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Trypanosoma cruzi Circulating in the Southern Region of the State of Mexico (*Zumpahuacan*) Are Pathogenic: A Dog Model

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Abstract

Here we describe clinical and pathologic evidence of Chagas disease caused in dogs by circulating *Trypanosoma cruzi* from a newly recognized endemic area in Mexico. We show that the *Zumpahuacan* isolate, although less virulent than the *Sylvio-X10* reference strain that caused acute myocarditis and death, was pathogenic in dogs. Dogs infected with the *Zumpahuacan* isolate exhibited electrocardiographic alterations, left- and right-ventricle dilation, and hydropericardium. Histologically, diffused perimysial and endomysial lymphoplasmacytic cell infiltration, cardiomyocyte necrosis, and amastigote nests were noted in *Zumpahuacan*-infected dogs. These findings suggest that the risk of *T. cruzi* infection and Chagas disease is present in the State of Mexico, and further research is needed to identify the *T. cruzi* bio-types circulating in southern State of Mexico.

INTRODUCTION

With an estimated 18 million people infected with *Trypanosoma cruzi*, and 25% of the population at risk, Chagas disease is endemic in Latin America.[1–3] In Mexico, ~5.8 million people might be infected with *T. cruzi*.[4,5] The State of Mexico, located in the central highlands

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of the country, was considered free of *T. cruzi* until 1998. However, we documented *T. cruzi*–specific antibodies in 7.1% of humans and 21% of dogs from rural areas of the State of Mexico[6] and noted that detailed epidemiologic information is needed to understand the true impact of Chagas disease in these areas.

In this study, we isolated *T. cruzi* from triatomines collected from Zumpahuacan municipality of the State of Mexico and aimed to evaluate its virulence in dogs. We examined physical, clinical, and histopathologic aspects in dogs that were naturally infected or experimentally infected with the *Zumpahuacan* isolate of *T. cruzi*. We chose dog as an experimental model because they are an important domestic reservoir host[7,8] and should be an excellent model to study Chagas disease.[9–15]

MATERIALS AND METHODS

Animals

Twelve of 57 mongrel dogs collected in Zumpahuacan municipality tested positive for anti-*T. cruzi* antibodies by indirect hemagglutination (IHA) test and enzyme-linked immunosorbent assay (ELISA) and were considered naturally infected (21% prevalence). Apart from cardiomyopathy, no signs of infections related to other cardiomyopathic diseases (e.g., ringworm) were noticed during clinical evaluation, necropsy, and histologic studies, and these dogs were considered affected by Chagas disease only.

For experimental infection, dogs (2 months old) were acquired locally and kept at the research center until used for challenge infection at 8 months of age. Dogs were confirmed serologically negative for *T. cruzi*–specific antibodies by IHA and ELISA and treated with anti-helminthics and vaccines against regional infectious diseases (canine distemper, parvovirus, canine hepatitis, leptospirosis, and rabies). All dogs received water *ad libitum* and commercial dog food according to age and development requirements. Experimental protocols were conducted according to Norma-Official-Mexicana (NOM-0062-ZOO-1999) technical specifications for the care and use of laboratory animals.

Parasites

Trypanosoma cruzi (*Sylvio-X10*) was purchased from American Tissue Culture Collection. A native *T. cruzi* isolate was obtained from *Triatoma pallidipennis* collected from El Zapote village of Zumpahuacan municipality, located in the southern State of Mexico (Figure 1). Triatomines feces were examined for epimastigotes by light microscopy,[16] and positive samples were diluted in PBS and intraperitoneally injected in Balb/C mice. Blood trypomastigotes were collected by cardiac puncture at Day 14 post-infection (pi). Parasites were purified by Ficoll gradient, seeded on a monolayer of NIH3T3 cells, and cultured and propagated at 37°C, 5% CO_2 in DMEM media (pH 7.2) supplemented with 2% FBS, 8 μg/mL ampicillin, and 0.1 mg/mL pyruvate (Gibco/Invitrogen, Grand Island, NY). The native isolate was named "*Zumpahuacan*."

Experimental infection

Seronegative dogs ($N = 10$) were blindly distributed. Four dogs were infected with *Zumpahuacan* and four with *Sylvio-X10* (3,500 trypomastigotes/kg body weight). Two dogs served as controls. Dogs were observed daily for general physical condition and every week for serology, clinical condition, and cardiac function.

Serology

Blood samples (7 mL) were obtained by venepuncture (once per week, until 10 weeks pi) and immediately processed, and sera were stored at −20°C. Sera samples were analyzed for anti-

T. cruzi antibodies by IHA (Polychaco, Laboratorio-Lemos SRL, Buenos Aires, Argentina) and ELISA (Laboratorio-Lemos SRL, Buenos Aires, Argentina) Chagas diagnostic kits, following the manufacturer's instructions. The horseradish peroxidase (HRP)-labeled anti-human-IgG in ELISA kit was replaced with anti-dog-IgG (Koma Biotech, Seoul, Korea). The cut-off value for IHA (positive titer at ≥ 1:8 serum dilution) and ELISA (OD_{450nm} ± 2 SD) tests were set using sera from four healthy dogs.[17,18] The seropositive and seronegative status of the control dogs was confirmed by InDre (national reference center for diagnosis of *T. cruzi* infection) by Serodia-Chagas (Fujirebio, Tokyo, Japan) and Chagas STAT-PAK (Chembio, Medford, NY).

Electrocardiography

Changes in cardiac rhythm and conduction were monitored each week (for 10 weeks pi). The EK-8 electrocardiographic machine (Burdick Stylus, Milton, WI) was set at 120 V, 60 H, 20 amps, and 25 W, and six leads were considered at 25 mm/s at 1 mV, standardized to 1 cm for this study.

Necropsy and histologic studies

Necropsy was performed when dogs died because of infection or after humanitarian sacrifice, and macroscopic and microscopic analysis of affected organs was performed. Animals were euthanized according to the Norma Official Mexicana guidelines (NOM-033-ZOO-1995). Tissue samples were fixed in 10% formalin, dehydrated, and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin and evaluated by light microscopy.

RESULTS

Clinical evaluation

The *Zumpahuacan*- and *Sylvio-X10*–infected experimental dogs developed a brief febrile episode during 5–16 dpi. No other signs of clinical illness were apparent in experimentally infected and healthy control dogs during the daily physical exam. The naturally infected dogs showed no fever during the observation period. Dogs infected with *Sylvio-X10* died at day 29 and 30 pi.

Serology

Trypanosoma cruzi–specific antibodies were evident in experimentally infected dogs by IHA and ELISA at 4 weeks pi. *Sylvio-X10*–infected dogs died during the Week 5 pi, and no further serology could be performed. Dogs infected with the *Zumpahuacan* isolate exhibited a gradual increase in anti-*T. cruzi* antibodies during 4–10 weeks pi. Naturally infected dogs exhibited highest level of parasite-specific antibodies. Control dogs remained seronegative throughout the study (Table 1).

Electrocardiography

Electrocardiograms were obtained at weekly intervals. The *Zumpahuacan*-infected dogs exhibited discrete EKG abnormalities. These included, at 4 weeks pi, a reduction in S-T segment below 0.2 mV and a premature ventricular contraction (Figure 2C), indicative of ischemia and arrhythmia, respectively, that evolved toward EKG normalization by 8 weeks pi. *Sylvio-X10*–infected dogs exhibited sinus tachycardia at 4 weeks pi (Figure 2D) and died thereafter. Naturally infected dogs showed the most evident EKG alterations, including a reduced R-wave voltage, a deep S-wave, a ST-segment increase > 0.15 mV, and a T-wave increment that are indicative of pericardial effusion, right-atrium enlargement, and ischemia. Some naturally infected dogs also exhibited reduced P-R and R-R intervals and sinus

tachycardia (Figure 2A and B). No EKG abnormalities were observed in healthy controls (Figure 2E).

Anatomo- and histo-pathologic findings

The anatomopathologic findings in infected dogs included dilated cardiomyopathy, cardiomegaly, hydropericardium, and focal and diffused myocarditis. Naturally infected dogs showed mild-to-severe bi-ventricle dilation that resulted in large cardiomegaly (Figure 3A and B). The *Zumpahuacan*- and *Sylvio-X10*–infected dogs displayed mild to moderate cardiopathic changes (e.g., hydropericardium and right ventricle dilation associated with moderate cardiomegaly; Figure 3C–F; Table 2). A healthy dog's heart is shown for comparison (Figure 3G and H).

Histopathologic studies showed diffused and multifocal necrotic myocarditis, characterized by necrosis and fragmentation of myocardial fibers and perimysial and endomysial histiocytic and lymphoplasmacytic infiltration in the heart of naturally and experimentally infected dogs (Figure 4; Table 2). The extent of inflammatory infiltrate and tissue necrosis corresponded to the macroscopic pale zones (whitish lines) macroscopically noted in the heart of infected dogs (Figure 3). Severity of microscopic pathological findings, specifically diffused myocarditis, was more evident in experimentally infected than naturally infected dogs. Myocardial amastigote nests were detected in all infected dogs (Figure 4). Parasite foci were significantly higher in heart tissue of *Zumpahuacan*-infected dogs than naturally and *Sylvio-X10*–infected dogs (Table 2). Healthy control dogs exhibited no anatomo- and histo-pathologic abnormalities or parasite foci in the heart.

DISCUSSION

We showed that experimental infection with the *Zumpahuacan* isolate produced an acute to indeterminate stage of infection that was also noted in naturally infected dogs from the rural areas of the State of Mexico. Infection of dogs with the *Sylvio-X10* strain resulted in a lethal phenotype.

We performed an initial characterization of a canine model of acute chagasic myocarditis and indeterminate asymptomatic disease. We used *Sylvio-X10* as a reference strain because it has been widely characterized in mouse models and proven to be pathogenically stable after many passages in *in vitro* culture.[19,20] Infection of dogs with *Sylvio-X10* was highly pathogenic; the symptoms of acute myocarditis and sudden death in *Sylvio-X10*–infected dogs resembled what has been noted in other hosts infected with virulent *T. cruzi* strains.[21,22] Dogs experimentally infected with the *Zumpahuacan* isolate exhibited acute myocardial disturbances evidenced by electrocardio-graphic analyses. Naturally infected dogs showed clinical and pathologic signs of chronic Chagas disease. These data suggest that *T. cruzi* strains circulating in southern State of Mexico are able to produce various stages of Chagas disease.[14]

Dogs experimentally infected with *Sylvio-X10* and *Zumpahuacan* strains developed anti-*T. cruzi* antibodies by 4 weeks pi. Our observations are in agreement with other reports where IgG response in infected dogs was noted at 3 weeks pi, and IgGs remained elevated with indeterminate to chronic progression of disease phase.[13,15] Cardiac parasitic foci were maximum in *Zumpahuacan*-infected and moderate to none in *Sylvio-X10*–infected dogs. The elicitation of comparable IgGs and detection of fewer parasitic foci in myocardium of *Sylvio-X10*–infected dogs compared with *Zumpahuacan*-infected dogs suggests that sudden death of *Sylvio-X10*–infected dogs was not caused by the inability to mount anti-parasite antibodies or uncontrolled tissue parasite burden.

The interpretation of dog EKG readings according to Tilley[23] and Sisson and others[24] provides clues to clinical outcome of infected dogs. The *Zumpahuacan*-infected dogs exhibited conduction abnormalities (myocardial hypoxia, premature ventricular contraction, and sometimes sinus tachycardia) at 4 weeks pi that were normalized by 8 weeks pi, indicating the development of a clinically asymptomatic indeterminate stage. In comparison, *Sylvio-X10*–infected dogs exhibited sinus tachycardia and cardiac hypertrophy that likely contributed to diastolic insufficiency and myocardial hypoxia. We surmise that inability of the heart to compensate for conduction abnormalities resulted in acute myocardial hypoxia and sudden death in *Sylvio-X10*–infected dogs.

The notion of differential EKG abnormalities in dogs infected with various *T. cruzi* strains is supported by others. For example, no EKG alterations and pathologic changes were detected in dogs infected with *147* and *SC-1* isolates,[15,25] whereas *12SF*-, *Colombian*-, or *Be-78*–infected dogs exhibited bradycardia, T-wave inversion, low-voltage QRS, S-T irregularities, or right bundle branch block (RBBB).[26–28] Others showed decreased QRS voltage, repolarization disturbance, axis deviation, and indeterminate-rhythm in some, but not all, infected dogs.[13] In our study, naturally infected dogs exhibited pericardiac effusion, right-ventricle enlargement, and myocardial hypoxia or right-ventricle and -atrium enlargement, accessory electric conduction defect, and sinus tachycardia. The extent of cardiomegaly and right-ventricle dilation was moderate in *Sylvio-X10*– and *Zumpahuacan*-infected dogs and mild-to-severe in naturally infected dogs. Overall, these data support the view that parasite strains, route of delivery, and number of injected parasites contribute to the differential clinical outcome of disease in *T. cruzi*–infected dogs.

Lesions related to cardiomyocyte fibrosis/necrosis are routinely detected in acutely infected dogs.[13,29] Similarly, lesions consisting in damage to cell membrane, phlogistic aspect of cardiomyocytes, and fibrosis/necrosis have been reported in dogs[28] and human patients in the indeterminate infection phase. We noted necrotic lesions representative of degenerative processes in cardiac tissue of infected dogs. Perimysial and endomysial lymphoplasmacytic infiltration in the heart of infected dogs was probably caused by evolution of adaptive immune responses and inflammatory processes that induce hyalinization and fibrosis. Others have reported the presence of myocardial necrosis[14,15] and inflammatory infiltrate[27,28] associated with fibrosis in dogs in indeterminate phases of *T. cruzi* infection and disease, thus providing support to our observations.

In summary, we showed that acute myocarditis and symptoms of indeterminate chronic Chagas disease develop in dogs experimentally or naturally infected with *T. cruzi* isolates from southern State of Mexico. Further studies evaluating the pathogenicity of *Zumpahuacan* and other isolates in dogs would advance the development of a canine model of Chagas disease and enhance our understanding of the risk of *T. cruzi* infection and its potential impact on inhabitants in this region of Mexico.

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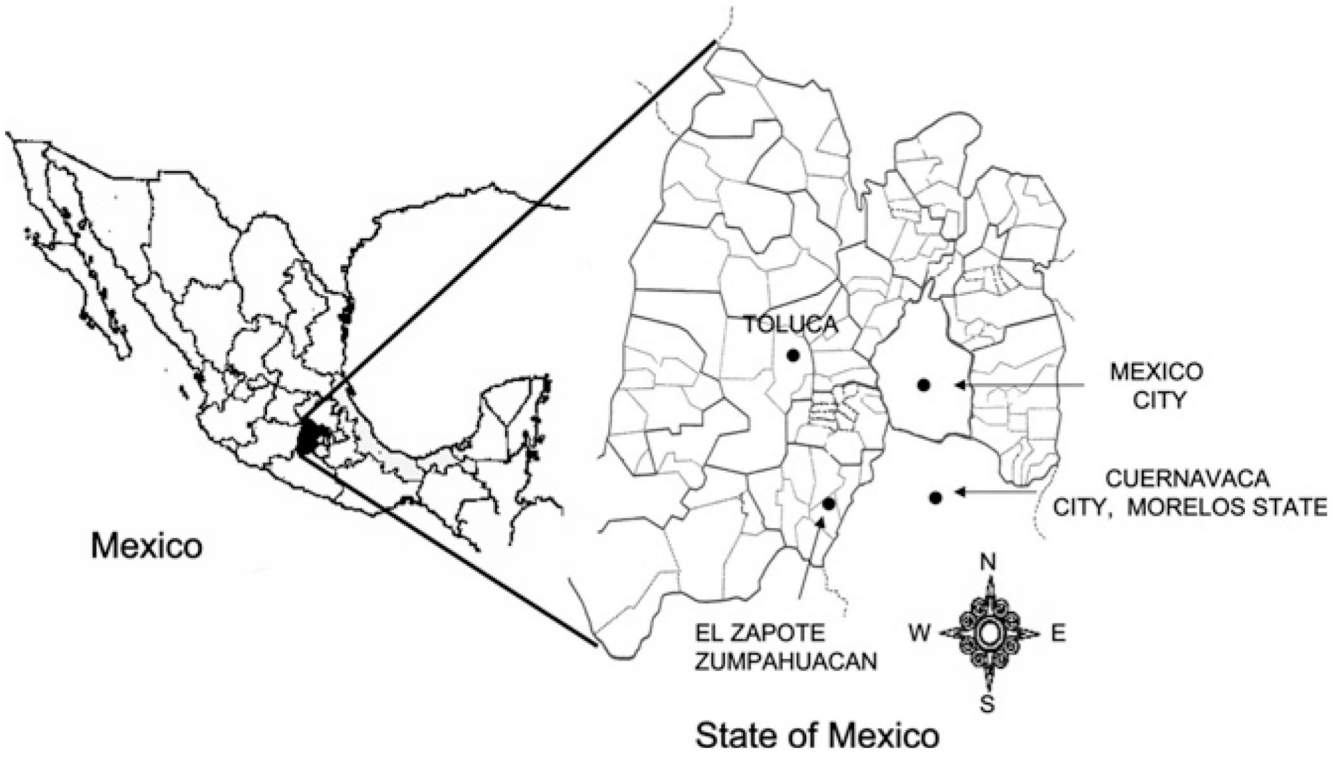

FIGURE 1.
Study site in Mexico. The *Zumpahuacan* isolate was obtained from infected triatomines collected from El Zapote village in the Zumpahuacan municipality of the State of Mexico. Dogs naturally infected with *T. cruzi* were also obtained from same region.

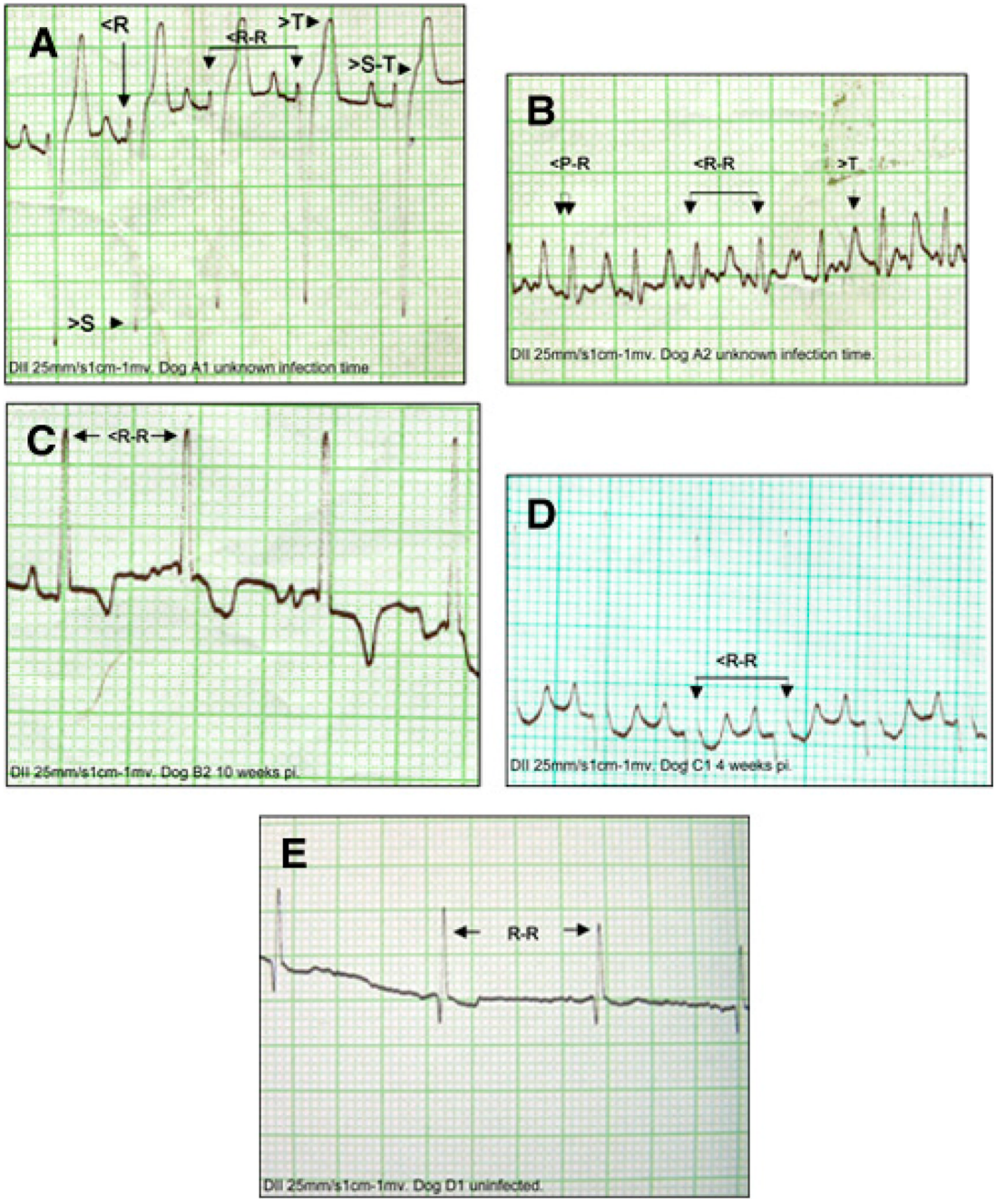

FIGURE 2.

Electrocardiographic recordings taken at 25 mm/s. 1 mm = 0.04 seconds, 1 cm = 1 mV. Shown are lead II recordings from naturally infected dogs (**A** and **B**), *Zumpahuacan*-infected dog at 4 weeks pi (**C**), *Sylvio-X10*–infected dog at 4 weeks pi (**D**), and non-infected dog (**E**). EKGs show some of the conduction alterations found in infected dogs (interpretation can be seen in Table 2). R-R, normal R-R interval; >S-T, increased S-T segment; >T, increased T wave;<R, decreased R wave; >S, increased deep S wave; <R-R, decreased R-R interval; <P-R, reduced P-R interval. This figure appears in color at www.ajtmh.org.

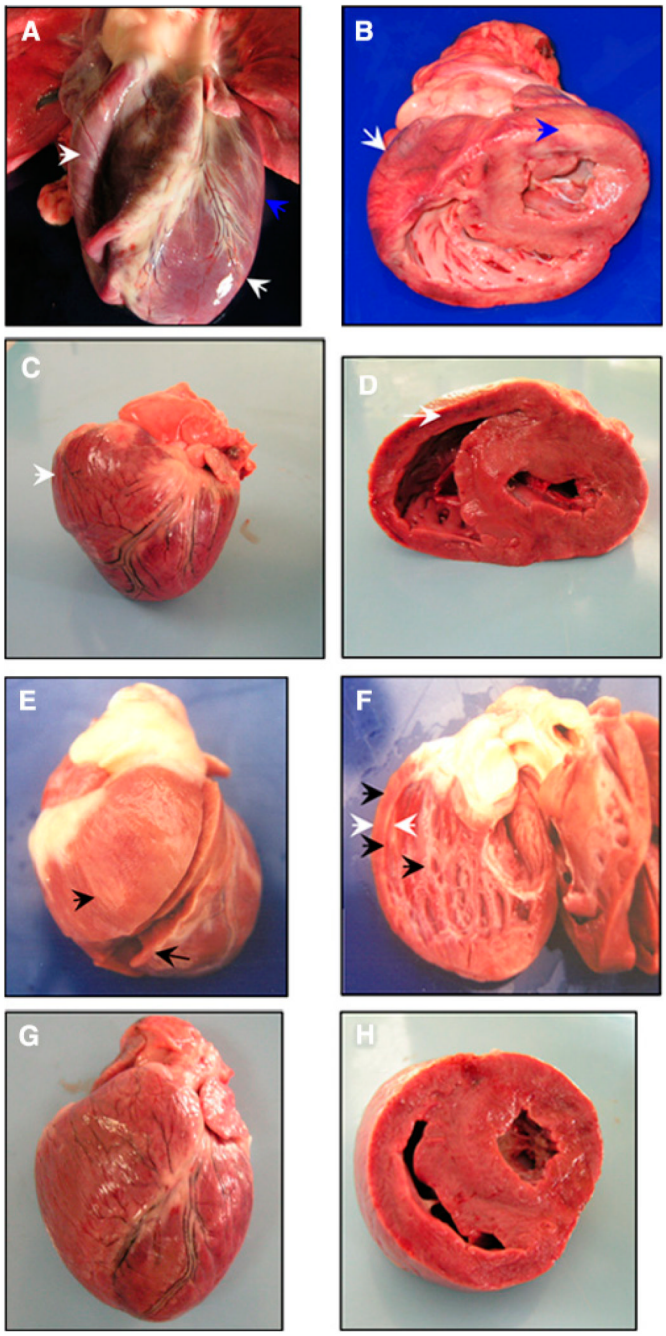

FIGURE 3.

Morphologic alterations of the Chagasic hearts from naturally and experimentally infected dogs. **A** and **B**, Heart morphology of a naturally infected dog with biventricular dilated cardiomyopathy (white arrows) and presence of pale striated epicardium (blue arrows). **C** and **D**, Heart from a *Zumpahuacan*-infected dog shows right-ventricle dilated cardiomyopathy and heart enlargement (white arrows). **E** and **F**, Heart from a *Sylvio-X10*–infected dog shows right-ventricle dilated cardiomyopathy with thin walls (white arrows) characterized by a rounded heart appearance and presence of whitish striates in epicardium, myocardium, and endocardium (black arrows). **G** and **H**, Heart from a healthy, non-infected dog. This figure appears in color at www.ajtmh.org.

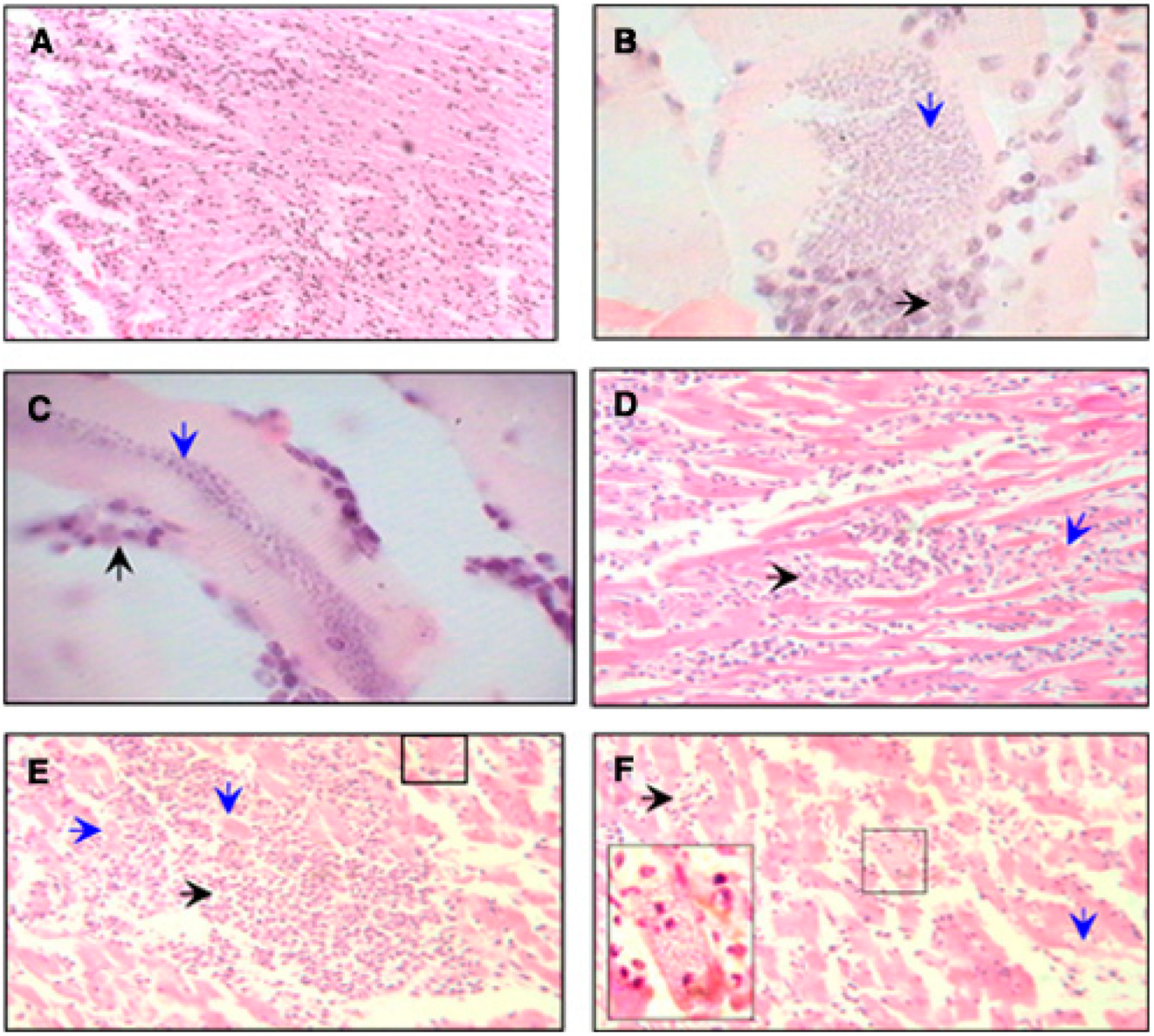

FIGURE 4.

Histopathological findings in naturally and experimentally infected dogs. Heart tissue sections (5 μm) were stained with hematoxylin and eosin. **A**, Note the infiltration of lymphoplasmacytic cells in myocardial section from a naturally infected dog (magnification: ×200). **B** and **C**, Transversal (**B**) and longitudinal sections (**C**) of muscular fiber from *Zumpahuacan*-infected dogs show the presence of large amastigote nests. In **B**, downward arrow shows the basophilic granular appearance of amastigotes inside of a muscular fiber and horizontal arrow shows the leukocyte inflammatory reaction against the infested fiber (×1,000). In **C**, the downward arrow shows the basophilic granular appearance of amastigotes in the middle of a muscular fiber, and the upward arrow shows the leukocyte inflammatory reaction against the infested fiber (magnification: ×1,000). **D–F**, Tissue sections from *Sylvio-X10*–infected dogs. **D**, Severe necrotic myocarditis characterized by leukocyte infiltration (horizontal arrow) with multifocal myofiber segmental necrosis (downward arrow) is evident. This infiltration corresponded to the macroscopic pale zones (whitish striates) observed in the heart. **E**, Dense focal interstitial

leukocyte infiltration with severe multifocal myofiber segmental necrosis, loss of myocytes, and presence of a nest of amastigotes into a myofiber (small square). **F**, Moderate leukocyte infiltration (horizontal arrow) with multifocal myofiber segmental necrosis (downward arrow), and presence of a nest of amastigotes inside a myofiber (inset) (magnification: ×400). This figure appears in color at www.ajtmh.org.

TABLE 1

Serologic analysis of dogs naturally or experimentally infected with *T. cruzi*

T. cruzi infection	Weeks post-infection					
	Week 0	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 10**
IHA						
Natural (A)	+	+	+	+	+	+
Zump (B)	−	−	+	+	+	+
Sylvio (C)	−	−	+	+	+	ND
None (D)	−	−	−	−	−	−
ELISA—OD_{450nm} ± SD						
Natural (A)	1.85 ± 0.07	1.81 ± 0.03	1.88 ± 0.05	1.89 ± 0.02	1.88 ± 0.02	1.92 ± 0.05
Zump (B)	0.11 ± 0.03	0.14 ± 0.01	0.16 ± 0.05	0.25 ± 0.04	0.38 ± 0.07	1.05 ± 0.61
Sylvio (C)	0.09 ± 0.03	0.18 ± 0.03	0.16 ± 0.04	0.28 ± 0.04	0.53 ± 0.2	ND
None (D)	0.13 ± 0.01	0.13 ± 0.02	0.17 ± 0.03	0.13 ± 0.003	0.14 ± 0.02	0.15 ± 0.01
Cut-off (CV)	0.23 (0.21–0.26)	0.23 (0.28–0.25)	0.27 (0.25–0.30)	0.23 (0.28–0.25)	0.24 (0.22–0.27)	0.25 (0.22–0.27)

Sera samples were collected from dogs with natural *T. cruzi* infection (Group A) and dogs that were experimentally infected with *Zumpahuacan* (*Zump*, Group B) or *Sylvio-X10* (*Sylvio*, Group C) isolates of *T. cruzi* at 1, 2, 3, 4, and 10 weeks post-infection. Normal, uninfected dogs (none, Group D) were used as controls. The sera level of anti-*T. cruzi* antibodies was determined by IHA and ELISA. IHA: +, positive at least at a 1/8 serum dilution; −, negative. The + and − represent mean of triplicate observations from four dogs in each group. ELISA: cut-off = average of negative controls +0.1 (±10%). Absorbance (OD_{450nm}) ± SD values are representative of mean of triplicate observations per dog, four dogs per group, with variation coefficient subtracted at 10% level.

CV = coefficient of variation; ND = not determined because dogs died, and no samples were available.

TABLE 2

Necropsy and histopathology alterations in dogs naturally or experimentally infected with *T. cruzi*

	***T. cruzi* infection (group)**			
	Natural (A)	**Zump (B)**	**Sylvio (C)**	**None (D)**
Anatomopathologic parameters				
Cardiomegaly	+ to +++	++	++	−
Hydropericardium	+ to ++	+	+ to ++	−
Left ventricular dilatation	− to +	−	−	−
Right ventricular dilatation	+ to +++	++	++	−
Splenomegaly	+ to +++	+ to ++	−	−
Hepatomegaly	− to +	+ to ++	−	−
Hydroperitoneum	− to +	− to +	− to +	−
Histopathologic lesions				
Focal MLI	+ to ++	−	−	−
Diffused MLI	−	+++	+++	−
Necrosis of cardiomyocytes	+ to +++	+ + to +++	++	−
PEH	+	+ + to +++	++	−
Amastigote nests	− to +	+ + to +++	− to +	−

Naturally infected dogs (A), and dogs experimentally infected with *Zumpahuacan* (B) or *Sylvio-X10* (C) isolates were examined. Healthy, seronegative dogs (D) were used as controls. The representative findings from four dogs per group are documented here.

+ = mild; ++ = moderate; +++ = severe; − = no alteration detected; MLI = myocardial lymphoplasmacytic inflammation; PEH = perimysial and endomysial histolytic lymphoplasma-cytic cell infiltration.