para su difusión pública respetando los derechos de autor.

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Quito, 3 de abril de 2018

CAMILA CILVETI RODRÍGUEZ RIGLOS
CC.# 1713168878