Molecular Characterization of Tandem Repeat Protein 36 Gene of *Ehrlichia canis* Detected in Naturally Infected Dogs from Peru

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Abstract. *Ehrlichia* spp. are emerging infectious pathogens, especially in the Americas. Although *Ehrlichia canis* is primarily a parasite of dogs, polymerase chain reaction-confirmed human infections have been reported from Mexico, Venezuela, and Costa Rica. This study reports the presence of *E. canis* DNA in 13.7% of 205 dogs from urban areas in Peru and of those, five were analyzed for phylogenetic variation using the Tandem Repeat Protein 36 (TRP36) gene. The use of the TRP36 gene for such analysis was validated against 16S rRNA and heat shock protein genes using Shannon's entropy bioinformatic approach. When compared with other *E. canis* strains previously reported, three unique and novel *E. canis* strains were detected. In addition, the TRP36 amino acid tandem repeat sequences of the Peruvian strains share close similarity to an *E. canis* strain detected from four human blood bank samples in Costa Rica. This study reports for the first time domestic dogs infected with *E. canis* strains closely related to a zoonotic strain, which may be of public health concern as dogs can be chronically infected with this pathogen.

INTRODUCTION

Tick-borne pathogens (TBP) are well-established threats to human and other mammalian species worldwide.^{1–3} *Ehrlichia canis* is a rickettsial TBP with global distribution and is highly endemic in South America.⁴ This pathogen is the causative agent of canine monocytic ehrlichiosis but often maintains chronic subclinical infection in dogs. *Ehrlichia canis* is mainly transmitted by *Rhipicephalus sanguineus* ticks, for which dogs are the natural host.^{5,6} This tick is found to be able to survive in multiple ecological zones.⁷ Although *R. sanguineus* inhabits both rural and urban environments, infestations are often observed near or inside human settlements because of the close proximity of their canine host.^{7,8}

Ehrlichia canis was first identified in Brazil in 2002 and has been documented throughout South America in countries such as Colombia, Chile, Argentina, Peru, and Venezuela.^{9–11} In addition, *E. canis* have been found in both dogs with subclinical and clinical symptoms in Peru.^{6,12,13} Although its role as a human pathogen is not fully characterized, *E. canis* should be considered a potential agent of human illness in areas where dogs are frequently infected.¹⁴ The first case of human monocytic ehrlichiosis (HME) caused by *E. canis* was reported in Venezuela in 1996.¹⁵ In 2001, an *E. canis* strain detected from a Venezuelan human was 99.9% identical to the strain isolated both from a dog and an *R. sanguineus* tick found in Venezuela.¹⁶ Well-characterized human cases of ehrlichiosis have not yet been reported from Peru; however, a recent study reported HME seroprevalence in four separate and unique rural communities.¹⁷ The distribution and preferred habitat of

R. sanguineus along with the close proximity of dogs and humans in confined spaces could be significant to the higher incidences of HME in Peruvian communities.

Although initial phylogenetic analyses based on 16S rRNA and GroEL genes resulted in an apparent lack of *E. canis* diversity, the introduction of the Tandem Repeat Protein 36 (TRP36) gene in 2013 for phylogenetic diversity analysis provided a more refined classification of *E. canis* strains.¹⁸ Using such approach, a correlation between the high prevalence of *E. canis* and recombination of the unique strains was demonstrated in South America.^{18,19} There may be genotypic differences within individual genotypes not only in South America but within other regions as well.^{18,20,21} Genotypic variations of *E. canis* has also recently been observed in human blood donors in Costa Rica with serological evidence of zoonotic transmission that share a resemblance to the Brazilian strains.²² The possibility of distinct genotypes of *E. canis* causing different clinical manifestation could lead to difficulty in diagnosing canine or human ehrlichiosis.^{19,20}

Based on the close proximity between domestic dogs and humans, dogs may serve as a sentinel for zoonotic vector-borne diseases in Peru as similarly described in Venezuela.^{16,23} The objective of this study is to report the molecular and serologic prevalence of *E. canis* in asymptomatic dogs from four distinct Peruvian settlements and to describe novel Peruvian *E. canis* strains closely related to a pathogen recently detected in human blood donors in Central America.²²

MATERIAL AND METHODS

Study sites. This cross-sectional study was conducted in three small communities and five cities in Peru. San Juan de Miraflores is a district of the Lima Province and located at the center of the coast at an altitude of 141 m (463 ft) with a

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population of approximately 336,000 people. Paita is located northwest of Lima at sea level with a population of approximately 98,000 people. Huaraz, in the Ancash region, is located northeast of Lima at an altitude of 3,052 m (10,013 ft) with a population of approximately 53,000 people. Caraz is also located in the Ancash region and is 460 km (285 miles) northeast of Lima at an altitude of 2,256 m (7,402 ft) with a population of approximately 20,000 people. Ondores is in the Junin region and located 230 km (143 miles) east of Lima at an altitude of 4,105 m (13,541 ft) with an estimated population of 2,571 people. Canchayllo, Pachacayo, and San Juan de Pachacayo are three small villages in the Junin region located approximately 200 km (124 miles) from Lima at an altitude of 3,671 m (12,043 ft) with a combined population of 1,774 people.²⁴

Study population. Domestic dogs were volunteered for this study by their owners. A sample size of 219 dogs was included. In December 2009, 122 dogs from Ondores, Canchayllo, Pachacayo, and San Juan de Pachacayo were enrolled in this study. In addition, in December 2010, 97 dogs from Lima, Paita, Huaraz, and Caraz were enrolled. Exclusion criteria were defined as follows: aggressive dogs, small puppies, and dogs exhibiting clinical signs of disease. Ethylenediaminetetraacetic acid anticoagulated and/or whole blood samples were aseptically collected from the jugular or cephalic veins, aliquoted, and stored at -20°C until analysis. Serum samples were not available from 111 dogs (nine dogs from Pachacayo, five dogs from Canchayllo, 30 dogs from Lima, 39 dogs from Caraz, and 28 dogs from Paita). The average age was 2.5 years (standard deviation: 2.75 years, 95% confidence interval: 2.1–2.9 years, median: 1.5 years, and range: 2 months to 14 years). Males represented 53.9% (118/219) of the dogs, whereas females represented 38.4% (84/219), with 7.8% (17/219) of unknown gender. Samples were shipped frozen on dry ice to the College of Veterinary Medicine at Western University of Health Sciences, Pomona, CA, under the import permit number 2009-12-105 from the Center for Disease Control and Prevention.

DNA extraction and quality control. DNA samples from Ondores, Canchayllo, Pachacayo, and San Juan were purified using a column-based method (Quick-gDNA Blood MiniPrep; Zymo[®] Research, Irvine, CA) according to the manufacturer's instructions at the Western University of Health Sciences College of Veterinary Medicine. DNA samples from San Juan de Miraflores, Paita, Huaraz, and Caraz were purified at Universidad Peruana Cayetano Heredia using a phenol-chloroform method. For each sample, DNA was quantified by spectrophotometry (GE Nanovue; GE Healthcare Biosciences, Pittsburgh, PA) and purity was determined by 260/280 ratio. DNA samples from 14 dogs were not available (six from Canchayllo and eight from Pachacayo).

Serology. Antibodies against *Anaplasma* sp., *Borrelia burgdorferi*, and *E. canis*, as well as antigens of *Dirofilaria immitis* were detected using the enzyme-linked immunosorbent assay (ELISA) SNAP[®] 4Dx[®] as per manufacturer instructions (IDEX[®] Laboratories, Westbrook, ME). The SNAP[®] test was chosen to for being a quick in-clinic diagnostic assay available to veterinarians and being available at no cost for this study by the manufacturer. Serum samples were not available from 111 dogs (five from Canchayllo, 39 from Caraz, 30 from Lima, nine from Pachacayo, and 28 from Paita); therefore, 94 samples had concomitant PCR and serology results, with 14 samples having only serology results.

PCR amplification and DNA sequencing. Two hundred and five DNA samples were screened for the presence of *E. canis* DNA by conventional PCR targeting the heat shock protein (GroEL) gene, using primers GroEL-643s 5'-ACT GAT GGT ATG CAR TTT GAY CG-3' and GroEL-1236 as 5'-TCT TTR CGT TCY TTM ACY TCA ACT TC-3' as described previously.²⁵ Five PCR-positive samples, four from Lima and one from Paita, were further characterized by the amplification of the TRP36, using primers TRP-F2 (5'-TTTAAAACAAAATTAACAC ACTA-3') and TRP36-R1 (5'-AAGATTAACCTAATACTCAATA TTA-3').¹⁸ The negative control consisted of molecular-grade water with DNA from *E. canis* strain Jake as a positive control (CP000107). Amplification was performed with conventional PCR in a 25-μL final volume reaction containing 1X PCR mix (Premix Ex Taq [Perfect Real Time], Takara Bio Inc., Shiga, Japan), 12.5 pmol of each primer, and 5.0 μL of DNA template. PCR products were analyzed by 1.5% agarose gel electrophoresis with UV exposure. Amplicons were compared using 1 kb Plus DNA Ladder with amplicons ranging from 800 to 1,000 bp were considered positive for *Ehrlichia* species.¹⁸ Nested PCR was performed with TRP36-DF (5'-CACACT AAAATGTATAATA-AAGC-3') and TRP36-R1 (5'-AAGATTA ACTTAATACTCAATATTACT-3').²¹ Amplicons were considered positive for *Ehrlichia* sp. at a range of 600–900 bp.²¹ Sample extraction, reaction setup, PCR amplification, and amplicon detection were performed in separate areas of the laboratory to prevent contamination.

Amplicons were purified from PCR products according to the manufacturer's instructions (MiniElute kit; Qiagen, Valencia, CA). The amplicons were then sequenced with a fluorescence-based automated sequencing system (Eurofins MWG Operon, Huntsville, AL). Sequence evaluation and alignment was performed using Contig Express and AlignX software (Vector NTI Suite 10.1; Invitrogen Corp., Carlsbad, CA). The GenBank database was used to define bacteria species and strains via the Basic Local Alignment Search Tool.²⁶

The MEGA7 software was used for phylogenetic analysis based on the maximum likelihood method with the Kimura two-parameter model.^{27,28} Bootstrap replicates were performed to estimate the node reliability of the phylogenetic trees. Values were obtained from 1,000 randomly selected samples of the aligned sequence data. DNA amplification and sequencing analysis were performed at the Western University of Health Sciences, College of Veterinary Medicine, Pomona, CA.

Entropy-based score for multiple sequence alignments (MSAs). All the TRP36 (or gp36), 16S, and GroEL nucleotide sequences used in this assay were downloaded from the two curated databases IMG (<https://img.jgi.doe.gov>) and PATRIC (<https://www.patricbrc.org>) (see Supplemental Table 1 for accession numbers). Multiple sequence alignments were obtained by running muscle v3.8.31.²⁹ After an MSA visual inspection using JalView,³⁰ MSA were trimmed for the described length of each gene based on the coverage of the consensus sequence. An ad hoc R script was written to calculate the overall Shannon's entropy score for the three genes.³¹ Specifically, all nucleotide sites presenting entropy greater than zero in the MSAs were counted to get the number of informative sites and its rate (informative sites/total columns in MSA) for each gene. The "diversity" function from the "vegan" R package was used for this purpose.³²

Overall Shannon's entropy was presented as violin plots using ggplot2 R library.³³ Wilcoxon's test with Bonferroni's

FIGURE 1. Tandem Repeat Protein 36 (TRP36) displays a higher Shannon's Entropy Index compared with both 16S rRNA and GroEL. The violin plots depict the overall entropy score for all nucleotide sites on the multiple sequence alignments (MSAs) for each gene. Entropy of zero means no variability at a specific position in the MSA. The overall mean is represented by filled circles, whereas the median is shown as open circles. Bonferroni-adjusted Mann-Whitney-Wilcoxon test P value < 0.001 for all three pairwise comparisons (***).

correction was used to compare the overall Shannon's entropy among 16S rRNA, GroEL, and TRP36 genes, within the statistical package R and a significance level of < 0.05 .³⁴

RESULTS

From 205 dogs tested by GroEL PCR, *E. canis* DNA was detected in 28 dogs (13.7%). DNA sequencing of the GroEL amplicons confirmed the presence of *E. canis* from those dogs. There was one PCR-positive dog from Pachacayo, 10 dogs from Lima, and 17 dogs from Paita. Of those 28 dogs infected with *E. canis*, five of them were further characterized by TRP36 PCR followed by DNA sequencing. The amplicons of the five samples varied from 611 to 905 bp after deletion of the primer sequence. When compared against sequences available from GenBank database, samples Lima PA-26 (444/445 bp) and Paita CO-1 (528/529 bp) were 99% identical to the Costa Rica Human (CRH) *E. canis* isolate (KU 194227.1). The

other three remaining samples Lima PA-8, Lima PA-16, and Lima PA-18 were highly conserved among each other and were 96% (406/421 bp) identical to the Israel Ranana strain (ABV02078.1). The sequences generated from this study using TRP36 gene were deposited in GenBank under Lima PA-26 (MF095617), Lima PA-8 (MF095618), and Paita CO-1 (MF095619).

To assess the phylotyping robustness of TRP36 sequence, we compared *Ehrlichia* spp. only MSA of TRP36 against the MSA of the two most commonly used genes (16S rRNA and GroEL). A nucleotide site with an entropy score of zero indicates lack of genetic variability at that position in the alignment. The comparison of the overall Shannon's entropy of each gene indicated that the TRP36 gene has a higher phylotyping discriminatory power (49.8%) when compared with both 16S rRNA (11.1%) and GroEL (19.4%) within the *Ehrlichia* genus ($P < 0.001$, Wilcoxon test, Figure 1).

On analysis of the amino acid sequences, the tandem repeats for the three sequences were found to be similar to the Costa Rica isolate sequence of "EASVVPAAEAPQPAQQTE DEFFSDGIEA" and a second tandem repeat sequence with a deletion of one alanine at the carboxyl terminus consisting of "EASVVPAAEAPQPAQQTEDEFFSDGIE."²² Paita CO-1, Lima PA-26, and Lima PA-8 strains had three, two, and one repeats as shown in Table 1. There was a second sequence of tandem repeats in the strains Paita CO-1 and Lima PA-26 that were similar to the second sequence found in the CRH strain with the number of repeats for each shown in Table 1. The phylogenetic relationship of the three Peruvian strains detected in this study was genetically distinct from *E. canis* strains described from the United States, Brazil, Africa, Europe, Israel, and Asia, forming a separate clade under the original classification suggested by Aguiar (Figure 2).¹⁸

In addition, a total of 108 domestic dogs were tested for the presence of antibodies against *E. canis*, *Anaplasma* spp., *B. burgdorferi*, and for antigens of *D. immitis* using the ELISA SNAP[®] 4Dx[®] test. All tested dogs being negative with exception of one dog from Canchaylo, which was seropositive for the *Anaplasma* spp. antigen. However, *Anaplasma* sp. DNA was not detected by GroEL PCR from this dog. Unfortunately, serum samples were not available from 27 of 28 PCR-positive dogs, with the only PCR-positive dog seronegative for *E. canis* on the ELISA SNAP[®] 4Dx[®] test.

DISCUSSION

Previously, a new strain of *E. canis* in Peru was characterized from symptomatic dogs by molecular diagnosis with 16S rRNA.⁶ We investigated the presence of the proposed strain with the recently introduced TRP36-based

TABLE 1
Distribution and comparison of repeated TRP36 amino acid sequences from the *Ehrlichia canis* clades

Country	<i>E. canis</i> strain	Source	No. of tandem repeats	Amino acid sequence
United States	Jake	Dog	12	TEDSVSAPA
Brazil	São Paulo	Dog	18	TEDSVSAPA
Brazil	Belem	Dog	6	ASVVPEAE
Israel	Ranana	Dog	10	TEDPVSATA
Costa Rica	CRH	Human	4	EASVVPAAEAPQPAQQTEDEFFSDGIEA
Peru	PA-26 and CO-1	Dog	2, 3	EASVVPAAEAPQPAQQTEDEFFSDGIEA
Peru Costa Rica	PA-8, PA-26, CO-1, and CRH	Dog	1	EASVVPAAEAPQPAQQTEDEFFSDGIE

CRH = Costa Rica human; TRP36 = Tandem Repeat Protein 36. The differences in amino acid sequences among strains are highlighted in bold.

phylogenetic analysis, which identified three novel amino acid sequences in the study population. The comparison of these sequences to others available from public databases indicated a high similarity with a DNA sequence obtained from four humans from a blood bank in Costa Rica.²² Therefore, our findings suggest that an *E. canis* strain closely related to a strain capable of infecting humans is present in the canine population in Peru. Considering that serologic evidence of human exposure to *Ehrlichia* spp. can be as high as 25% in some communities, our data suggest that dogs may be potential reservoirs for ehrlichiosis in Peruvian communities.^{16,17,35}

We have determined that 13.7% of 205 asymptomatic dogs in Peru were infected with *E. canis*. Our findings are consistent with a previous report of dogs with clinical signs confirmed to

be infected with *E. canis*.⁶ Collectively, these findings indicate that *E. canis* is established in the canine population in Peru, although historical trends in exposure or infection are not yet available for human or canine populations. Several previous studies and reviews have characterized the endemic nature of *E. canis* in dogs in other countries bordering Peru, including Ecuador, Colombia, Brazil, and Bolivia.^{10,11,36–38}

Historically, several genes have been targeted for the genetic characterization of *E. canis* strains, including the 16S rRNA and GroEL.^{39–41} It has been shown that the TRP36 provides better discriminatory power in the phylogenetic analysis of this species.^{18,20,21} Most of the differences in the TRP36 gene between *E. canis* strains ranged in the number of tandem repeats from 5 to 16. However, in the last few years, an increasing amount of genetically diverse strains of *E. canis*,

FIGURE 2. Phylogenetic relationship between novel Peruvian *Ehrlichia canis* genotypes (PA-8, PA-26, and CO-1) and *E. canis* Tandem Repeat Protein 36 (TRP36) amino acid sequence clade system. The alignments, percent identity, and phylogenetic comparison were analyzed with MEGA 7.²⁸ Internal branches are numbered to represent the 1000 bootstrap values. GenBank accession numbers are provided in parenthesis. Nucleotide accession number sequences were translated to the correct TRP36 open reading frame (ORF) prior to running the multiple sequence alignment with other protein sequences.

in terms of amount of repeats and the tandem repeat sequence, has been documented.^{18,42–45} In this study, the entropy of TRP36, 16S rRNA, and GroEL were calculated and compared, where entropy is determined to be the amount of variability within the gene. Our findings of the entropy of TRP36 and its higher discriminatory abilities of 49.8% as opposed to 11.05% from 16S rRNA and 19.4% from GroEL validates the current and further use for phylotyping of *E. canis* (Figure 2). This study is the first to identify why TRP36 has a higher divergence among *E. canis* strains using Shannon's entropy and MSA, indicating that future investigation using bioinformatic analysis of the TRP36 gene and *E. canis* may lead to valuable information about TRP36 and host interactions.

The classification system initially introduced using TRP36 separates *E. canis* strains in clades based on the number of tandem repeat and C-terminal amino acid sequences.¹⁸ When the Peruvian *E. canis* strains from this study were included in such clade system, they formed a separate clade that includes the *E. canis* strain from humans in Costa Rica (KU194227) with > 90% of amino acid sequence similarity (Clade D, Figure 2).²² With this in mind, the fact that none of the infected dogs in this study presented clinical signs supports the need for further investigation into *E. canis* as a hidden zoonotic disease in urban areas of Peru.

The lack of seroreactivity to *E. canis* in the Peruvian population is an intriguing finding in this study because 28 dogs were PCR positive for *E. canis*. All 108 dogs tested for *E. canis* exposure by SNAP[®] serology were negative. Unfortunately, most of the PCR-positive dogs (27 of 28) did not have a serum sample available, so we cannot infer about the status of the immune response of the *E. canis*-infected dogs. These negative serological findings differ from a previous study in the same country where 25 dogs showing clinical signs of ehrlichiosis were positive for *E. canis* by SNAP[®] 4Dx[®], with 11 tested positive for a new strain of *E. canis* based on 16S rRNA sequence analysis.⁶

The fact that our study targeted asymptomatic dogs may have increased the likelihood of selecting nonexposed dogs; however, the significant number of PCR-positive dogs in the same population challenges this hypothesis. Other possible hypotheses for such differences in serology versus molecular diagnosis in our study include the sensitivity of the dot-ELISA assay used, pathogen modulation of host immunity, stage of infection, or antigenic variation of the *E. canis* strains.¹² Further research is required to see if current in-clinic diagnostic methods, such as the SNAP[®] test, can identify multiple strains of *E. canis*.²⁰

This study had limitations that deserve discussion. A different serologic assay such as immunofluorescence assay or ELISA was not used to confirm the results from the SNAP[®] 4Dx[®] test in this study. The SNAP[®] test was used to mimic the in-clinic experience of veterinarians, as well as being available at no cost by the manufacturer. Sample collection and gDNA extraction were performed in 2009–2010 as part of another study. While serum samples were tested in 2010, DNA samples were stored in –80°C for a long period and subjected to several freeze–thaw cycles. In addition, TRP36 gene sequences were generated from only five subjects; therefore, further genetic diversity of *E. canis* in Peru may exist. However, the good quality of the chromatograms obtained from these five samples supported the characterization of three new *E. canis* strains and their classification into a new clade.

In conclusion, we have documented asymptomatic Peruvian dogs infected with strains of *E. canis* genetically distinct from strains described from other countries around the world. As a result, our results further expand the molecular and epidemiologic knowledge of *E. canis*. In addition, our results suggest that novel *E. canis* strains similar to a known zoonotic strain are present in the canine population in Peru, which may pose at risk for human infections. Vector control in dogs and environments shared between dogs and humans is highly recommended. A larger molecular and epidemiologic study is necessary to further characterize such potential risk and support further public health actions.

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REFERENCES

1. Dantas-Torres F, Chomel BB, Otranto D, 2012. Ticks and tick-borne diseases: a one health perspective. *Trends Parasitol* 28: 437–446.
2. Eremeeva ME et al., 2007. Bacteremia, fever, and splenomegaly caused by a newly recognized *Bartonella* species. *N Engl J Med* 356: 2381–2387.
3. Arraga-Alvarado CM, Qurollo BA, Parra OC, Berrueta MA, Hegarty BC, Breitschwerdt EB, 2014. Molecular evidence of *Anaplasma platys* infection in two women from Venezuela. *Am J Trop Med Hyg* 91: 1161–1165.
4. Allison RW, Little SE, 2013. Diagnosis of rickettsial diseases in dogs and cats. *Vet Clin Pathol* 42: 127–144.
5. de Castro MB, Machado RZ, de Aquino LP, Alessi AC, Costa MT, 2004. Experimental acute canine monocytic ehrlichiosis:

- clinicopathological and immunopathological findings. *Vet Parasitol* 119: 73–86.
6. Vinasco J, Li O, Alvarado A, Diaz D, Hoyos L, Tabachi L, Sirigireddy K, Ferguson C, Moro MH, 2007. Molecular evidence of a new strain of *Ehrlichia canis* from South America. *J Clin Microbiol* 45: 2716–2719.
 7. Gray J, Dantas-Torres F, Estrada-Pena A, Levin M, 2013. Systematics and ecology of the brown dog tick, *Rhipicephalus sanguineus*. *Ticks Tick Borne Dis* 4: 171–180.
 8. Uspensky I, Ioffe-Uspensky I, 2002. The dog factor in brown dog tick *Rhipicephalus sanguineus* (Acari: Ixodidae) infestations in and near human dwellings. *Int J Med Microbiol* 291 (Suppl 33): 156–163.
 9. Eiras DF, Craviotto MB, Vezzani D, Eyal O, Baneth G, 2013. First description of natural *Ehrlichia canis* and *Anaplasma platys* infections in dogs from Argentina. *Comp Immunol Microbiol Infect Dis* 36: 169–173.
 10. Vieira RF et al., 2011. Ehrlichiosis in Brazil. *Rev Bras Parasitol* 20: 1–12.
 11. McCown ME, Monterroso VH, Cardona W, 2014. Surveillance for *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, and *Dirofilaria immitis* in dogs from three cities in Colombia. *J Spec Oper Med* 14: 86–90.
 12. Diniz PP et al., 2013. Infection of domestic dogs in Peru by zoonotic *Bartonella* species: a cross-sectional prevalence study of 219 asymptomatic dogs. *PLoS Negl Trop Dis* 7: e2393.
 13. Huerto-Medina E, Damaso-Mata B, 2015. Factors associated with *Ehrlichia canis* infection in dogs infested with ticks from Huanuco, Peru. *Rev Peru Med Exp Salud Publica* 32: 756–760.
 14. Diniz PPVP, Schwartz DS, De Moraes HSA, Breitschwerdt EB, 2007. Surveillance for zoonotic vector-borne infections using sick dogs from southeastern Brazil. *Vector Borne Zoonotic Dis* 7: 689–697.
 15. Perez M, Rikihisa Y, Wen B, 1996. *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. *J Clin Microbiol* 34: 2133–2139.
 16. Unver A, Perez M, Orellana N, Huang H, Rikihisa Y, 2001. Molecular and antigenic comparison of *Ehrlichia canis* isolates from dogs, ticks, and a human in Venezuela. *J Clin Microbiol* 39: 2788–2793.
 17. Moro PL, Shah J, Li O, Gilman RH, Harris N, Moro MH, 2009. Short report: serologic evidence of human ehrlichiosis in Peru. *Am J Trop Med Hyg* 80: 242–244.
 18. Aguiar DM et al., 2013. Genetic diversity of *Ehrlichia canis* in Brazil. *Vet Microbiol* 164: 315–321.
 19. Harrus S, Waner T, 2011. Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): an overview. *Vet J* 187: 292–296.
 20. Aguiar DM, Zhang X, Braga IA, Taques II, McBride JW, 2016. Detection of genotype-specific *Ehrlichia canis* exposure in Brazilian dogs by TRP36 peptide ELISA. *Ticks Tick Borne Dis* 7: 142–145.
 21. Aguiar DM et al., 2014. A novel *Ehrlichia* genotype strain distinguished by the TRP36 gene naturally infects cattle in Brazil and causes clinical manifestations associated with ehrlichiosis. *Ticks Tick Borne Dis* 5: 537–544.
 22. Bouza-Mora L, Dolz G, Solorzano-Morales A, Romero-Zuniga JJ, Salazar-Sanchez L, Labruna MB, Aguiar DM, 2017. Novel genotype of *Ehrlichia canis* detected in samples of human blood bank donors in Costa Rica. *Ticks Tick Borne Dis* 8: 36–40.
 23. Diniz PP et al., 2010. High prevalence of tick-borne pathogens in dogs from an Indian reservation in northeastern Arizona. *Vector Borne Zoonotic Dis* 10: 117–123.
 24. INEI, 2007. *Instituto Nacional de Estadística e Informática, Censo Nacionales 2007*. Lima, Peru: Presidencia del Consejo de Ministros.
 25. Barber RM et al., 2010. Evaluation of brain tissue or cerebrospinal fluid with broadly reactive polymerase chain reaction for *Ehrlichia*, *Anaplasma*, spotted fever group *Rickettsia*, *Bartonella*, and *Borrelia* species in canine neurological diseases (109 cases). *J Vet Intern Med* 24: 372–378.
 26. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *J Mol Biol* 215: 403–410.
 27. Kimura M, 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111–120.
 28. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739.
 29. Edgar RC, 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32: 1792–1797.
 30. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ, 2009. Jalview version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25: 1189–1191.
 31. Shenkin PS, Erman B, Mastrandrea LD, 1991. Information-theoretical entropy as a measure of sequence variability. *Proteins* 11: 297–313.
 32. Jari Oksanen et al., 2017. *Ordination Methods, Diversity Analysis and Other Functions for Community and Vegetation Ecologists*. Available at: <https://cran.r-project.org/web/packages/vegan/index.html>. Accessed June 1, 2017.
 33. Wickham H, 2016. *Use R! ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer.
 34. R Core Team, 2017. *R: A language and Environment for Statistical Computing*. Available at: <http://www.R-project.org>. Accessed June 1, 2017.
 35. Perez M, Bodor M, Zhang C, Xiong Q, Rikihisa Y, 2006. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. *Ann N Y Acad Sci* 1078: 110–117.
 36. Bronson E, Emmons LH, Murray S, Dubovi EJ, Deem SL, 2008. Serosurvey of pathogens in domestic dogs on the border of Noel Kempff Mercado National Park, Bolivia. *J Zoo Wildl Med* 39: 28–36.
 37. Lopez J, Abarca K, Mundaca MI, Caballero C, Valiente-Echeverria F, 2012. Molecular identification of *Ehrlichia canis* in a dog from Arica, Chile. *Rev Chilena Infectol* 29: 527–530.
 38. McCown ME, Opel T, Grzeszak B, 2013. Vector-borne disease surveillance in Puerto Rico: pathogen prevalence rates in canines? Implications for public health and the U.S. Military? Applying the one health concept. *J Spec Oper Med* 13: 59–63.
 39. Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR, 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and ‘HGE agent’ as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 51: 2145–2165.
 40. Walker DH, Dumler JS, 1996. Emergence of the ehrlichioses as human health problems. *Emerg Infect Dis* 2: 18–29.
 41. Sumner JW, Nicholson WL, Massung RF, 1997. PCR amplification and comparison of nucleotide sequences from the groESL heat shock operon of *Ehrlichia* species. *J Clin Microbiol* 35: 2087–2092.
 42. Hsieh YC, Lee CC, Tsang CL, Chung YT, 2010. Detection and characterization of four novel genotypes of *Ehrlichia canis* from dogs. *Vet Microbiol* 146: 70–75.
 43. McBride JW, Doyle CK, Zhang X, Cardenas AM, Popov VL, Nethery KA, Woods ME, 2007. Identification of a glycosylated *Ehrlichia canis* 19-kilodalton major immunoreactive protein with a species-specific serine-rich glycopeptide epitope. *Infect Immun* 75: 74–82.
 44. Cárdenas AM, Doyle CK, Zhang X, Nethery K, Corstvet RE, Walker DH, McBride JW, 2007. Enzyme-linked immunosorbent assay with conserved immunoreactive glycoproteins gp36 and gp19 has enhanced sensitivity and provides species-specific immunodiagnosis of *Ehrlichia canis* infection. *Clin Vaccine Immunol* 14: 123–128.
 45. Yu X-j, McBride JW, Walker DH, 2007. Restriction and expansion of *Ehrlichia* strain diversity. *Vet Parasitol* 143: 337–346.