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Transcriptional Analysis of *p30* Major Outer Membrane Protein Genes of *Ehrlichia canis* in Naturally Infected Ticks and Sequence Analysis of *p30-10* of *E. canis* from Diverse Geographic Regions

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***Rhipicephalus sanguineus* ticks transmit *Ehrlichia canis*, the etiologic agent of canine ehrlichiosis. In experimentally infected ticks, only *p30-10* transcript was detected among 22 *p30* paralogs encoding immunodominant major outer membrane P30 proteins of *E. canis*. The present study revealed transcription of *p30-10* by *E. canis* in naturally infected ticks and sequence conservation of *p30-10* genes for *E. canis* from diverse geographic regions.**

Ehrlichia canis is the etiologic agent of canine monocytic ehrlichiosis (CME) (5, 9). CME has been recognized as a significant canine disease worldwide and at a higher frequency in tropical and subtropical regions (5, 9). *E. canis* is primarily transmitted to dogs by nymph and adult stages of the brown dog tick *Rhipicephalus sanguineus* (2). CME develops in three consecutive phases: acute, subclinical, and chronic. The acute phase lasts 2 to 4 weeks and is characterized by fever, oculonasal discharge, depression, anorexia, weight loss, and lymphadenomegaly with laboratory findings of thrombocytopenia, leukopenia, mild anemia, hypergammaglobulinemia, and increases in serum aminotransferase activities and acute-phase proteins (5, 11). In the subclinical phase of persistent ehrlichial infection, dogs appear normal clinically but abnormalities in laboratory findings may persist. Mild thrombocytopenia and hypergammaglobulinemia may be seen. In the chronic phase, in addition to the clinical signs and laboratory findings of the acute phase, hemorrhages, epistaxis, edema, and hypotensive shock may occur, which are often exacerbated by superinfection with other organisms (5, 9). Without or often even with antibiotic treatment, dogs infected with *E. canis* remain infected (3, 18). There is no vaccine available for this disease.

E. canis 30-kDa proteins (P30s) are immunodominant major outer membrane proteins that are strongly recognized by sera from naturally and experimentally infected dogs (3, 6, 10). P30s are encoded by a multigene family (6, 7). We found that *p30-10* is the only *p30* transcribed by the *E. canis* Oklahoma type strain in experimentally infected *R. sanguineus* ticks, whereas in the blood of experimentally infected dogs, *p30-10* is transcribed with eight other *p30* paralogs during the 2-month period after infection (15). However, transcriptional analysis has so far been limited to a single laboratory-maintained strain (Oklahoma strain) and the sequence of the *p30-10* gene was determined only in this strain. It is important to examine transcriptional profiles of *E. canis* in naturally infected ticks and learn the genetic diversity of *E. canis* strains infecting dogs to design an effective vaccine that will prevent the transmission of *E. canis* by ticks in the future. Therefore, the present study investigated the expression of *p30-10*, *p30*, and *p30-3* paralogs by *E. canis* in naturally infected ticks and analyzed the complete base sequence of *p30-10* genes of *E. canis* in the blood of naturally infected dogs from diverse geographic regions.

A total of 226 *R. sanguineus* ticks were removed from 59 dogs in 23 different veterinary clinics in Phoenix, Ariz., from October 2001 to March 2002 in a random survey. One to 18 live nymph and/or adult engorged and/or unengorged ticks from each dog were divided into 59 groups. Ticks were kept at room temperature at 95 to 100% relative humidity with a 12-h photoperiod for more than 10 days to digest blood meals prior to specimen preparation. The ticks were ground with a pellet pestle in RLT buffer (Qiagen Inc., Valencia, Calif.), and RNA was extracted from the tick pools by using an RNeasy Protect kit (Qiagen). RNA was treated with DNase and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen, San Diego, Calif.). To find *E. canis*-infected ticks, cDNA was used as the template for *E. canis*-specific nested PCR amplification of 16S rRNA as previously described (18). Expression of *p30-10*, *p30*, and *p30-3* was analyzed in five *E. canis* 16S rRNA-positive tick pool samples. Template cDNA was subjected to PCR by using 10 pmol of *p30-*, *p30-3-*, and *p30-10*-specific primer pairs (15).

Peripheral blood mononuclear cells were isolated from dog blood samples as described elsewhere (1). Dog blood samples from different clinics were sent to our laboratory for diagnosis. Four *E. canis*-specific PCR (18)-positive DNA samples from blood of dogs in Arizona, California, New Mexico, and Hawaii were used for this study. An *E. canis* VDE strain from a naturally infected dog in Venezuela was isolated in our laboratory (16). The strain was cultivated in the DH82 dog macrophage cell line as previously described (16). A QIAmp DNA blood mini kit (Qiagen) was used for extraction of DNA from infected DH82 cells and dog peripheral blood mononuclear cells. To amplify the entire *p30-10* gene, several degenerate

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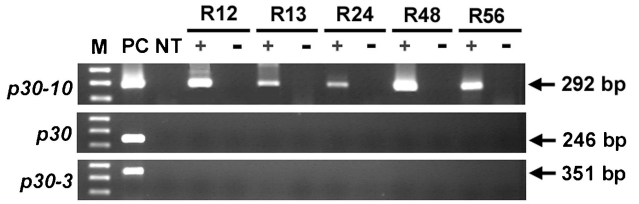

FIG. 1. mRNA expression of the *p30-10*, *p30*, and *p30-3* genes of *E. canis* in naturally infected *R. sanguineus* tick groups. Total RNA was extracted and subjected to reverse transcription-PCR. The amplified products were separated on agarose gel containing ethidium bromide. Lanes: M, molecular size marker (1 Kb Plus DNA Ladder; Invitrogen); PC, product amplified by PCR with DNA from *E. canis* Oklahoma as a template and respective primer pairs, used as a positive control for PCR and as a determinant of amplicon size on the gel; NT, no template as a negative control. Sizes of amplified products are indicated on the right. Symbols: +, reverse transcription-PCR with reverse transcriptase; −, without reverse transcriptase. Tick groups: R12 (one engorged female), R13 (two engorged females, one unengorged male), R24 (one male, one semiengorged female), R48 (two males, one semiengorged female), and R56 (two unengorged females).

primers were designed on the basis of a comparison of corresponding regions of *E. chaffeensis* and *E. canis* (7), since levels of conservation of intergenic regions flanking the *p30-10* gene were unknown among strains of *E. canis*. After experimental evaluation of a combination of these primers, a DP30-10F1 (TCTTTTATAAAAGGTTTATTAACATG)–DP30-10R1 (AAACTTGAAATAACTTCACATTTTN) combination was selected for amplification of the *p30-10* gene. Ten microliters of each template sample was amplified in a 50-μl reaction mixture containing 5 μl of 10× PCR buffer (10 mM Tris-HCl [pH 8.4]–50 mM KCl), 5 μl of 50 mM $MgCl_2$, 1 μl of 10 mM deoxynucleoside triphosphate mixture, 2.5 U of *Taq* DNA polymerase (Invitrogen), and 10 pmol of primers DP30-10F1 and DP30-10R1. Amplification was performed in a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) with a three-step program (5 min of denaturation at 94°C; 40 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C; and a final extension at 72°C for 10 min). The 954-bp PCR products were extracted from the gel, and DNA was cloned into the PCRII TA cloning vector (Invitrogen). Three clones were sequenced for each sample and each strand.

We focused on ticks attached to dogs, since this population is more likely to transmit *E. canis* than are ticks field collected by flagging. CME is enzootic in Phoenix, Ariz. (18). Five (8.5%) of 59 groups of *R. sanguineus* ticks (a total of 11 ticks) were found to be positive for *E. canis* 16S rRNA. Three groups of ticks removed from dogs in Venezuela were all infected with *E. canis* (16). Murphy et al. reported that *E. canis* PCR was positive in two (33.3%) of six groups of *R. sanguineus* ticks collected from PCR-positive dogs (4). There is no other report on the *E. canis* infection rate of field-collected ticks. All five groups of naturally infected ticks, including males and females, engorged or unengorged, expressed *p30-10* (Fig. 1). The two other most prevalent *p30* transcripts found in experimentally infected dog blood, *p30* and *p30-3* (15), were not detected in the tick specimens (Fig. 1).

The full-length *p30-10* genes of *E. canis* from four dog blood DNA samples from several different states of the United States and one VDE DNA culture from Venezuela were amplified, and 954-bp PCR products were sequenced. These sequences were compared to the Oklahoma type strain *p30-10* gene sequence. Five *p30-10* sequences were 100% identical to the sequence from *E. canis* Oklahoma. The Hawaii strain contained three base differences resulting in two deduced amino acid changes (Table 1).

TABLE 1. Nucleotide and deduced amino acid differences among *p30-10* genes of *E. canis* in the blood of dogs from various geographic regions

Source[b]	Nucleotide at position:			Amino acid at position:	
	465	564	759	155	253
Oklahoma	T	A	T	D	N
Arizona[c]	—[a]	—	—	—	—
California[c]	—	—	—	—	—
New Mexico[c]	—	—	—	—	—
Hawaii[c]	G	G	G	E	K
Venezuela[d]	—	—	—	—	—

[a] —, Position conserved relative to the sequence of the Oklahoma strain.
[b] The GenBank accession numbers for the new *p30-10* genes of *E. canis* from dogs in Arizona, California, Hawaii, New Mexico, and Venezuela are AF528511, AF528512, AF528513, AF528514, and AF528515, respectively.
[c] Dog blood sample.
[d] Culture isolate from dog blood.

Several arthropod-transmitted pathogens use antigenic variation for effective transmission and persistence in reservoir mammals (12–14, 17). Antigenic variation presents a challenge in the development of vaccines against vector-borne pathogens. P30s are immunodominant major surface antigens of *E. canis* encoded by a *p30* multigene family. Twenty-two *p30* paralogs have been described (7). *p30-10* is the only *p30* expressed in experimentally infected *R. sanguineus* ticks. It is expressed in the tick salivary glands and midguts of in both adult female and male ticks and nymphs prior to and after a blood meal, but several *p30* paralogs are expressed in dog blood (15). Like *E. canis*, *Borrelia burgdorferi* changes its outer surface during its alternating infections in ticks and mammals (14). OspA is abundantly expressed in *B. burgdorferi* in the guts of blood-feeding ticks. OspA is not expressed in mammals. The current licensed OspA-based Lyme disease vaccine prevents transmission of *B. burgdorferi* to mammals by neutralizing borreliae in the tick gut after the tick has taken a blood meal (8). We speculated, by analogy, that P30-10 might be a vaccine candidate for the prevention of *E. canis* transmission from ticks to dogs.

E. canis strain diversity appears to be limited, since 16S rRNA genes were only up to five bases different among the different strains compared (16). Antigenic diversity among different isolates is an important factor in the selection of vaccine candidates. It would be ideal if antigenic epitopes were conserved to protect against all strains. In the present study, *p30-10* sequences appeared to be highly conserved among the strains examined. These results suggest that P30-10 is a potential vaccine candidate for the prevention of tick transmission of *E. canis*, but this remains to be tested.

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