Parasite-Induced Genetically Driven Autoimmune Chagas Heart Disease in the Chicken Model

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Short Abstract:

Birds are refractory to the *T. cruzi* infections as a consequence of innate immunity present after eight days of the embryo growth. The inoculation of a few virulent typanosomes in fertile eggs prior to incubation results in the integration of the parasite mitochondrion kDNA minicircle into the genome of embryo cells. Crossbreeding of the kDNA-mutated *Gallus gallus* reveals the vertical transfer of the kDNA mutations to the progeny. The employment of the targeted-prime TAIL-PCR technique documented the kDNA mutations in coding regions at several chromosomes. The kDNA-mutated chickens died of an inflammatory heart disease similar to Chagas disease in humans. This is the first experimental reproduction of an autoimmune human disease in a transkingdom model showing parasite-induced genome modifications.

Long Abstract:

The *Trypanosoma cruzi* acute infections acquired in infancy and childhood can be asymptomatic but approximately one third of the chronically infected cases may die of Chagas disease usually three decades later. Autoimmunity and parasite persistence are main theories trying to explain the pathogenesis of Chagas disease [1]. To separate roles played by parasite persistence and autoimmunity in Chagas disease we inoculated the *T. cruzi* in the air chamber of embryonated eggs. The mature chicken immune system is a tight biological barrier against *T. cruzi*, and the infection is eradicated upon development of its immune system by the end of the first week of growth [2]. The chicks are parasite-free at hatching, but they retained the parasitic mitochondrial kinetoplast DNA minicircle in their genome, and these mutations are transferred to their progeny. The documentation of the kDNA minicircle integration in the chicken genome is obtained by a targeted prime TAIL-PCR, Southern hybridizations, cloning and sequencing [2, 3]. The kDNA minicircle integrations rupture open reading frames for transcription and immune system factors, phosphatase (GTPase), adenylate cyclase and phosphorylases (PKC, NF-Kappa B activator, PI-3K) associated with cell physiology, growth, and differentiation [2, 4-6], and several other genes. Severe myocarditis due to rejection of target heart fibers by effector cytotoxic lymphocytes is seen in the kDNA-mutated chickens, showing an inflammatory cardiomyopathy similar to that seen in Chagas disease. Interestingly, heart failure and skeletal muscle weakness were present in adult chickens with the kDNA mutations and rupture of the dystrophin gene in chromosome 1 [7]. Genotype alterations resulting from transfers of the parasitic DNA were associated with the tissue destruction carried out by effectors $CD45^{+}$, $CD8\gamma\delta^{+}$, CD8α lymphocytes. This research provides insights about a protozoan infection that can induce genetically driven autoimmune disease. Experimental treatment of the inflammatory autoimmune cardiomyopathy in kDNA-mutated chickens may require drug suppression of bone marrow progenitor of specific T-cell phenotype infiltrating the myocardium, and transplantation of histocompatible healthy bone marrow to prevent the rejection of self-tissue.

Protocol Text:

1.)Growth of parasites

Trypomastigote forms of *T. cruzi* Berenice and the β-galactosidase-expressing Tulahuen *T. cruzi* MHOM/CH/00 C4 are grown in murine muscle cell (L6) cultivated in Dulbecco minimal essential medium with 10% FSB, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 250 nM L-glutamin (pH 7.2), 5% CO_2 at 37^{o}C. The free-swimming trypomastigotes in the supernatant medium is used to inoculate chicken eggs.

2.) Parasite inoculation in embryonated chicken eggs

2.1) A suspension of 100 trypomastigotes in 10 µl of culture medium is inoculated through a 2-mm diameter hole into the air chamber of stage X fertile eggs. The invasion and replication of the virulent parasites into the embryo cells are shown in Video S1.

2.2) Holes are sealed by adhesive tape.

3.3) The *T. cruzi*-infected eggs as well mock and uninfected control samples are incubated at 37.5ºC and 65% humidity for 21 days.

3.4) The chicks that hatch are kept in incubatory for 24 h and thereafter at 32ºC for three weeks.

3.) Obtaining samples for DNA extraction

3.1) Peripheral blood mononuclear cells and solid tissues from chickens hatched from *T. cruzi* inoculated eggs and from control and mock (shells pierced but not *T. cruzi* or kDNA inoculated) chickens are processed for DNA extraction.

3.2) DNA is also extracted from semen collected from roosters, and from no fertile eggs (< 5mm) from hens hatched from eggs inoculated with *T. cruzi* [2, 3].

3.2) The *T. cruzi* mitochondrial kDNA is obtained from the parasite forms grown in liquid medium [3].

4.) Primers and probes used

The primers used for PCR amplifications and the thermal conditions are shown in **Table 1.** The probes used in Southern blot hybridizations were:

4.1) Wild-type kDNA (~1.4 kb) minicircle sequences purified from *T. cruzi* epimastigote forms;

4.2) kDNA minicircle fragments (362 bp) obtained by *Nsi*I digests of wild-type kDNA;

4.3) nDNA repetitive sequence (188 bp) obtained by amplification of the parasite DNA with the Tcz1/2 primers. The probes were purified from 1% agarose gels [2].

5.) PCR analyses

5;1) Genomic DNAs from infected chicks and uninfected controls are templates for PCR with specific *T. cruzi* nDNA Tcz1/2 [8] and kDNA primers s35/s36 [9] used in a standard PCR procedure

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5.1) The reaction mix is made of 100 ng template DNA, 0.4 µM of each pair of primers, 2 U Taq DNA polymerase, 0.2 mM dNTP and 1.5 mM $MgCl_2$ in a 25 µL final volume.

5.2) Set termocycler programe for 95 ºC for 5 min, 30 cycles of 30 secs at 95 ºC/30 secs at 68 ºC/1 min at 72 ºC with 5 min final extension before refrigeration.

5.3) Analysis of the amplification products in 1.3% agarose gel, which is transferred to a positively-charged nylon membrane (GE Life Sciences) by the alkaline method for hybridization with specific probes labeled with [α-^{32}P] dATP using Random Primer Labeling Kit (Invitrogen, Carlsbad, CA).

6.) Genomic Southern blots:

6.1) Use *Mbo*I and/or with *Eco*RI (Invitrogen) enzymes that make single cuts in minicircles integrated into DNA samples of body tissues.

6.2) Digest DNA from uninfected control chickens and from chickens hatched from eggs inoculated with virulent *T. cruzi* forms.

6.3) Subject the digests of DNA from *T. cruzi* and from chicken test samples to electrophoresis in 0.8% agarose gel at 50 V overnight at 4ºC.

6.4) Transfer separated DNA bands to positively charged nylon membrane.

6.5) Hybridize the DNA bands with radio labeled kDNA probe.

6.6) Wash the membrane twice for 15 min at 65 ºC with 2X SSC and 0.1% SDS, twice for 15 min at 65 ºC each with 0.2X SSC and 0.1% SDS, and autoradiograph for variable periods of time.

7.) Targeted prime TAIL-PCR

7.1) The amplification of the kDNA minicircle integrated into the chicken genome is obtained by a modified TAIL-PCR technique, which combined kDNA primers with specific primer sets [2] in three walk-in cycles nested PCR, as shown in **Fig. 1**:

7.1) Primary cycle: Each reaction included 200 ng template DNA, 2.5 mM $MgCl_2$, 0.4 µM of kDNA primers (S34 or S67), 0.2 mM dNTPs, 2.5 U *Taq* Platinum (Invitrogen, Carlsbad, CA). The kDNA primers are used in combination with 0.04 µM of *Gg* primers (*Gg*1 to *Gg*6, **Table 1**), separately. The targeting primers annealing temperatures ranged from 57.9 to 60.1 ºC for kDNA primers, and from 59.9 to 65.6 ºC for CR-1 primer sets. These temperatures are higher than those (~45 ºC) required for the arbitrary degenerated primers used in the TAIL-PCR [10]. The temperature and cycles used (MyCycle Termocycler, Bio-Rad Laboratories, Hercules, CA) are described in a previous paper [2].

7.2) Secondary cycle: The PCR products from primary cycle were diluted 1:40 (v/v) in water. kDNA primers S35 and S35 antisense replaced the previous ones, along with the same *Gg* primers.

7.3) Terciary cycle: The PCR products from secondary cycle were diluted 1:10 (v/v) in water and the *Gg* primers were combined in the reaction with S67 antisense or S36, separately.

Table 1. Primers used in the targeted-primeTAIL-PCR amplifications

Primer	Target DNA	Sequence	Tm*
S 34	*T. cruzi* kDNA	5' ACA CCA ACC CCA ATC GAA CC 3'	57,9
S 67	*T. cruzi* kDNA	5' GGT TTT GGG AGG GG(G/C) (G/C)(T/G)T C 3'	60,1
S 35	*T. cruzi* kDNA	5' ATA ATG TAC GGG (T/G)GA GAT GC 3'	59,4
S 36	*T. cruzi* kDNA	5' GGT TCG ATT GGG GTT GGT G 3'	57,9
*Gg*1	*Gallus gallus*	5' AGC TGA TCC TAA AGG CAG AGC 3'	60.1
Gg2	*G. gallus*	5' CTG AGC CTC TGC TTT GAA A 3'	56.8
Gg3	*G. gallus*	5' TTT CAA AGC AGA GGC TCG G 3'	60.1
Gg4	*G. gallus*	3' GCT CTG CCT TTA GGA TCA GCT 5'	64.2
Gg5	*G. gallus*	3' AGC AAC TCA GCG TCC ACC TT 5'	62.3
Gg6	*G. gallus*	3' CTG TTA GCA TGA GGC TTC ACA A 5'	60.4
XeCRs-1[a]	*G. gallus*	5' ATW TCW GTS TTT GCA GAT GAC ACA 3'	60.4
XeCRs-2	*G. gallus*	5' CTT WGT TGC CCT YCT CTG KAC YCT CTC YA	66.6
XeCRs-3	*G. gallus*	5' TGT GTC ATC TGC AAA SAC WGA WAT 3'	65.3
XeCRs-4	*G. gallus*	5'TRG AGA GRG TMC AGA GRA GGG CAA CWA T 3'	67.9

* Tm = average annealing temperature ^{o}C. Reprinted from reference 2.

7.4) Cloning of the PCR terciary cycle products: The last amplification products that hybridize with kDNA probe were cloned directly in pGEM T easy vector (Promega, Madison, WI).

7.5) Clones selected by hybridization with kDNA probe are sequenced commercially.

7.6) The validation of the *tp*TAIL-PCR is determined in a mix of 300 pg of kDNA from *T. cruzi* with 200 ng of DNA from control birds never exposed to kDNA. The temperature and amplification cycles are the same used for the test birds' DNA.

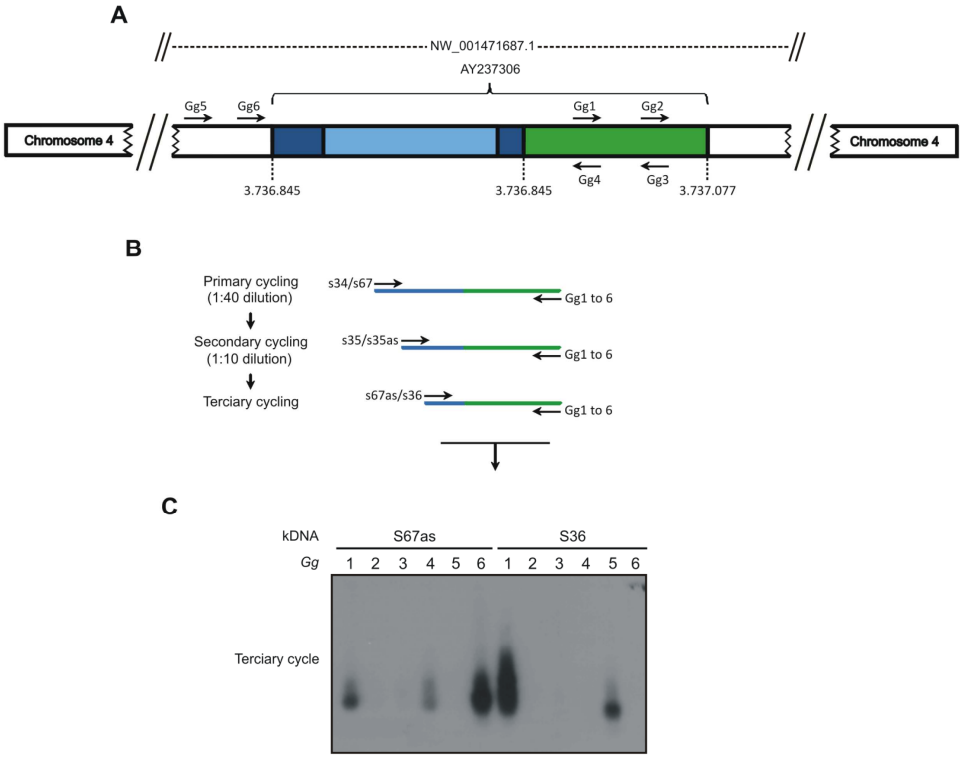

Fig. 1. The *tp*TAIL-PCR strategy used to detect *Trypanosoma cruzi* kDNA integration into the *Gallus gallus* genome. A) A chimera sequence with a fragment of kDNA minicircle conserved (dark blue) and variable (light blue) regions integrated in the locus NW_001471687.1 at chromosome 4 (AY237306) of the chicken [10] genome (green) was used to obtain the host specific primer sets (*Gg*1 to *Gg*6). B) The *tp*TAIL-PCR amplifications were initiated (primary cycle) by annealing of the kDNA-specific S34 or S67 primers in combination with chicken-specific *Gg*1 to *Gg*6 primers. Diluted products provided template for the secondary cycle with the S35 (sense/antisense) primers and the combinations of *Gg* primers. In the tertiary cycle a dilution of the secondary products was subjected to amplification with kDNA S36 or S67 antisense primers in combination with the *Gg* primers. C) These amplification products were separated in 1% agarose gels and transferred to nylon membrane, hybridized with the specific kDNA probe, then cloned and sequenced. The combinations of kDNA and targeted *Gg1* to *Gg6* are shown on top of the gel. The sequential PCR reactions amplified target kDNA-host DNA sequences with kDNA minicircles (blue) and the avian sequence (green). (Reprinted from reference 2).

8.) Chagas disease clinic manifestation

8.1) Growth and development of chickens hatched from *T. cruzi* infected eggs and of healthy controls hatched from non-infected eggs are monitored daily for mortality and weekly for disease manifestations.

8.2) Clinical abnormalities in those chickens are detected by clinical (**Fig. 2**) and ECG recordings that evaluated the electrical axes, heart rates and arrhythmias [2].

8.3) kDNA-mutated and controls chickens are submitted monthly to ECG recordings of frontal leads AVR, AVL and AVF, and assessment of deviation of mean electrical axis to the left, which is suggestive of heart enlargement [2].

Fig. 2. Clinical manifestation of impaired heart function in a chicken genetically modified by the integration of the mitochondrion kDNA minicircle from *Trypanosoma cruzi.* The poor blood oxigenation of the kDNA mutated chicken showing purple comb (left) contrasts with the bright red comb of the control chicken (right) not showing heart damage.

9.) Pathology and immunochemical analyses

9.1) Heart and body weight indexes were obtained after natural deaths of kDNA-mutated chickens. For each experimental case, a control (kDNA- the negative) chicken of the same age and gender is sacrificed, and the heart weight (g)/ body weight (kg) indexes are obtained (**Fig. 3**)

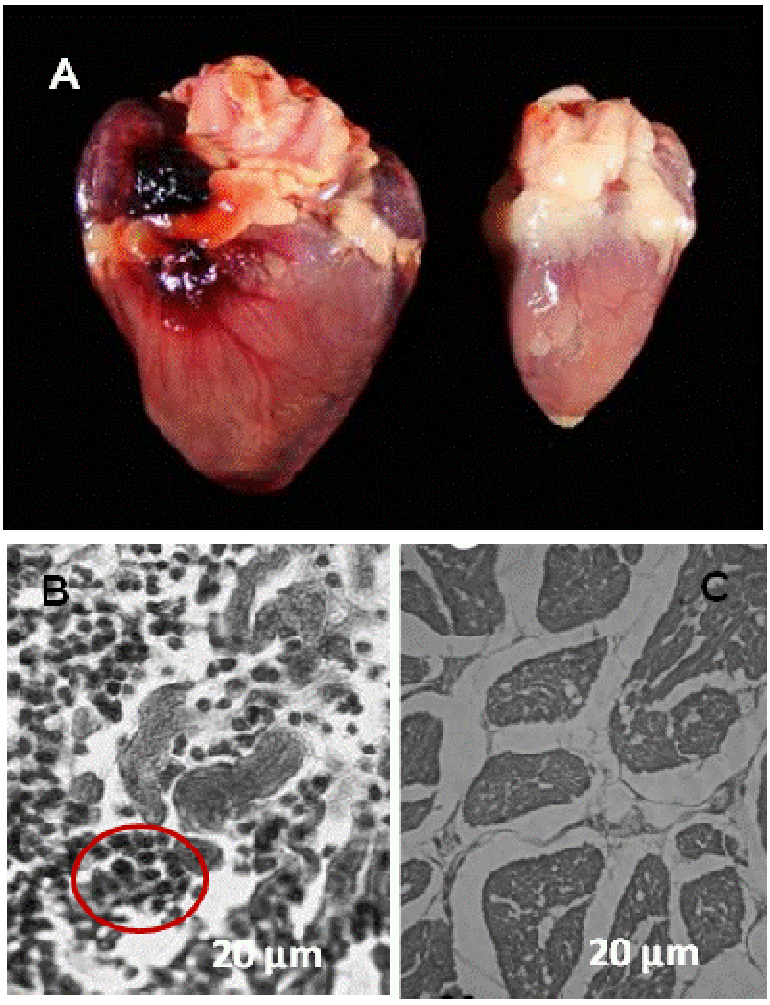

Fig. 3. Pathological findings in *Gallus gallus* with *Trypanosoma cruzi* kDNA mutations. **A**) Cardiomegaly (30 g) in a nine month-old hen (right) that died of heart failure, and control heart (8 g, left) from a nine month-old hen. B) Diffuse myocarditis showing immune system mononuclear cell infiltrates and lysis of target heart cells. The red circle depicts a minimal rejection unit whereby effectors lymphocytes destroy a target heart cell. C) Histology of control chicken heart.

9.2) Sections are taken from the heart, esophagus, intestines, skeletal muscle, lungs, liver, and kidneys. (Modified from reference 2)

9.3) Histological analyses of tissues fixed in buffered 10% formalin (pH 7.4), embedded in paraffin and cut to 4 µm thick sections for Hematoxylin-Eosin (HE) staining (**Fig 4**).

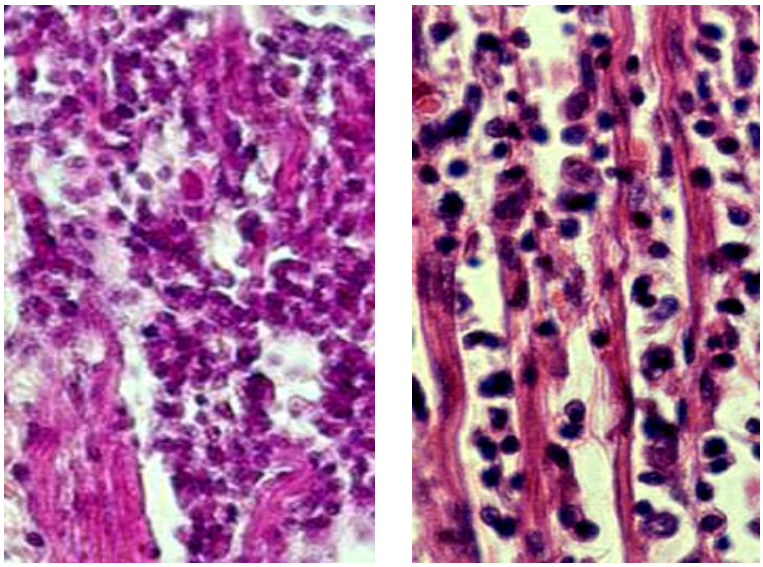

Fig. 4. Severe myocarditis in kDNA-mutated chicken (right) and in the human Chagas heart disease (right). Hematoxilin and Eosin, 100x magnification. (Reprinted from reference 11)

9.4) Tissues harvested from embryos and from chicks at set times are bisected so that half is fixed in 0.02% glutaraldehyde dissolved in phosphate buffered saline (pH 7.2) and stained with X-Gal [2].

9.5) Parasite expressed β-galactosidase X-Gal-stained tissues are fixed in paraformaldehyde.

9.6) Paraffin embedded tissues sections are mounted by standard methods for microscopic examination.

9.7) Sections showing X-Gal-stained blue cells are subjected to first incubation with a human Chagas diseased antiserum with specific anti-*T. cruzi* antibody 1:1024.

9.8) Wash sections trice with PBS, pH 7.4, 5 min each.

9.9) Stain blue cells in the embryo tissues by second incubation with a fluorescein-conjugated rabbit anti-human IgG.

9.10) Wash sections with PBS (step 8) and observe the blue cells light-up green upon examination under UV light at 502nm wavelength, 200x magnification, for colocalizing *T. cruzi* in embryo cells.

10.) Phenotyping immune system cells in heart lesions

10.1) Tissue sections of heart from kDNA-positive and from control kDNA-negative chickens are separated for phenotype immune effectors cells.

10.2) The slides containing tissue sections embedded in paraffin are placed at 65°C for 30 min to melt wax previous to submission to four baths in 100% to 70% xylene and then in absolute ethanol PBS for 5 min each.

10.3) The slide rinsed in distilled water is air dried and treated with specific monoclonal antibodies:

10.4) The fluorescein- or R-phycoerythrin-conjugated monoclonal antibodies are obtained from SouthernBiotech, Birmingham, AL.

a) Mouse anti-chicken Bu-1 (Bu-1^a and Bu-1^b alleles, Mr 70-75 kDa) Mab AV20 recognizing monomorphic determinant on the B cell antigens of inbred chickens.

b) Mouse anti-chicken CD45, Ig isotype IgM1κ specific to chicken thymus lineage cells (Mr 190 to 215-KDa variant).

c) Mouse anti-chicken TCRγδ (Mr 90-kDa heterodimer) Mab specific to thymus dependent $CD8^+$γδ T cells.

d) Mouse anti-chicken Mab CD-8 specific to chicken α chain (Mr 34 kDa) recognizing the CD8 cells in thymocytes, spleen and peripheral blood.

e) Mouse anti-chicken KuL01 exclusively recognizing monocytes/macrophages of the phagocyte system.

10.5) After incubation with specific anti-phenotype antibody for 90 min in a moist chamber, the slide is washed three times with O.1 M PBS, pH 7.4, 5 min each.

10.6) The slide is assembled with buffered glycerin for exam under a fluorescent light microscope with emission filter of wavelength 567 and 502nm, respectively, to detect red and green fluorescence-labeled cells (**Fig 5**).

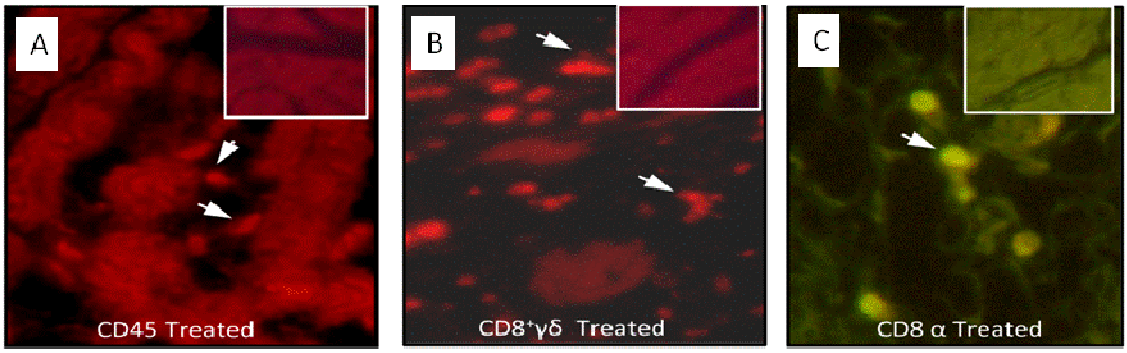

Fig. 5. Immunocytochemical analyses of the immune system cells infiltrating the heart of kDNA-mutated chicken shown in Fig. 3. A) $CD45^+$ lymphocytes identified (arrows) in heart lesions by a phycoerythrin-labeled specific monoclonal antibody. B) $CD8^+\gamma\delta$ immune lymphocytes (arrows) involved in severe destruction of the heart. C) Abundant $CD8\alpha^+$ T cells present in severe lesions with heart cell lysis. The inserts show absence of immune system cells in the control uninfected chicken heart (Reprinted from reference 2).

11) Data analyses

11.1) The chicken genome database (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9031) is used for BLASTn sequence analyses.

11.2) CLUSTALW alignments are performed and statistical significance ($p < 0.001$) are determined for scores (e-values) recorded.

11.3) The GIRI repeat masking algorithm CENSOR (http://girinst.org/censor/index.php) is employed for localization of different classes of repeats in chimeric sequences.

11.4) The Kinetoplastid Insertion and Deletion Sequence Search Tool (KISS) were employed to identify potential gRNAs in the kDNA sequences [2].

11.5) The KISS database comprises *Trypanosoma brucei* and *Leishmania tarentolae* minicircle and maxicircle as well as a work bench for RNA editing analysis in kinetoplastids [50, 51] with the aid of WU-Blastn-modified-matrix [2].

11.6) *T. cruzi* sequences (http://www.biomedcentral.com/content/supplementary/1471-2164-8-133-s1.fas) are used to search-in gRNAs in the kDNA-host DNA chimera sequences.

11.7) Student's *t* and the Kolmorov-Smirnov tests are used, respectively, to detect significant differences between deviations of electric axes and between heart/body weight indexes obtained in the experimental and control groups, and to detect mortality ratios significant differences between groups of chickens hatched from *T. cruzi* inoculated eggs and from controls.

References:

1. Teixeira ARL, Nitz N, Guimaro MC, Gomes C, Santos-Buch CA (2006) Chagas disease. Postg Med J 82 (974):788-798.
2. Teixeira AR, Gomes C, Nitz N, Sousa AO, Alves RM, Guimaro MC, Cordeiro C, Bernal FM, Rosa AC, Hejnar J, Leonardecz E, Hecht MM (2011) Trypanosoma cruzi in the Chicken Model: Chagas-Like Heart Disease in the Absence of Parasitism. PLoS Negl Trop Dis. 5(3):e1000.
3. Hecht MM, Nitz N, Araujo PF, Sousa AO, Rosa Ade C, Gomes DA, Leonardecz E, Teixeira AR (2010) Inheritance of DNA transferred from American trypanosomes to human hosts. PLoS One. 5(2):e9181.
4. Xing Z, Cardona CJ, Anunciacion J, Adams S, Dao N (2010) Roles of the ERK MAPK in the regulation of proinflammatory and apoptotic responses in chicken macrophages infected with H9N2 avian influenza virus. J Gen Virol 91(2): 343-351.

5. Kim HB, Evans I, Smallwood R, Holcombe M, Qwarnstrom EE (2010) NIK and IKKbeta interdependence in NF-kappaB signalling--flux analysis of regulation through metabolites. Biosystems 99 (2):140-149.

6. Karakhanova S, Meisel S, Ring S, Mahnke K, Enk AH (2010) ERK/p38 MAP-kinases and PI3K are involved in the differential regulation of B7-H1 expression in DC subsets. Eur J Immunol 40(1):254-266.

7. Finsterer J, Stöllberger C (2003) The heart in human dystrophinopathies. Cardiology 99(1): 1-19.

8. Moser DR, Kirchhoff LV, Donelson JE (1989) Detection of Trypanosoma cruzi by DNA amplification using the polymerase chain reaction. J Clin Microbiol 27 (7): 1477-1482.

9. Sturm NR, Degrave W, Morel C, Simpson L (1989) Sensitive detection and schizodeme classification of Trypanosoma cruzi cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease. Mol Biochem Parasitol 33: 205-214.

10. Nitz N, Gomes C, de Cássia Rosa A, D'Souza-Ault MR, Moreno F, Lauria-Pires L, Nascimento RJ, Teixeira AR (2004) Heritable integration of kDNA minicircle sequences from Trypanosoma cruzi into the avian genome: insights into human Chagas disease. Cell 118(2):175-86.

11. Teixeira ARL, Nascimento R, Sturm NR (2006) Evolution and Pathology in Chagas Disease. Mem Inst Oswaldo Cruz 101(5): 463-491.