Original Contribution

Molecular Epidemiology of Trypanosomatids and *Trypanosoma cruzi* in Primates from Peru

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Abstract: We determined the prevalence rate and risk of infection of *Trypanosoma cruzi* and other trypanosomatids in Peruvian non-human primates (NHPs) in the wild ($n = 126$) and in different captive conditions ($n = 183$). Blood samples were collected on filter paper, FTA cards, or EDTA tubes and tested using a nested PCR protocol targeting the 24S α rRNA gene. Main risk factors associated with trypanosomatid and *T. cruzi* infection were genus and the human–animal context (wild vs captive animals). Wild NHPs had higher prevalence of both trypanosomatids (64.3 vs 27.9%, $P < 0.001$) and *T. cruzi* (8.7 vs 3.3%, $P = 0.057$), compared to captive NHPs, suggesting that parasite transmission in NHPs occurs more actively in the sylvatic cycle. In terms of primate family, Pitheciidae had the highest trypanosomatid prevalence (20/22, 90.9%) and Cebidae had the highest *T. cruzi* prevalence (15/117, 12.8%). *T. cruzi* and trypanosomatids are common in Peruvian NHPs and could pose a health risk to human and animals that has not been properly studied.

Keywords: Chagas, *Trypanosoma cruzi*, Non-human primates, Trypanosomatids, Epidemiology, Prevalence

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INTRODUCTION

Trypanosoma cruzi, etiological agent of Chagas disease, infects approximately 8 million people worldwide and more than 25 million people are at risk of infection (WHO 2014). This neglected tropical disease is endemic in 21 Latin American countries, including Peru with 964 cases reported between 2000 and 2015 (MINSA 2015). However, the number of cases is probably underestimated as the disease is often asymptomatic. Transmission typically occurs via a

triatomine bug vector, but it can also occur via blood transfusion, organ transplantation, congenital transmission, the oral route, and accidental infection (Luquetti and Schmunis 2010). Recent outbreaks reported in Latin America have been caused by foodborne transmission of *T. cruzi* due to ingestion of contaminated beverages (Yoshida 2008; Coura and Junqueira 2015). The consumption of infected animal blood or raw or undercooked meat also represents a risk of infection due to the presence of blood trypanomastigotes and tissue amastigotes (Pereira et al. 2010; Rueda et al. 2014). This route of infection may be more common in tropical forests where wild animals are consumed or traded into urban areas.

The diagnosis of Chagas disease is complex due to the dynamics of parasitemia in the two successive phases of the disease: an acute phase diagnosed by the direct detection of the parasite circulating in the bloodstream (microscopy) where the parasite is abundant and a chronic phase where the parasite is hidden in host tissues and diagnosis is usually made via the detection of antibodies against *T. cruzi* (serology) (WHO 2014). In many prevalence studies, microscopy has commonly been used to detect trypanosomes in non-human primates (NHPs) (Sousa et al. 1974; D'Alessandro et al. 1986; Ziccardi and Lourenco-de-Oliveira 1997). However, this technique has a low sensitivity in the chronic phase, and species identification may be difficult due to the interspecific morphological similarities and the high intraspecific variability of trypanosomes (Ziccardi and Lourenco-de-Oliveira 1998). Alternatively, although PCR shows a higher specificity and sensitivity (Kirchhoff et al. 1996; Ndao et al. 2000; Cox et al. 2010), most PCR protocols for the detection of trypanosomes focus specifically on *T. cruzi* in human blood, overlooking other trypanosomatids probably present in wild animals that may have veterinary and public health importance. Thus, a nested PCR protocol is a convenient option that enables the detection of trypanosomatids in a first reaction, while a second reaction allows for the identification of *T. cruzi*.

Since non-human primates (NHPs) can act as reservoirs for human pathogens, they may serve as sentinels for epidemiologic studies (Wolfe et al. 1998), providing important information for the understanding of the epidemiology, transmission dynamics, and emergence risk of various anthroponoses. Close contact between these mammals and human beings may result in transmission risks in both urban and rural settings, possibly leading to emerging zoonoses. Communities in the Amazon frequently hunt and poach wildlife and may inadvertently

introduce infectious parasites into new areas. Thus, epidemiological and prevalence studies that address these issues are necessary. By estimating the prevalence rate and risk of infection of trypanosomatids and *T. cruzi* from different genera of neotropical peruvian NHPs, we aimed to identify which NHP families and species may pose the highest risk of infection to humans, and to contribute with relevant data as studies in Peruvian primates is scarce.

MATERIALS AND METHODS

Sample Collection

Convenience sampling was used based on the availability of captive ($n = 183$) and wild ($n = 126$) NHPs. From November 2011 to May 2012, captive NHPs from seven Peruvian cities were sampled from zoos, wildlife rescue centers, wet markets, and households (Fig. 1). A 1–3-ml blood sample was collected from the femoral vein of 183 primates in EDTA-containing tubes and an aliquot was applied to Flinders Technology Associates (FTA) classical cards. Blood samples were refrigerated during field collection and stored at -80°C with a silica gel desiccant until PCR analysis. In 82 of these NHPs, blood smears were also collected for microscopy screening of hemoparasites.

From 2007 to 2012, blood samples were collected from 126 wild NHPs by subsistence hunters from two remote indigenous communities in the Northeastern Peruvian Amazon: Nueva Esperanza (S $04^{\circ}19.53$ W $71^{\circ}57.33$) and Sol Naciente (S $03^{\circ}38.26$ W $73^{\circ}12.57$) (Fig. 1). A Whatman filter paper No. 3 was impregnated with blood from the jugular vein during the postmortem management of each NHP. This material was sealed in individual plastic bags with desiccant and kept in indoor conditions before transfer to storage at -80°C in Lima.

The research protocol was approved by the Peruvian Forestry and Wildlife Agency (0411-2010-AG-DGFFS-DGEFFS, 0618-2011-AG-DGFFS-DGEFFS, 0350-2012-AG-DGFFS-DGEFFS) and the Institutional Animal Care and Use Committee (IACUC) of UC Davis School of Veterinary Medicine (IACUC PREDICT-WCS #16027).

Nested PCR Protocol

DNA from parasite cultures and filter paper samples was extracted using QIAamp DNA Mini kit (Qiagen), while DNA from whole blood in EDTA tubes was extracted using QIAamp Blood Mini kit (Qiagen) and quantified using a

Figure 1. Map of Peru showing the locations where captive ($n = 183$, circles) and wild ($n = 126$, triangles) NHPs were sampled: Nueva Esperanza ($n = 118$), Yurimaguas ($n = 68$), Lima ($n = 28$), Pucallpa ($n = 26$), Puerto Maldonado ($n = 26$), Cuzco ($n = 18$), Moyobamba ($n = 16$), Sol Naciente ($n = 8$) and Iquitos ($n = 1$). Biological samples from wild NHPs were collected in two indigenous communities in the Northern Peruvian Amazon.

NanoDrop 1000 spectrophotometer. Blood samples applied to FTA cards were processed following the manufacturer's instructions (GE Healthcare Life Sciences).

The nested PCR protocol was optimized using primers for the 24S alpha subunit rRNA gene. Primers D75/D76

target the conserved flanking sequences of the D7 alpha domain in trypanosomatids, while primers D71/D72 target an internal region in the same domain that is specific to *T. cruzi* (Souto and Zingales 1993; Souto et al. 1999). PCR was performed in a Gene Amp PCR System 9700 thermocycler

(Applied Biosystems, Foster City, CA) in a final volume of 30 μ l.

The first PCR reaction had 3 μ l of template DNA, and a final concentration of 1X PCR buffer (Invitrogen), 0.1 mM of each primer (D75 and D76), 1 U Taq DNA Polymerase (Thermo Scientific), 2.0 mM $MgCl_2$, and 0.15 mM of each dNTP. The PCR protocol included an initial denaturation step at 94°C for 5 min; 35 cycles at 94°C for 40 s to denature the template, annealing at 57°C for 40 s, extension at 72°C for 40 s; and a final extension step at 72°C for 5 min. Amplicons were analyzed on a 2% agarose gel with a 100 bp DNA ladder (Invitrogen).

The second PCR reaction contained 1 μ l of PCR product from the first reaction, and a final concentration of 1× PCR buffer (Invitrogen), 0.2 mM of each primer (D71 and D72), 1 U Taq DNA Polymerase (Thermo Scientific), 1.5 mM $MgCl_2$, and 0.25 mM of each dNTP. The initial denaturation step of 94°C for 4 min, was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min; and a final extension step at 72°C for 5 min. PCR products were analyzed in 2.5% agarose gels with a 50 bp size standard (Thermo Scientific).

Twelve parasite strains were used as reference, including *T. rangeli*, four *T. cruzi* strains (Tulahuen, Y, AQP 300, Viera Roque), and seven *Leishmania* species (*L. (V.) braziliensis*, *L. (V.) peruviana*, *L. (V.) guyanensis*, *L. (V.) panamensis*, *L. (V.) lainsoni*, *L. (L.) amazonensis*, and *L. (L.) mexicana*). DNA extracted from human and primate (*Aotus nancymaae*) blood infected with *Plasmodium falciparum* was used to assess cross-reactions with the host and *Plasmodium*. The analytical sensitivity of the protocol was evaluated by determining the minimum amount of *T. cruzi* DNA required for its successful PCR amplification. Serial 1:10 dilutions of *T. cruzi* DNA in AE buffer were prepared and a total of nine different concentrations were assayed (ranging from 0.5 ng/ μ l to 0.15 fg/ μ l).

Statistical Analyses

Diagnosis was based on microscopy and PCR. Results were grouped as correct positive diagnosis (*a*), false positive diagnosis (*b*), false negative diagnosis (*c*), correct negative diagnosis incorrect (*d*). From these values, the sensitivity ($100 \times a/(a + c)$) and specificity ($100 \times d/(c + d)$) of trypanosomatids and *T. cruzi* diagnosis were calculated (Ndao et al. 2000).

Prevalence rates of infection were calculated for both trypanosomatids and *T. cruzi* by expressing the percentage of

infected individuals with respect to the number of analyzed monkeys. This analysis was performed considering primate taxonomy and the human-animal context in which infection could occur (wild and captive interfaces). Prevalences were compared with Chi-squared and Fisher's exact tests (significance level $P < 0.05$). We used a recursive modeling approach with classification trees to identify major risk factors linked to the presence of trypanosomatids and *T. cruzi* using the package "rpart" version 4.1-9 of the statistical software R version 3.2.1 (Therneau et al. 2015). Recursive partitioning is the most appropriate technique for epidemiological studies based on complex relationships including nonlinear and interacting risk factors (Zhang and Singer 2010). The presence of trypanosomatids or *T. cruzi* was the dependent variable, and genus and the human-animal context (interface) were the independent categorical variables considered in the study as possible risk factors for prior trypanosomatid or *T. cruzi* infection.

RESULTS

Nested PCR Optimization

All trypanosomatids generated bands of approximately 280 bp with primers D75/D76, while *T. cruzi* strains produced bands of approximately 120 bp with primers D71/D72. The nested PCR protocol did not show evidence of cross-reactions or non-specific amplification with *A. nancymaae* nor *P. falciparum* DNA control samples. Primers D71/D72 were specific for *T. cruzi* and did not amplify neither *T. rangeli* nor any of the seven *Leishmania* species assayed.

The detection limit of the PCR using primers D75/D76 for detecting trypanosomatids was 1.5 pg of parasite DNA, while the detection limit for the PCR using primers D71/D72 for detecting *T. cruzi* was 15 fg. Compared to PCR, microscopy had a sensitivity of 82.9% (29/35, 95% CI 66–93.0%) and a specificity of 97.9% (46/47, CI 89.1–99.9%).

PREVALENCE OF TRYPANOSOMATIDS AND *T. CRUZI*

Tables 1 and 2 show the prevalence of trypanosomatids and *T. cruzi* in the 16 species (11 genera, 5 families) of the 309 sampled NHPs screened. Table 3 shows the prevalence of trypanosomatids and *T. cruzi* in the NHPs, according to their holding facility.

Table 1. Prevalence of Trypanosomatids and *T. cruzi* According to NHP ($n = 309$) Family and Interface (Wild/Captive).

Family	Genus	Species	Trypanosomatids			<i>T. cruzi</i>		
			Captive	Wild	Total	Captive	Wild	Total
Aotidae	<i>Aotus</i>	<i>A. nigriceps</i>	0/1 (0.0)	0/0 (0.0)	0/1 (0.0)	0/1 (0.0)	0/0 (0.0)	0/1 (0.0)
		<i>Aotus sp.</i>	2/4 (50.0)	0/0 (0.0)	2/4 (50.0)	0/4 (0.0)	0/0 (0.0)	0/4 (0.0)
Pitheciidae	<i>Cacajao</i>	<i>C. calvus</i>	0/0 (0.0)	12/12 (100.0)	12/12 (100.0)	0/0 (0.0)	0/12 (0.0)	0/12 (0.0)
		<i>Callicebus C. cupreus</i>	0/0 (0.0)	3/3 (100.0)	3/3 (100.0)	0/0 (0.0)	1/3 (33.3)	1/3 (33.3)
		<i>C. oenanthe</i>	0/1 (0.0)	0/0 (0.0)	0/1 (0.0)	0/1 (0.0)	0/0 (0.0)	0/1 (0.0)
		<i>Total Callicebus</i>	0/1 (0.0)	3/3 (100.0)	3/4 (75.0)	0/1 (0.0)	1/3 (33.3)	1/4 (25.0)
Atelidae	<i>Pithecia</i>	<i>P. monachus</i>	0/0 (0.0)	5/6 (83.3)	5/6 (83.3)	0/0 (0.0)	0/6 (0.0)	0/6 (0.0)
		<i>Alouatta A. seniculus</i>	1/10 (10.0)	7/9 (77.8)	8/19 (42.1)	0/10 (0.0)	0/9 (0.0)	0/19 (0.0)
		<i>Ateles A. belzebuth</i>	1/7 (14.3)	0/0 (0.0)	1/7 (14.3)	0/7 (0.0)	0/0 (0.0)	0/7 (0.0)
		<i>A. chamek</i>	0/20 (0.0)	6/18 (33.3)	6/38 (15.8)	0/20 (0.0)	0/18 (0.0)	0/38 (0.0)
	<i>Total Ateles</i>		1/27 (3.7)	6/18 (33.3)	7/45 (15.6)	0/27 (0.0)	0/18 (0.0)	0/45 (0.0)
	<i>Lagothrix</i>	<i>L. cana</i>	1/3 (33.3)	0/0 (0.0)	1/3 (33.3)	0/3 (0.0)	0/0 (0.0)	0/3 (0.0)
		<i>L. lagotricha</i>	21/55 (38.2)	0/0 (0.0)	21/55 (38.2)	0/55 (0.0)	0/0 (0.0)	0/55 (0.0)
		<i>L. poeppigii</i>	0/0 (0.0)	31/39 (79.5)	31/39 (79.5)	0/0 (0.0)	1/39 (2.6)	1/39 (2.6)
	<i>Total Lagothrix</i>		22/58 (37.9)	31/39 (79.5)	53/97 (54.6)	0/58 (0.0)	1/39 (2.6)	1/97 (1.0)
	<i>Cebus</i>	<i>C. albifrons</i>	0/13 (0.0)	9/12 (75.0)	9/25 (36.0)	0/13 (0.0)	5/12 (41.7)	5/25 (20.0)
		<i>Sapajus S. macrocephala</i>	2/18 (11.1)	3/21 (14.3)	5/39 (12.2)	1/18 (5.6)	1/21 (4.8)	2/39 (5.1)
		<i>Saimiri S. boliviensis</i>	1/6 (16.7)	4/5 (80.0)	5/11 (45.5)	0/6 (0.0)	2/5 (40.0)	2/11 (18.2)
	<i>Saimiri</i>	<i>S. sciureus</i>	22/41 (53.7)	1/1 (100.0)	23/42 (54.8)	5/41 (12.2)	1/1 (100.0)	6/42 (14.3)
		<i>Total Saimiri</i>	23/47 (48.9)	5/6 (83.3)	28/53 (52.8)	5/47 (10.6)	3/6 (50.0)	8/53 (15.1)
Callitrichidae	<i>Saguinus</i>	<i>S. fuscicollis</i>	0/4 (0.0)	0/0 (0.0)	0/4 (0.0)	0/4 (0.0)	0/0 (0.0)	0/4 (0.0)
Total NHPs			51/183 (27.9)	81/126 (64.3)	132/309 (42.7)	6/183 (3.3)	11/126 (8.7)	17/309 (5.5)

The number of infected animals with respect to the total number of examined animals for each category. The numbers in parentheses are the calculated prevalences (%).

Table 2. Prevalence of Trypanosomatids and *T. cruzi* According to NHP ($n = 309$) Family in Captive and Wild Individuals.

Family	Trypanosomatids			<i>T. cruzi</i>		
	Captive	Wild	Total	Captive	Wild	Total
Aotidae	2/5 (40.0)	0/0 (0.0)	2/5 (40.0) ^{abc}	0/5 (0.0)	0/0 (0.0)	0/5 (0.0) ^{ab}
Pitheciidae	0/1 (0.0)	20/21 (95.2) ^a	20/22 (90.9) ^c	0/1 (0.0)	1/21 (4.8) ^a	1/22 (4.5) ^a
Atelidae	24/95 (25.3)	44/66 (66.7) ^b	68/161 (42.2) ^a	0/95 (0.0)	1/66 (1.5) ^a	1/161 (0.62) ^a
Cebidae	25/78 (32.1)	17/39 (43.6) ^c	42/117 (35.9) ^{bd}	6/78 (7.7)	9/39 (23.1) ^b	15/117 (12.8) ^b
Callitrichidae	0/4 (0.0)	0/0 (0.0)	0/4 (0.0) ^{ad}	0/4 (0.0)	0/0 (0.0)	0/4 (0.0) ^{ab}
Total NHPs	51/183 (27.9)	81/126 (64.3)	132/309 (42.7)	6/183 (3.3)	11/126 (8.7)	17/309 (5.5)

a, b, c, d values appearing in total prevalence ratios per NHP family with different letters are significantly different ($P < 0.05$).

Overall prevalence was 42.7% (132/309) for trypanosomatids and 5.5% (17/309) for *T. cruzi*. In decreasing order, the prevalence of trypanosomatids by NHP family was: Pitheciidae (90.9%), Atelidae (42.2%), Aotidae (40%) and Cebidae (35.9%) ($P < 0.001$). The highest prevalence

of *T. cruzi* by family was observed in Cebidae (12.8%) and Pitheciidae (4.5%) ($P < 0.001$). Wild NHPs had a higher prevalence of both trypanosomatids (64.3 vs 27.9%, $P < 0.001$) and *T. cruzi* (8.7 vs 3.3%, $P = 0.057$) compared to captive NHPs (Table 1). Within the captive interface,

wet markets were the most important holding facility for the presence of both trypanosomatids ($P < 0.001$) and *T. cruzi* ($P < 0.008$).

Table 3. Trypanosomatid and *T. cruzi* Infection Rates of NHPs Considering Their Holding Facility.

Interface	Trypanosomatid +/n (%)	<i>T. cruzi</i> +/n (%)
Captive		
Wild	81/126 (64.3) ^b	11/126 (8.7) ^b
Household	6/32 (18.8) ^a	0/32 (0.0) ^a
Zoo	6/34 (17.6) ^a	1/34 (2.9) ^{ab}
Wet market	22/38 (57.9) ^b	5/38 (13.2) ^{ab}
Rescue center	17/79 (21.5) ^a	0/79 (0.0) ^a
Total	51/183 (27.9)	6/183 (3.3)

^a, ^b values appearing in columns with different letters are significantly different ($P < 0.05$).

In NHPs hosts, the most parsimonious tree model explained 35.1% and 23.1% of the observed variability of presence of trypanosomatids (Fig. 2) and *T. cruzi* (Fig. 3), respectively. The best tree size for each model was selected using cross-validation: for the trypanosomatids model, a tree of size 5 (N splits = 4) with a complexity parameter CP = 0.0065; and for the *T. cruzi* model, a tree of size 6 (N splits = 5) with a complexity parameter CP = 0.0165. The trees showed that genus and interface are risk factors associated with trypanosomatids and *T. cruzi*.

DISCUSSION

Since the close contact between humans and NHPs infected with trypanosomes poses an important risk of infection, this study aimed to determine the prevalence rate of both trypanosomatids and *T. cruzi* in different genera of Neotropical primates in Peru. Trypanosomatid and *T. cruzi*

Figure 2. Most parsimonious tree build using recursive partitioning to identify major risk factors linked to the presence of trypanosomatids in NHPs. Wild NHPs and NHPs from wet markets, and genera *Alouatta*, *Lagothrix*, *Cacajao*, *Pithecia*, *Cebus*, *Saimiri* and *Callicebus* have a significantly higher risk of trypanosomatid infection. The most parsimonious tree model explained 35.1% of observed variability.

Figure 3. Most parsimonious tree build using recursive partitioning to identify major risk factors linked to the presence of *Trypanosoma cruzi* in NHPs. Wild NHPs, and genus *Cebus*, *Saimiri* and *Callicebus* have a significantly higher risk of *T. cruzi* infection. The most parsimonious tree model explained 23.1% of observed variability.

prevalence rates were significantly higher in wild primates compared to captive animals, suggesting that parasite transmission in NHPs occurs more actively in the sylvatic cycle. Primates in the wild are probably exposed to a greater number of infection routes, such as triatomine vectors that may dwell and feed on primate resting sites, but also via oral route (i.e., ingesting infected insects) and other mechanisms such as vertical transmission or sexual contact (Jansen and Roque 2010; Rocha et al. 2013). In addition, there is a greater number of potential reservoir species in the wild which increases the probability of transmission within wild NHP populations. *T. cruzi* is a multihost parasite that exhibits remarkable biological and genetic diversity, as well as complex transmission cycles (Xavier et al. 2012; Rocha et al. 2013). Therefore, sylvatic cycles should be understood as dynamic webs, with parasite transmission occurring by different routes in cycles in the forest, understory, or canopy that may or may not overlap (Jansen and Roque 2010).

Neotropical primates can be naturally infected with several species of trypanosomes, including *T. cruzi*, *T. rangeli*, *T. evansi*, *T. simiae*, *T. minasense*, *T. saimiri*, *T. lambrechtii*, *T. devei*, *T. sanmartini*, *T. diasi*, *T. prowazecki*,

and *T. mycetiae* (Ziccardi and Lourenco-de-Oliveira 1997; Ndao et al. 2000). Trypanosomes have been commonly reported in the NHP families Callitrichidae (tamarins and marmosets), Cebidae (squirrel monkeys and capuchins), Aotidae (night monkeys), and Atelidae (spider monkeys and howler monkeys) (Sousa et al. 1974; Ziccardi and Lourenco-de-Oliveira 1997; Ziccardi et al. 2000; Maia da Silva et al. 2008).

In our study, the observed variability in presence of trypanosomatids and *T. cruzi* was mainly explained by intrinsic (genus) and extrinsic (interface) factors. Among wild NHPs, Pitheciidae (Titi Monkeys, Sakis, and Uakaris) had the highest trypanosomatid prevalence (95.2%), and Cebidae had the highest *T. cruzi* prevalence (23.1%). Within captive NHPs, specimens from wet markets had the highest trypanosomatid and *T. cruzi* prevalences, which underscores the possibility that extracted primates from the wild may introduce the parasite into urban areas. The wet market interface probably has the closest prevalence rate to the one found in the wild, since recently captured animals from the jungle are brought into cities for trade in markets (Shanee et al. 2015). Primates recovered from this illegal

traffic and from wildlife and biodiversity conservation programs are usually reintroduced to the wild without an exhaustive disease screening. Thus, availability of efficient protocols for trypanosome detection could mitigate risks to naïve resident populations exposed to reintroduced or translocated animals (Lisboa et al. 2004).

High mortality during transport and captivity could explain the lower rate of infection observed in captive NHPs. Another possibility for low prevalence is the absence of vectors in the area. However, transmission can still occur in closed NHP colonies by vector-independent mechanisms such as trauma, blood exposure, saliva, sexual activity and transplacental transmission (Ndao et al. 2000; Kunz et al. 2002; Lisboa et al. 2004; Dorn et al. 2012).

Feeding behavior of NHPs is an intrinsic factor associated with genus that might contribute to the rate of infection. Oral transmission of *T. cruzi* is probably the most frequent mechanism among animals in the wild cycle, considering that several species of wild mammals frequently ingest insects, including infected triatomines (Coura 2015). In addition, stercorarial transmission may not be as efficient as the oral route because infected feces of triatomines are probably deposited in dense animal fur and not directly on skin (Roque et al. 2008).

Most human Chagas cases in Peru occur in the region of Arequipa (682 cases since 2004), where we could not sample NHPs. However, we did find *T. cruzi* positive animals in regions with very low numbers of human cases reported, such as Ucayali (6 cases since 2004) and Amazonas (13 cases) (MINSA 2015). This could represent the introduction of *T. cruzi* by NHPs to new areas, and future vectorial transmission cannot be ruled out since Peru has at least 19 species (9 genera) of triatomine bugs, which are present in all the regions sampled in this study (Lima, Cuzco, Loreto, San Martín, Madre de Dios, and Ucayali) (Chávez 2006; Náquira and Cabrera 2009).

Prevalence studies in Peruvian NHPs are scarce, and *T. cruzi* infection rates varied from 3.6 to 12.9% (Dunn et al. 1963; Sullivan et al. 1993), although some studies reported a difference in prevalence depending on the method used: 6.1% using microscopy, 10.4% using ELISA, and 26.5% using PCR (Ndao et al. 2000). Studies in free ranging primates of Central and South America show variable but usually high trypanosome prevalence. In Brazil, trypanosome prevalences of 47–51% (D'Alessandro et al. 1986), 67.9% (Ziccardi and Lourenco-de-Oliveira 1997), and 46.8–87.5% have been reported (Maia da Silva et al. 2008); in French Guiana, trypanosome prevalence varied

between 16 and 51% depending on the primate species (de Thoisy et al. 2001); and in Panama, the trypanosome prevalence was 10.1% (Sousa et al. 1974). However, there is need for caution when comparing prevalences from different studies because of the different sampling techniques and the different diagnostic methods used (with varying sensitivities). For instance, the infection rates of *T. cruzi* in Amazonian monkeys ranged from 10.3% using parasitological methods (Ziccardi and Lourenco-de-Oliveira 1997; Ziccardi et al. 2000) to 46% using serology (Lisboa et al. 2006). Furthermore, in studies using PCR several other aspects must be taken into account, such as sample type, sample volume and conservation, DNA extraction method, parasite sequences targeted, primers, reagents, and thermocycling conditions (Luquetti and Schmunis 2010).

In our study, whole blood was collected in EDTA tubes whenever possible, but samples were also collected on FTA cards and filter paper. Dried blood spot sampling overcame the limitations of cold chain in rural areas and remote locations (Luquetti and Schmunis 2010). However, natural inhibitors present in samples and the uneven distribution of parasite DNA on FTA cards may decrease the assay's sensitivity (Pizarro et al. 2007; Ahmed et al. 2011). We were able to analyze only one disk (punch) per sample, which could have resulted in an underestimation of the real prevalences, as it has been observed that *Trypanosoma spp.* prevalence increases dramatically when a higher number of FTA sections are analyzed (Cox et al. 2010). Further studies should consider analyzing multiple FTA sections and a complementary PCR protocol that targets host DNA to avoid false-negatives (Molaei et al. 2008; Jercic et al. 2010; Schijman et al. 2011).

In terms of sensitivity, the first PCR reaction amplifies up to 1.5 pg of parasite DNA, whereas the second reaction detects up to 15 fg of parasite DNA. Thus, the second step is more sensitive and may detect *T. cruzi* infections that are not evident with only the first step of the protocol. For this reason, even when a sample is negative for trypanosomatids, *T. cruzi* infection cannot be discarded and the second step should be performed. Estimating the exact number of parasites detected with the nested PCR is not completely straightforward. Preparing samples with very few parasites may not be accurate, as parasite DNA from partially degraded organisms may be present in the liquid portion of the samples (Moser et al. 1989; Kirchhoff et al. 1996). Thus, we opted to prepare serial dilutions of known amounts of parasite DNA. However, the total amount of DNA in *T. cruzi* cells varies among different strains (Santos et al. 1997), and the absolute amount of total DNA (nu-

clear + kinetoplast) varies from 0.12 to 0.33 pg per *T. cruzi* cell (Souza et al. 2011). Considering this range, the nested PCR protocol detects from 4.5 to 12.5 parasites in the first reaction, and 0.045 to 0.12 parasites in the second step.

In our study, we found the highest *T. cruzi* prevalence in NHPs in the wild and wet markets. Since NHPs may act as reservoirs for this parasite, their handlers are at risk of accidental infection (Ndao et al. 2000; Strait et al. 2012). Trade of wildlife for exotic pet markets results in close contact between humans and NHPs, providing new opportunities for disease emergence (Wolfe et al. 1998). Accidental infections have been reported to occur not only by needlestick injuries, but by exposure of mucous membranes to contaminated fluids and skin lesions while handling infected animals (Hofflin et al. 1987; Kinoshita-Yanaga et al. 2009; Pereira et al. 2010; Kirchhoff 2011). This may be a frequent threat in rural communities where wild animals are hunted for subsistence, because carcasses are handled with bare hands. Indeed, wildlife subsistence hunting in the Amazon region is a traditional source of food for rural populations (FitzGibbon 1998), and primates represent an important source of meat in the region (Puertas 2004; Bowler et al. 2014; Mayor et al. 2015). Furthermore, meat in these rural settings may be ingested raw or undercooked, and parasite pseudocysts and blood form trypomastigotes in the hunted animals could represent a high-risk of infection (Pereira et al. 2009).

In summary, *T. cruzi* and trypanosomatids frequently infect both wild and captive Peruvian NHPs, posing a risk to human and animal health that has not been properly studied. The main risk factors associated with trypanosomatids and *T. cruzi* infections were genus and interface. Prevalence rates were also especially high in wild primates, suggesting that parasite transmission in NHPs occurs more actively in the sylvatic cycle. NHPs are regularly hunted, manipulated, consumed, and introduced into urban areas; hence, further studies are necessary to better understand pathogen transmission and public health implications.

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COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST The authors declare that no competing interests exist.

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