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Short communication

Detection of genotype-specific *Ehrlichia canis* exposure in Brazilian dogs by TRP36 peptide ELISA

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ABSTRACT

We recently characterized a novel genotype of *Ehrlichia canis* based on the tandem repeat (TR) sequence of the *TRP36* gene in Brazil. The TR amino acid sequence of the Brazilian (Br) genotype (ASVVPEAE) was divergent from the previously described US genotype (TEDSVSAPA) of *E. canis*. In this study, we developed an ELISA based on TRP36 TR synthetic peptides from both Br and US *E. canis* TRP36 genotypes to serologically detect and distinguish infections caused by these genotypes. Sera from 30 Brazilian dogs naturally infected with *E. canis*, sera from dogs experimentally infected *E. canis* (Jake and Cuiabá #1 strains) and *E. chaffeensis* (Arkansas strain) and 12 seronegative *E. canis* dogs were evaluated. Fifteen naturally infected Brazilian dogs had antibodies that reacted with the US TRP36 ($n=9$) or Br TRP36 ($n=6$) only, and 13 dogs had antibodies that reacted with both TPR36 peptides suggesting that these dogs were exposed to both genotypes. Most dogs ($n=28$) had antibodies that reacted with the highly conserved *E. canis* TRP19 peptide; however, two dogs had antibodies to *E. canis* TRP19, but did not have TRP36 antibodies, raising the possibility that another novel TRP36 genotype is circulating in Brazil. Our results demonstrate that synthetic peptides based on the TR region of *E. canis* TRP36 can be used to serologically distinguish infections or identify coinfections by different genotypes, and to determine the seroprevalence of various *E. canis* genotypes in Brazil.

1. Introduction

Ehrlichia canis is the etiologic agent of the globally distributed tick-borne disease, canine monocytic ehrlichiosis (CME), a chronic and sometimes lethal disease of dogs (Zweygarth et al., 2014). Despite the worldwide distribution of *E. canis*, 16S rRNA gene sequences are 99.4–100% identical among isolates from dispersed countries and provide little information regarding the overall diversity of this organism. Immunoreactive proteins including the OMP-1 family, thiodisulfide oxidoreductase (Dsb), and the tandem repeat proteins (TRP) 19 and 140 have also been found to be conserved in geographically dispersed strains (Zhang et al., 2008; Yu et al., 2007; Aguiar et al., 2008; Kamani et al., 2013). However, differences in the *TRP36* gene have been reported, indicating substantial degree of *E. canis* diversity (Doyle et al., 2005; Zhang et al., 2008; Hsieh et al., 2010; Aguiar et al., 2013; Zweygarth et al., 2014).

The TRP19 and TRP36 of *E. canis* are major immunoreactive proteins and primary targets of host antibody response during infection. The sensitivities and specificities of these proteins for species-specific immunodiagnosis have been determined using recombinant proteins and synthetic peptide based assays (Doyle et al., 2006; Cárdenas et al., 2007; McBride et al., 2007). The TRP19 is highly conserved among the known *E. canis* strains (McBride et al., 2007; Zhang et al., 2008). Conversely, variability in the TRP36 gene among isolates from the US, Israel, Taiwan, and Brazil has indicated that this gene can be utilized to define *E. canis* strain diversity since a variable amino acid tandem repeat sequences have been reported. For example, the TR amino acid sequence of the US TRP36 has been detected on different continents (Doyle et al., 2005; Hsieh et al., 2010; Kamani et al., 2013; Zweygarth et al., 2014), while the different TR amino acid sequences have been reported in Israel and Brazil (Zhang et al., 2008; Aguiar et al., 2013).

Major antibody epitopes have been characterized in the tandem repeat regions of TRPs from *E. canis* and *E. chaffeensis*, and these

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epitopes are molecularly distinct enabling detection of species-specific antibodies that allow serologic differentiation of the infecting agent (Doyle et al., 2006; McBride et al., 2007; Luo et al., 2008). In this study, we developed an ELISA-based peptide assay to detect and distinguish *E. canis* infections by two defined TRP36 genotypes in Brazil, and provide serologic evidence of genotype-specific infections as well as coinfections or multiple infections with both TRP36 genotypes.

2. Materials and methods

2.1. Dog sera

Sera from 30 dogs naturally infected with *E. canis* were obtained during serological surveys in the Brazilian States of Rondonia, Pará, Paraná, São Paulo and Mato Grosso, which antibody status to *E. canis* was previously determined by IFA (titers ranging from 160 to 327,680) (Aguiar et al., 2007; Melo et al., 2011). *E. canis* was previously isolated and molecularly characterized from six of these dogs (MN#24, Cba#1, Cba#16, Ld#1, PP#14 and BL#6) (Aguiar et al., 2013). Positive control sera from dogs experimentally infected with *E. canis* US TRP36 genotype (Jake strain), *E. canis* Brazilian TRP36 genotype (Br TRP36, Cba #1 strain) and *E. chaffeensis* (Arkansas strain) (dogs #2995, #B2015 and #2251 respectively) and sera from 12 dogs from the US and Brazil that did not have *E. canis* antibodies determined by IFA were also included in the experiment as positive and negative controls.

2.2. Synthetic peptides and ELISA

Peptides corresponding to the repeat regions of TRP36 from US *E. canis* strain (18-mer, TEDSVSAPATEDSVSAPA) (Doyle et al., 2006), the Brazilian *E. canis* strain (24-mer, ASVVPEAEASVVPEAEASVVPEAE) (Aguiar et al., 2013) and the TRP19 *E. canis* epitope-containing region (24-mer, HFTGPTF-SEVNLSEEEKMELQEVS) (McBride et al., 2007) were synthetized (Bio-Synthesis Inc., Lewisville, TX). A peptide corresponding to the TRP36 C-terminal region from the *E. canis* Israeli strain (IS36-C-V, 15-mer, NPTGLKFLDLYTQLTL) (Zhang et al., 2008) was used as a negative peptide control. All peptides (lyophilized) were resuspended in molecular grade water at 1 mg/mL.

ELISA plates (MaxiSorp; Nunc, Roskilde, Denamark) were coated (1.0 μg/well) with respective synthetic peptides suspended in phosphate-buffered saline (pH 7.4) and the assay was performed as previously described (Luo et al., 2010). The color development was determined on a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA), and data were analyzed by using SoftMax Pro v4.0 (Molecular Devices). Optical density (OD; A_{650}) readings represent the mean for three wells (±standard deviations) after subtracting the OD value of the non-reactive control peptide (IS36-C-V). All sera IFA negative for *E. canis* and the *E. chaffeensis*-infected dog had mean OD readings of <0.020 and <0.100, respectively; therefore a positive sample threshold was set at >0.300 OD units above the negative control absorbance. Specificity value for the ELISA was calculated according to Thrusfield (2007) and the confidence interval of 95% were calculated for the results of serum samples of naturally infected dogs by the EpiInfo 7.0 software for windows.

3. Results

All Brazilian dog samples had antibodies that reacted against at least one *E. canis* peptide. The OD values of naturally *E. canis* infected dogs are shown in Table 1. Nineteen (63.3%, 95% IC: 43.8–80.0%) sera had antibodies specific for the Br TRP36 peptide, 22 (73.3%, 95% IC: 54.1–87.7%) dogs had antibody that reacted with US TRP36 peptide, and 28 (93.3%, 95% IC: 78.0–99.2%) dogs had antibody to the TRP19 peptide. Six (20%) dogs had antibodies to the Br TRP36 peptide, but not the US TRP36, and 9 (30%) dogs (30%) had antibodies that reacted with the US TRP36 peptide, but not the Br TRP36 peptide. Antibodies that reacted with both TRP36 peptides were detected in 13 (43.3%) samples. One dog (61C4) had antibodies specific only for US TRP36 peptide, and the dog 49C1 had antibodies specific only for Br TRP36 peptide. The dogs 82C2 and R69 had antibodies against the TRP19 peptide, but not for either Br or US TRP36 peptides. The mean OD value of Br TRP36 peptides of positive dogs was 1.630, US TRP36 positive dogs was 1.994 and TRP19 positive dogs was 1.705. The mean OD value of IS36-C peptides was 0.101. The results of dogs naturally infected with *E. canis* in Brazil were shown in Fig. 1.

Table 1
Reactivity of synthetic peptides from TRP36 (Brazilian and US genotype) and TRP19 with antibodies in sera of dogs naturally infected with *E. canis* by ELISA.

Dog	IFA titers	Optical density (OD; A_{650})[a]		
		TRP19	Br TRP36	US TRP36
#22	20,480	0.798	0.144	2.295
#37	20,480	2.040	0.138	2.712
42C1	20,480	0.571	0.191	1.818
45C1	20,480	0.544	0.002	2.576
49C1	20,480	0.222	0.382	0.020
61C4	1280	0.039	0.080	0.828
82C2	1280	0.542	0.070	0.079
#93	5120	0.332	0.116	0.781
#107	10,240	0.598	1.837	0,142
U#4	2560	2.509	0.653	1.964
U#8	2560	3.015	0.241	1.580
U#37	2560	2.139	0.161	2.849
U#45	160	1.828	0.364	1.793
U#72	20,480	3.258	2.864	2.752
R#49	327,680	2.861	0.347	1.768
R#55	327,680	2.689	2.261	2.060
R#57	40,960	2.721	1.876	2.263
R#62	81,920	0.840	1.656	0.169
R#69	1280	0.579	0.111	0.038
Dani	10,240	1.870	1.739	2.829
SPT	2560	1.876	0.003	0.407
BL#1	40,690	0.913	3.082	1.748
BL#3	640	0.300	1.112	0.248
BL#9	163,840	1.365	2.183	2.011
MN#24	81,920	2.269	1.955	2.081
Cba #1	81,920	2.660	2.326	1.049
Cba#16	10,240	0.385	0.369	2.499
LD#1	10,240	2.702	0.311	0.160
PP#14	640	2.869	3.698	3.202
BL#6	2560	2.671	2.007	0.009

[a] The OD readings represent the means for three wells with the OD of the control (IS36C-V) wells subtracted.

The synthetic peptides exhibited 100% specificity in the ELISA (Fig. 2). The results were compared to the results of IFA with sera from healthy (sera negative) dogs and US TRP36 *E. canis* (#2995), Br TRP36 *E. canis* (#2015) and *E. chaffeensis* (#2551) experimentally infected dogs. None of the IFA-negative dogs reacted positively with synthetic peptides. The serum from the US TRP36 *E. canis*-infected dog had a titer of 10,240 by IFA, and reacted against US TRP36 peptide with mean OD of 0.963 and against TRP19 with mean OD of 1.681. The mean values for Br TRP36 and IS36-C-V were 0.171 and 0.097 respectively. The serum of Br TRP36 *E. canis* infected dog had a titer of 20,480 by IFA and reacted against Br TRP36 peptide with mean OD of 2.113 and against TRP19 with mean OD of 2.097. The mean values for US TRP36 and IS36-C-V were 0.055 and 0.063 respectively. The sera of *E. chaffeensis* infected dogs had a titer of 10,240 by IFA (DH82 cells infected with *E. chaffeensis*) and showed mean OD values of 0.091 for Br TRP36, 0.077 for US TRP36, 0.091 for TRP19 and 0.069 for IS36-C-V peptides. All negative control sera (IFA < 64) were negative for all peptides and the mean ELISA OD value was 0.053.