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Determination of the Nucleotide Sequences of Heat Shock Operon *groESL* and the Citrate Synthase Gene (*gltA*) of *Anaplasma* (*Ehrlichia*) *platys* for Phylogenetic and Diagnostic Studies

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The 1,670-bp nucleotide sequence of the heat shock operon *groESL* and the 1,236-bp sequence of the citrate synthase gene (*gltA*) of *Anaplasma* (*Ehrlichia*) *platys* were determined. The topology of the *groEL*- and *gltA*-based phylogenetic tree was similar to that derived from 16S rRNA gene analyses with distances. Both *groESL*- and *gltA*-based PCRs specific to *A. platys* were also developed based upon the alignment data.

Anaplasma (*Ehrlichia*) *platys* is a bacterial parasite of dog platelets that causes infectious cyclic thrombocytopenia (8). *A. platys* has been shown to be closely related to *Anaplasma marginale*, *Anaplasma centrale*, and *Anaplasma phagocytophila,* including the former human granulocytic ehrlichia (HGE) agents *Ehrlichia equi* and *Ehrlichia phagocytophila*, based on 16S rRNA gene sequences (7); however, little information is available regarding the natural history of the pathogen. The 16S rRNA gene had been the only known gene sequence of *A. platys* before the heat shock protein gene (*groEL*) was sequenced recently (21). A *groEL* sequence analysis supported the phylogenetic relationship between *A. platys* and related species. The *groESL* operon contains a spacer region between *groES* and *groEL* which is thought to be more divergent than the coding regions (18, 19). However, the nucleotide sequence of the spacer region of *A. platys* has not been studied yet. Thus, the nucleotide sequences of *groES* and the spacer region between *groES* and *groEL* were analyzed for additional phylogenetic characterization of *A. platys*. More recently, we sequenced the citrate synthase gene (*gltA*) of 13 species, including *Ehrlichia*, *Anaplasma*, and *Neorickettsia*, for phylogenetic analyses and found higher variation than for the 16S rRNA gene (10). The topology of the *gltA*-based phylogenetic tree confirmed the reorganization of genera in the families *Rickettsiaceae* and *Anaplsmataceae* reported recently (7). However, the *gltA* sequence of *A. platys* has yet to be determined. Thus, the nucleotide sequences of *A. platys gltA* were also analyzed to support the phylogenetic relationship of *A. platys* among related species. We also propose to use sequence data from *groESL* and *gltA* with greater differences among species to develop an *A. platys*-specific PCR method. New PCR primers to specifically detect *A. platys* fragments were developed based on the alignment data of these two genes for a diagnostic assay.

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The *A. platys* DNA analyzed in this study was supplied from a dog infected in Somieres, France (2). The dog had a history of a clotting disorder. At the time of bleeding, the platelet count was 256,000/μl, which was in the normal range, and *A. platys* was observed within 58% of platelets on a Giemsa-stained peripheral blood smear. To evaluate the species-specific PCR designed in this study, DNA from other strains of *A. platys* were used. DNA from a dog infected with *A. platys* in Venezuela was kindly provided by E. B. Breitschwerdt, North Carolina State University (17). DNA from a dog infected with *A. platys* in Okinawa, Japan, was also used (9).

For amplification of the *groESL* operon of *A. platys*, EEgro1F and EEgro2R were used to amplify an approximately 1,700-bp fragment with an annealing temperature of 55°C (3) (Table 1). The amplification products were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and sequenced. Five primers shown in Table 1 were used to complete the sequence of *groESL*. Fluorescence-labeled dideoxynucleotide technology was used for DNA sequencing reactions (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.). Samples were then sequenced using a Perkin-Elmer ABI Prism 377 automated DNA sequencer at the DNA Core Facility of the Center for Gene Research, Yamaguchi University.

The strategy for determining the *gltA* sequence was similar to that used in our previous report (10). A partial sequence of *A. platys gltA* was first determined using two sets of degenerate primers, F1b and EHR-778R, and F1b and HG1085R (10, 15) (Table 1). These primers were designed based upon the sequence of *gltA* of *A. phagocytophila*, *A. marginale*, and *A. centrale*. The amplification conditions were the same as in the previous study (10), with an annealing temperature of 53°C. The amplification products were purified and sequenced as described above. After a partial determination of the sequence, the unknown areas of the 3′ and 5′ ends of the gene were determined using the Universal Genome Walker kit (Clontech Laboratories, Palo Alto, Calif.). Briefly, genomic DNA was digested with *Eco*RV, *Dra*I, *Pvu*II, *Stu*I, and *Sca*I. DNA fragments were ligated with a Genome Walker adaptor,

TABLE 1. Oligonucleotide sequences of primers used in this study

Primer name	Oligonucleotide sequence (5′→3′)[a]	Reference
PCR amplification for *groESL*		
EEgro1F	5-GAG-AGA-TGC-TTA-TGG-TAA-GAC-3	1
EEgro2R	5-CAG-CGT-CGT-TCT-TAC-TAG-GAA-C-3	1
Sequence primers for *groESL*		
SQ3F	5-ATT-AGC-AAG-CCT-TAT-GGG-TC-3	This study
SQ4R	5-CTT-TAG-GCT-ATC-AAG-AGA-TG-3	This study
SQ4F	5-CAT-CTC-TTG-ATA-GCC-TAA-AG-3	This study
SQ5F	5-TCA-GTG-TGT-GAA-GGA-AGT-TG-3	This study
SQ6R	5-TGC-TTC-CTA-TGT-TCT-TAT-CG-3	This study
PCR amplification of *gltA* fragments		
F4b	5-CCG-GGT-TTT-ATG-TCT-ACT-GC-3	8
F1b	5-GAT-CAT-GAR-CAR-AAT-GCT-TC-3	This study
EHR-CS779R	5-GCN-CCM-CCA-TGM-GCT-CG-3	14
HG-1085R	5-ACT-ATA-CCK-GAG-TAA-AAG-TC-3	8
gltA-specific primer for the Genome Walker method		
PLATYS-SPF	5-CTG-CCG-GAA-CAG-AGC-TAT-TC-3	This study
PLATYS-SPR1	5-TCG-GAT-GAC-AGA-GCA-TAG-TG-3	This study
PLATYS-SPR2	5-TTG-CTC-GAA-CAC-TGT-GTC-TG-3	This study
A. platys-specific primers		
PLA-HS475F	5-AAG-GCG-AAA-GAA-GCA-GTC-TTA-3	This study
PLAT-HS1198R	5-CAT-AGT-CTG-AAG-TGG-AGG-AC-3	This study
PLA-CSM136F	5-TTG-CAA-AAA-GTA-AGC-GGA-GC-3	This study
PLA-CS1359R	5-AAC-CAC-AGG-CTT-ATG-ACA-AC-3	This study

[a] K, G or T; R, G or A; M, C or A; N, G, C, A, or T.

which had one blunt end and one end with a 5′ overhang. A ligation mixture of the adaptor and ehrlichial genomic DNA fragments was used as a template for PCR. This PCR was performed using an adaptor primer supplied by the manufacturer and *A. platys gltA*-specific primers to walk downstream on the DNA sequence (Table 1). For the amplification, the conditions were as in our previous report (10).

The sequences of *A. platys* and the registered sequences of other related species deposited in GenBank were analyzed for phylogenetic relationships. Multiple alignment analysis, the calculation of distance matrices, and the construction of phylogenetic trees were performed with the ClustalW program (20) version 1.8 in the DNA Data Bank of Japan (Mishima, Japan; http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html). The distance matrices for the aligned sequences with all gaps ignored were calculated using the Kimura two-parameter method (11), and the neighbor-joining method was used for constructing a phylogenetic tree (16). The stability of the tree obtained was estimated by bootstrap analysis for 100 replications using the same program. Tree figures were generated using the TreeView program, version 1.61 (14).

A primer set, forward primer PLA-HS475F and reverse primer PLA-HS1198R, was designed based upon the alignment data to specifically amplify an *A. platys groESL* fragment. Another set of primers, PLA-CSM136F and PLA-CS1359R, was also designed based upon the alignment data of *gltA*. PCR conditions were the same as described above but with an annealing temperature of 58°C and the use of 40 cycles. The specificity of the reaction was tested with DNA extracted from the three strains of *A. platys* and related species, including *A. phagocytophila* (formerly the HGE strain Webster) (J. S. Dumler), *E. equi* strain California (J. E. Madigan), *E. phagocytophila* strain 1602 (A. Garcia-Perez), *A. marginale* strain Florida (G. H. Palmer), *E. canis* strain Oklahoma (J. Dawson), *Wolbachia pipientis* (M. Taylor), and *Neorickettsia helminthoeca* (Y. Rikihisa). The sensitivities of both PCR systems were also examined using DNA from *A. platys* strain France. The DNA was diluted 10-fold from 1:1 to 1:10,000 with distilled water.

A 1,670-bp *groESL* fragment of *A. platys* was determined to contain 41 bp of the partial *groES*, 51 bp of the spacer region, and 1,577 bp of the *groEL* coding region. The *groESL* operon was compared with that of other *Anaplasma* and *Ehrlichia* bacteria reported previously and was found to be closely related to the operon of *A. phagocytophila* and *A. marginale*, with 81.4 and 78.8% identity, respectively. The level of similarity among *groESL* sequences was much lower than that for the 16S rRNA gene sequence in the same species (98.6% with *A. phagocytophila* and 96.1% with *A. marginale*). The spacer length of 51 nucleotides was similar to that for related *Anaplasma* species: 52 bp for *A. phagocytophila* and 47 bp for *A. marginale*. The percent identities of the nucleotide sequence in the spacer region of *A. platys* compared to that of *A. phagocytophila* and to that of A. *marginale* were 74.5 and 72.3%, respectively, revealing a greater degree of divergence than for the entire *groEL* coding region.

After the initial identification of the 955-bp partial sequence of *A. platys gltA* using the degenerate PCR strategy, the full-length open reading frame extending from the ATG start codon to the TAA stop codon was determined using the Genome Walker PCR method. The length of the *gltA* open reading frame was 1,236 bp and encoded a protein of 411 amino acids. The complete *gltA* sequence was compared with that of

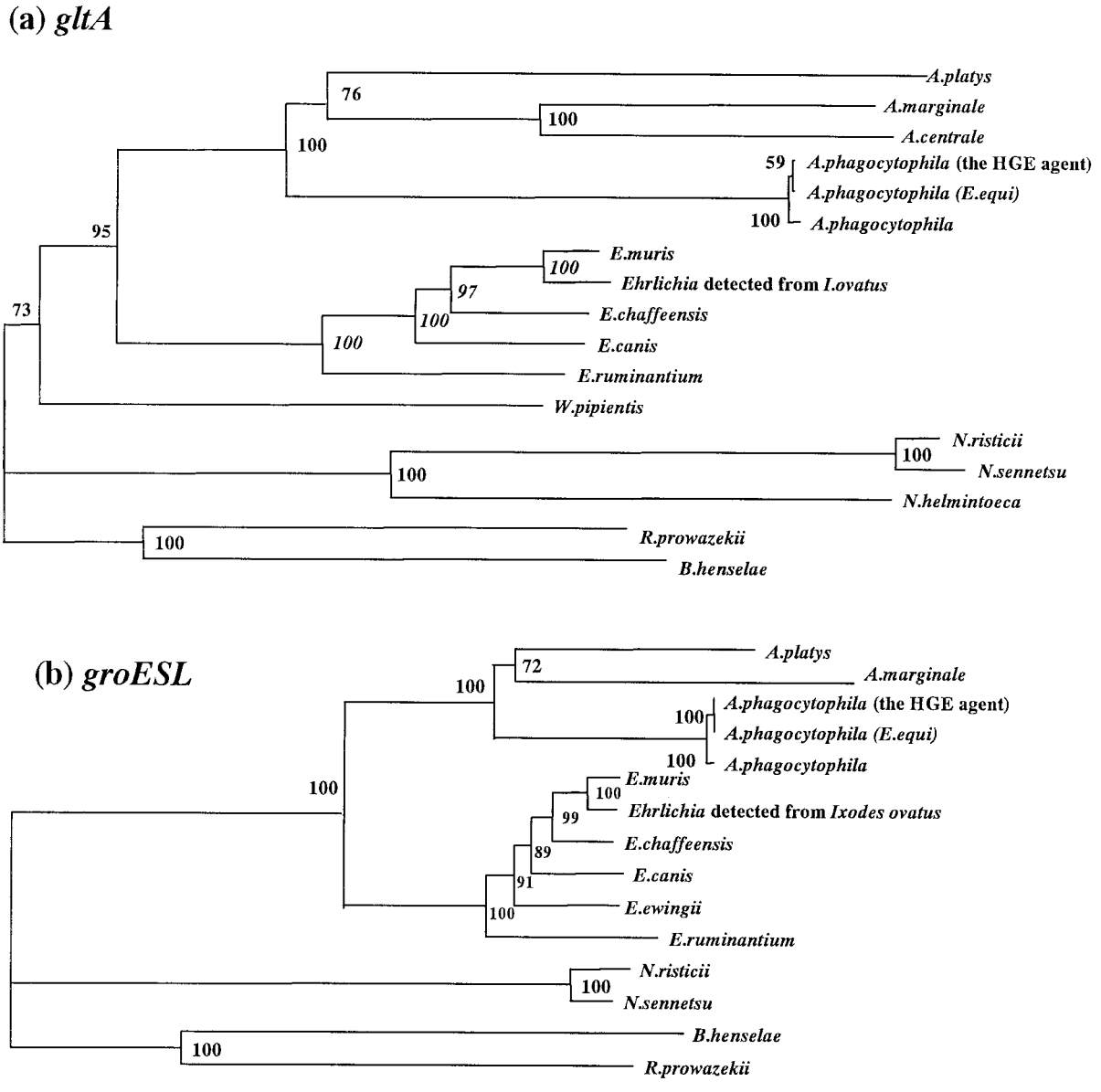

FIG. 1. Phylogenetic relationship of various *Anaplasma*, *Ehrlichia*, and *Neorickettsia* spp. based on the nucleotide sequences of *gltA* (a) and *groESL* (b) genes. The neighbor-joining method was used to construct the phylogenetic tree with the ClustalW program. The scale bar represents 10% divergence. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The GenBank accession numbers of the *groESL* sequences used to construct the phylogenetic tree and aligned data are as follows: *A. phagocytophila* (strain HGE agent), AF165812; *E. equi*, AF172162; *E. phagocytophila*, U96729; *A. marginale*, AF165812; *E. chaffeensis*, L1 0917; *E. canis*, U96731; *E. muris*, AF210459; *Ehrlichia* sp. detected from *I. ovatus*, AB032711; *Ehrlichia* (*Cowdria*) *ruminantium*, U13638; *Neorickettsia* (*Ehrlichia*) *risticii*, U96732; *Neorickettsia* (*Ehrlichia*) *sennetsu*, U88092; *Rickettsia prowazekii*, Y15783; *Bartonella henselae*, U96734. The GenBank accession numbers of the *gltA* sequences used for comparative analysis are as follows: *A. phagocytophila* (strain HGE agent), AF304136; *E. equi*, AF304137; *E. phagocytophila*, AF304138; *A. marginale*, AF304139; *A. centrale*, AF304141; *E. chaffeensis*, AF304142; *E. canis*, AF304143; *E. muris*, AF304144; *Ehrlichia* sp. detected from *I. ovatus*, AF304145; *E.* (*C.*) *ruminantium*, AF304146; *N.* (*E.*) *risticii*, AF304147; *N.* (*E.*) *sennetsu*, AF304148: *N. helminthoeca*, AF304149; *R. prowazekii*, M17149; *B. henselae*, U78514.

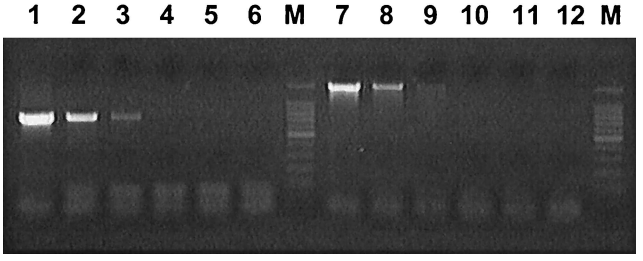

FIG. 2. The sensitivities of the *A. platys*-specific PCR based upon the *groESL* (lanes 1 to 6) and *gltA* (lanes 7 to 12) genes were evaluated. DNA equivalent to that from 375, 37.5, 3.75, 0.375, and 0.0375 infected platelets was used as a template for the amplicons demonstrated in lanes 1 and 7, 2 and 8, 3 and 9, 4 and 10, and 5 and 11, respectively. Distilled water was used in lanes 6 and 12 as a negative control. Lane M, 100-bp DNA ladder.

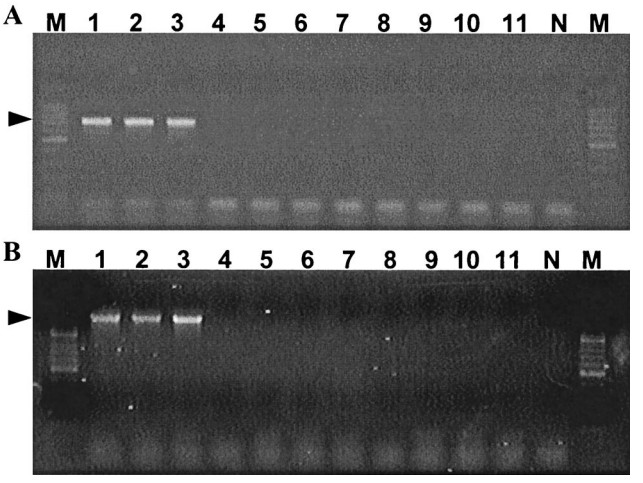

FIG. 3. *A. platys*-specific PCRs based upon the *groESL* (A) and *gltA* (B) genes were evaluated for their specificity with DNA from *A. platys* strains from Somieres, France (lane 1), Okinawa, Japan (lane 2), and Venezuela (lane 3), and from *A. marginale* (lane 4), *A. centrale* (lane 5), *A. phagocytophila* strain HGE agent (lane 6), *E. equi* (lane 7), *E. phagocytophila* (lane 8), *W. pipientis* (lane 9), *E. canis* (lane 10), and *N. helminthoeca* (lane 11), with distilled water as a negative control (lane N). Positive bands (arrows) were observed only with DNA of the three strains of *A. platys* in both *groESL*- and *gltA*-based PCRs.

other *Anaplasma* and *Ehrlichia* bacteria reported previously (10) and found to be closely related to those of *A. phagocytophila* and *A. marginale*, with 62.7 and 63.2% identity, respectively. The level of similarity among ehrlichial *gltA* was much lower than that for the 16S rRNA gene sequence in the same species. Thus, the sequence of *gltA* is much more variable among these species than is *groESL*. The length of the *gltA* sequence of *A. platys* (1,236 bp) is the same as that of *A. phagocytophila* but slightly shorter than that of *A. marginale* and *A. centrale* (1,254 bp).

In topology, the *gltA*-based phylogenetic tree (Fig. 1a) was very similar to the tree derived from analysis of the 16S rRNA gene analyses (3) and the *groEL*-based tree reported previously (21). However, the trees constructed from *gltA* and *groESL* nucleotide sequences showed more distance than the 16S rRNA-based trees. These findings also support the use of *gltA*-based and *groESL*-based comparisons in determining the phylogeny of *Anaplasma*, *Ehrlichia*, and *Neorickettsia* agents and strengthen the 16S rRNA- and *groESL*-based phylogeny reported recently (7). There have been reports of *A. platys*-like organisms identified based on 16S rRNA analysis, including bacteria from white-tailed deer in North America (6) and from a cow in South Africa (7). It would be interest to determine the phylogenetic position of these agents by analyzing the *gltA* and *groESL* gene sequences.

PCR is a powerful tool for epidemiological or diagnostic purposes because of its high sensitivity and specificity; however, there are few molecular tools available for *A. platys*. All PCR assays reported previously were developed based upon 16S rRNA gene sequences (4, 5, 9, 12, 13). In the present study, new PCR primers were designed to amplify *A. platys* DNA specifically and were based upon the alignment data of *groESL* and *gltA* of *A. platys* and closely related *Anaplasma* and *Ehrlichia* species. The PCR produced a fragment of 724 bp from *groESL* and 1,459 bp from *gltA* with the DNA from *A. platys* strain France (Fig. 2). The sensitivities of the PCR systems were examined by using diluted DNA. Both *groESL*- and *gltA*-based PCRs detected DNA diluted 1:100 (Fig. 2), similar to the 16S rRNA-based method (9). As 5 μl of the original DNA solution contained genomic DNA from approximately 375 platelets infected with *A. platys*, both PCR systems can detect DNA from 3.75 infected platelets in a reaction mixture. The specificity of both systems was also examined using DNA of three *A. platys* strains from different geographic locations, France, Japan, and Venezuela, and using DNA from related species, including *A. phagocytophila* and *A. marginale*. Figure 3 shows that both PCR systems were specific for *A. platys*. Furthermore, the *gltA*-based PCR amplifies the whole *gltA* sequence of *A. platys*, which contains both start and stop codons. As the sequences of *gltA* and *groESL* have greater variation than the sequence of 16S rRNA, the sequence analysis of PCR products may supply useful information for phylogenic studies of the agents. Our findings suggest that both *groESL*- and *gltA*-based PCRs are useful for the specific detection of *A. platys* DNA, and they would be additional molecular tools for both phylogenetic study and diagnosis in veterinary medicine.

Nucleotide sequence accession numbers. The nucleotide sequences of *groESL* and *gltA* of *A. platys* strain France determined herein have been deposited in the GenBank database under the accession numbers AY044161 and AB058782, respectively.

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