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Equine and Canine *Anaplasma phagocytophilum* Strains Isolated on the Island of Sardinia (Italy) Are Phylogenetically Related to Pathogenic Strains from the United States

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The presence of *Anaplasma phagocytophilum*, a tick-transmitted zoonotic pathogen, was investigated in Sardinia using a molecular approach. Phylogenetic analysis revealed that Sardinian strains are genetically distinct from the two lineages previously described in Europe and are closely related to strains isolated in different areas of the United States.

Anaplasma phagocytophilum (formerly *Ehrlichia phagocytophila*), a tick-transmitted bacterium able to infect bone marrow-derived cell lines of several animal species, including humans, is the causative agent of a tick-borne fever of ruminants, as well as equine granulocytic anaplasmosis, canine granulocytic anaplasmosis, and human granulocytic anaplasmosis (HGA) (5).

To date, only two HGA cases have been reported in continental Italy (14). However, serological and molecular findings demonstrated the occurrence of *A. phagocytophilum* infections in dogs and *Ixodes ricinus* ticks in this country (9, 15, 17). An average rate of 13.4 tick bite-related disease cases/year/100,000 inhabitants was reported for the island of Sardinia between 1992 and 1996, compared to the national average value of 2.1 cases/year/100,000 inhabitants. Moreover, 117 cases of tick bite-related disease whose etiology remains obscure were registered between 1995 and 2002.

Indirect immunofluorescence is commonly used for *A. phagocytophilum* diagnosis, but it should take into account coinfection, cross-reactivity among different pathogens (5, 7, 13, 16), and seroconversion (2). The diagnostic strategy has therefore focused on molecular methods based on PCR approaches (10). PCR methods should be highly specific, and a recently developed PCR assay for amplification of the *A. phagocytophilum* 16S rRNA gene resulted in coamplification of the *Anaplasma platys* gene (6).

In order to establish the presence of *A. phagocytophilum* in an area of Sardinia characterized by a high incidence of tick bite-related diseases in dogs and humans and to compare Sardinian strains with American and European isolates, symptomatic dogs and horses were tested by a molecular method able to distinguish *A. phagocytophilum* from *A. platys*. To accomplish this, a strategy based on *groEL* PCR-restriction fragment length polymorphism and sequence analysis, an alternative to the 16S rRNA-based PCR method, was used.

Between 2002 and 2004, veterinarians based in central Sardinia were instructed to collect blood samples when a suspected case of tick bite-related disease was found in their clinics. A total of 40 blood samples were collected from 40 dogs showing tick infestation and symptoms consistent with tick bite-related disease, such as fever, anorexia, depression, anemia, myalgia, and a reluctance to move. Clinical hematology indicated thrombocytopenia and anemia. Moreover, 20 blood samples were obtained from 20 horses showing tick infestation, hyperthermia, anemia, anorexia, jaundice, myalgia, and a reluctance to move.

Genomic DNA was extracted from the buffy coat obtained by centrifugation of 2 to 4 ml of blood as previously described (12). *A. phagocytophilum* genomic DNA (NCH-1 strain) was extracted from FA substrate slides (VMRD, Inc.) and used as a positive control in canine granulocytic anaplasmosis-specific PCRs. A DNA preparation of a southern Italy *A. platys* isolate was used as a positive control in infectious cyclic thrombocytopenia-specific PCRs. Based on an alignment of the heat shock protein *groEL* gene sequences available in the GenBank database for species belonging to the family *Anaplasmataceae*, four primers were generated and used in combination in two heminested PCRs. Primers EphplgroEL(569)F (ATGGTATG CAGTTTGATCGC) and EphplgroEL(1193)R (TCTACTCT GTCTTTGCGTTC) anneal to nucleotide strings conserved in *A. phagocytophilum* and *A. platys* and were designed to selectively amplify, in a first PCR round, 624 bp of the *groEL* gene of both species from clinical samples. EphgroEL(1142)R (TTG AGTACAGCAACACCACCGGAA) and EplgroEL(1084)R (C ATAGTCTGAAGTGGAGGAC) are specific for *A. phagocytophilum* and *A. platys*, respectively, and were alternatively coupled with primer EphplgroEL(569)F in two heminested PCRs in which the amplification products obtained from the first PCR round were used as DNA templates. The final 50-μl PCR mixture from the first PCR round contained 5 μl of the DNA extract, 1 μM primer EphplgroEL(569)F, 1 μM primer EphplgroEL(1142)R, and HotMaster *Taq* DNA polymerase (5

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TABLE 1. Identity of the 39 *groEL* sequence types used as OTUs in phylogenetic analyses

groEL sequence type	No.	Location(s) and host species	GenBank accession no.	Nucleotides at variable positions[b]																						
				593	613	660	662	674	700	708	726	732	774	840	849	933	939	969	972	979	996	1002	1022	1098	1113
Strains with proven pathogenicity[a]																										
1-HGA-Europe	5	Slovenia (human), Sweden (horse), Germany (tick), Switzerland (horse), Germany (horse)	AF033101, AY529490, AY281849, U96735, AF482760	A	A	T	T	A	C	A	T	A	G	C	G	C	T	A	C	A	G	T	A	T	A
2-HGA-USA	11	California (2 humans), New York (human), Minnesota (human), California (5 horses), California (woodrat), Missouri (dog)	AF172159/63, U96728, U72628, AF172158/60–62/U96727, AF173988, AY219849	●	●	C	●	●	●	●	●	●	●	●	A	●	●	G	A	●	T	●	●	●	G
3-Sweden.1	1	Sweden (horse)	AY529489	●	●	●	●	●	●	●	●	●	●	T	●	T	●	●	●	●	●	●	●	●	●
4-Norway.1	1	Norway (sheep)	AF548385	●	●	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	C	●	●	●
5-Norway.2	2	Norway (sheep), Scotland (sheep)	AF548386, U96730	●	●	C	●	●	●	●	●	G	T	T	●	●	●	●	●	●	●	C	●	●	●
6-Italy.1	1	Italy (dog, Pippo)	AY848751	●	●	C	C	●	●	●	C	●	●	●	A	●	●	G	A	●	T	●	●	C	G
7-Italy.2	1	Italy (horse, Quada)	AY848747	●	G	C	●	●	●	●	●	●	●	●	A	●	●	G	A	●	T	●	●	●	G
8-Italy.3	1	Italy (horse, Dollaro)	AY848748	●	●	C	●	T	T	G	●	●	●	●	A	●	●	G	A	G	T	●	●	●	G
9-Italy.4	1	Italy (dog, Stechi)	AY848750	●	●	C	●	●	●	●	●	●	●	●	A	●	A	G	A	●	T	●	●	●	G
10-Italy.5	1	Italy (horse, Sogno)	AY848749	T	●	C	●	●	●	●	●	●	●	●	A	●	●	G	A	●	T	●	●	●	G
11-Italy.6	1	Italy (dog, Perla)	AY848752	●	●	C	●	●	●	●	●	●	●	●	A	●	●	G	A	●	T	●	G	●	G
Strains with unknown pathogenicity[a]																										
12-Slovenia.794	1	Slovenia (roe deer)	AF478556																							
13-Slovenia.805	2	Slovenia (roe deer), Germany	AF478555, AY281827																							
14-Slovenia.806	1	Slovenia (roe deer)	AF478554																							
15-Switzerland.811	14	Switzerland (roe deer), Slovenia (2 roe deer), Germany (11 ticks)	AF383226, AF478551/59, AF281810/12/14-17/19/21-22/46/50																							
16-Germany.X17	1	Germany (tick)	AY281848																							
17-Scotland	3	Scotland (feral goat), Germany (2 ticks)	U96729, AY281844/31																							
18-Germany.L158	2	Germany (2 ticks)	AY281832/20																							
19-Germany.145	2	Germany (2 ticks)	AY281830/25																							
20-Germany.94	1	Germany (tick)	AY281828																							
21-Germany.22	1	Germany (tick)	AY281818																							
22-CzechRep.A41	2	Czech Republic (roe deer), Germany (tick)	AY220468, AY281824																							
23-Germany.D20	1	Germany (tick)	AY281815																							
24-Austria.A6	1	Austria (tick)	AY220470																							
25-Switzerland.56	2	Switzerland (roe deer), Germany (tick)	AF383225, AY28181																							
26-Austria.A4	1	Austria (roe deer)	AY220469																							
27-Austria.A1	1	Austria (roe deer)	AY220467																							
28-Switzerland.1	1	Switzerland (bank vole)	AF192796																							
29-California	1	California (tick)	AF173989																							
30-Slovenia.478	2	Slovenia (roe deer), Germany (tick)	AF478564, AY281836																							
31-Slovenia.474	1	Slovenia (red deer)	AF478563																							
32-Slovenia.707	1	Slovenia (red deer)	AF478557																							
33-Slovenia.473	1	Slovenia (red deer)	AF478562																							
34-Slovenia.832	1	Slovenia (red deer)	AF478553																							
35-Slovenia.812	1	Slovenia (red deer)	AF478552																							
36-Slovenia.472	3	Slovenia (roe deer), Germany (tick), Switzerland (roe deer)	AF478561, AY281813, AF383227																							
37-Slovenia.471	1	Slovenia (roe deer)	AF478560																							
38-Slovenia.921	16	Slovenia (roe deer), Germany (15 ticks)	AF478558, AY281823/26/29/33–35/37–43/45/47																							
39-Switzerland	1	Switzerland (tick)	AF202895																							

[a] Pathogenicity based on the 530-bp region considered in this study.

[b] Numbering based on the accession number AF033101 sequence. Dots indicate nucleotides identical to nucleotides in the HGA-Europe sequence. The underlined nucleotides are nucleotides that are shared by the USA-HGA sequence type and the sequence types obtained in this study (Italy.1 to Italy.6).

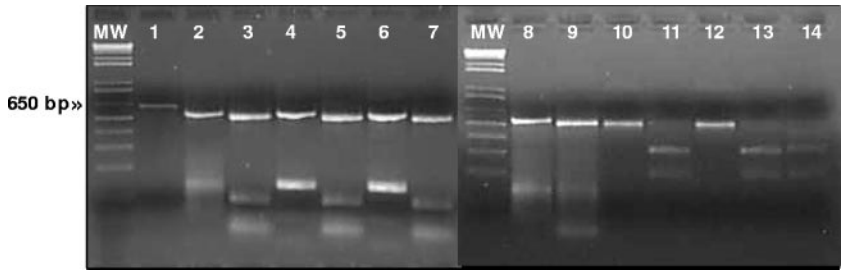

FIG. 1. Results of *groEL* PCRs and HindIII digestion. Lane MW contained molecular weight standards. Lane 1 contained amplicon obtained after the first PCR round starting with DNA extracted from the buffy coat of a symptomatic dog. Lanes 2, 4, 6, and 8 show *groEL* heminested PCR results obtained using internal primer EPhGroR, specific for *A. phagocytophilum*, with DNA extracted from the reference strain of *A. phagocytophilum* and from samples Stechi, Sogno, and Perla, respectively. To the right of each PCR result, the corresponding HindIII digestion result is shown (lanes 3, 5, 7, and 9). Lanes 10 and 12 show *groEL* heminested PCR results obtained using internal primer EPlGroR, specific for *A. platys*, with DNA extracted from the control strain of *A. platys* and from sample Lara, respectively. To the right of each PCR result, the corresponding HindIII digestion result is shown (lanes 11 and 13). Lane 14 shows HindIII digestion of the amplicon obtained after the first PCR (lane 1) and identification of the sample as *A. platys*.

U/μl; Eppendorf) according to the manufacturer's basic protocols for cycle profiling and mixture composition. Five microliters of the PCR products obtained from the first PCR round was used as the template DNA in each of the two heminested PCRs. The heminested PCR profiles were the same as the profiles in the first PCR round, except for the reverse primer used [EphgroEL(1142)R for the *A. phagocytophilum*-specific heminested PCR and EplgroEL(1084)R for the *A. platys*-specific heminested PCR]. Amplicons were digested with restriction endonuclease HindIII. The HindIII restriction pattern of the *A. phagocytophilum groEL* gene amplified region (three fragments, 525 bp, 21 bp, and 27 bp) is different from the restriction pattern produced after digestion of amplicons of the *A. platys groEL* gene (two fragments, 199 bp and 316 bp). An ABI PRISM Big Dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems) was used for direct cycle sequencing of the PCR products obtained according to the protocol supplied by the manufacturer. To ensure that polymorphisms did not represent PCR errors, all the amplicons obtained were cloned into the pCR2.1-TOPO vector (Invitrogen), and three clones per sample were sequenced using universal M13 primers. The sequences generated were edited with CHROMAS (Technelysium Pty. Ltd., Australia). Based on alignment of all the *A. phagocytophilum groEL* sequences available in the GenBank database with the sequences of *A. phagocytophilum* generated during this study (573 bp), 39 type sequences were identified (Table 1). The 39 *groEL* type sequences were used as operational taxonomic units (OTUs) for phylogenetic analyses, which were performed with MEGA, version 3.0 (8). Genetic distances among the OTUs were expressed as percent total nucleotide differences or by the Kimura two-parameter method and were used to construct neighbor-joining (NJ) trees. Statistical support for internal branches of the trees was evaluated by bootstrapping. Maximum-parsimony (MP) trees and consensus values were also generated.

Of the 40 dog samples and 20 horse DNA samples, 3 dog samples and 3 horse samples tested positive for the presence of *A. phagocytophilum*, whereas only one dog sample (Lara) resulted in *A. platys* DNA amplification. HindIII digestion confirmed the PCR diagnosis (Fig. 1). Sample Lara generated the same HindIII restriction pattern as the *A. platys* DNA used as a positive control, and this pattern also coincided with the HindIII pattern predicted for *A. platys*. The six *groEL A. phagocytophilum* amplicons obtained in this study generated a pattern identical to that of *A. phagocytophilum* NCH-1 and to the HindIII pattern predicted for *A. phagocytophilum*. A total of seven new *groEL* sequence types were obtained from three horse samples and four dog samples. Alignment of the 530 bp investigated (not including the primer regions) of the *groEL* gene of the six *A. phagocytophilum* Sardinian genetic variants with all the *groEL* type sequences identified as described above (for a total of 39 sequence types) (Table 1) revealed a variability pattern characterized by nucleotide substitutions (60 variable sites, including 29 parsimony informative polymorphic sites and 31 singleton polymorphic sites). Six parsimony informative polymorphic sites (Table 1) are shared by Sardinian sequences, by a sequence obtained from a wood rat in California, by sequences of pathogenic *A. phagocytophilum* isolated from four human patients, five horses, and a dog in different states of the United States (type sequence 2-HGAUSA [Table 1]), and by a sequence obtained from a tick in California (type sequence 29-IxodesCali), but not by *A. phagocytophilum* isolated from symptomatic hosts in different regions of Europe (type sequences 1-HGAEurope and 39-HorseSweden). Coinciding and statistically supported NJ and MP trees (Fig. 2) allowed us to distinguish two main groups of *groEL* sequence types (MP consensus value, 100; NJ bootstrap value, 100). The first group (group Europe2) contained only strains with unknown pathogenicity isolated from ticks and roe deer in different European countries (Germany, Austria, Slovenia, Switzerland, and Czech Republic [Table 1]). The second group comprised pathogenic strains and strains with unknown pathogenicity isolated from various hosts in numerous European countries and the United States. Surprisingly, Sardinian *groEL* sequence types segregated with sequence type 2-HGAUSA isolated from human patients in California, Minnesota, and New York, from horses in California, and from a dog in Missouri and with sequence type 29-IxodesCali, a strain isolated from *Ixodes pacificus* in California. Indeed, 100% of the MP trees and 95% of the bootstrapped NJ trees generated during this study allowed us to identify a United States-Sardinia group

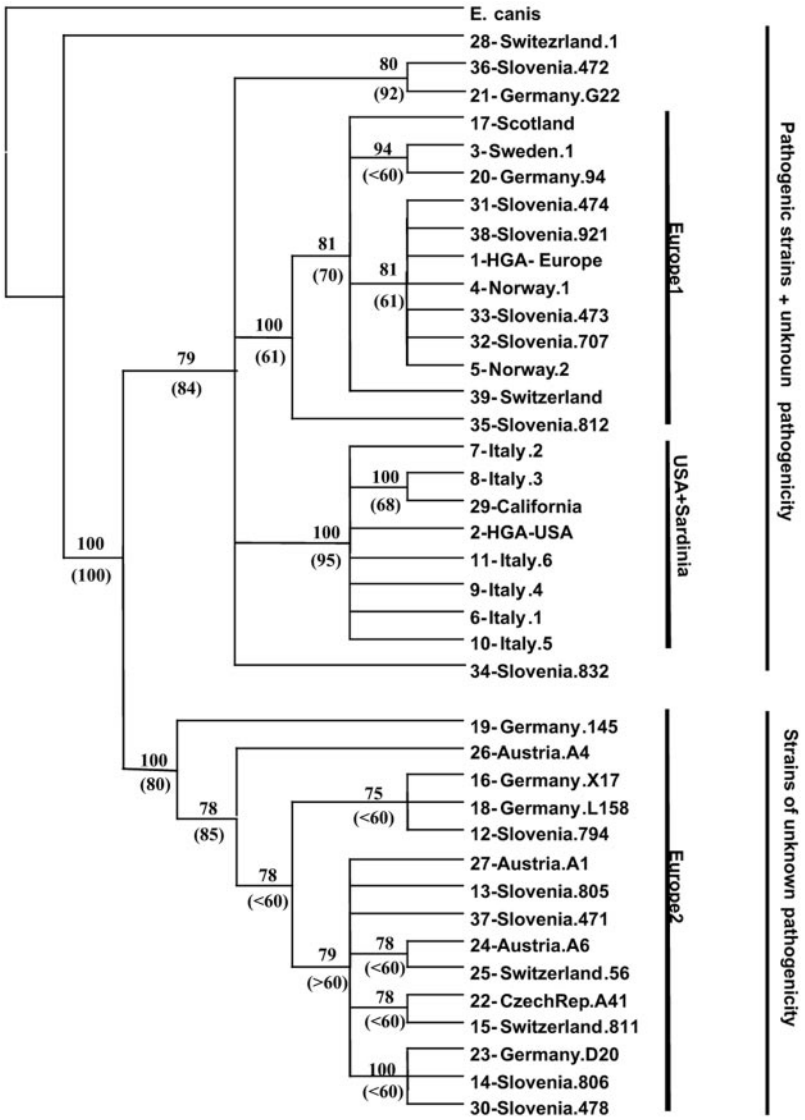

FIG. 2. Coinciding NJ and MP trees generated by considering 39 *A. phagocytophilum* sequence types OTUs for phylogenetic analyses. The numbers in parentheses are statistically significant bootstrap values for the branches obtained by testing the robustness of NJ trees. The numbers not in parentheses are statistically significant consensus values for branches obtained by testing MP trees.

that is genetically distinct from group Europe1, which contains *groEL* sequence types of European strains with unknown pathogenicity and pathogenic strains isolated from humans, horses, and sheep (Fig. 2 and Table 1). The average genetic distances calculated using MEGA highlight the low degree of genetic variation within groups (group Europe1, 0.005; group Europe2, 0.004; United States-Sardinia group, 0.006). Among groups, calculations of averages revealed greater genetic distances, especially between the two European lineages identified and between the United States-Sardinia group and group Europe2 strains (group Europe1 versus group Europe2, 0.031; United States-Sardinia group versus group Europe1, 0.013; United States-Sardinia group versus group Europe2, 0.029). None of the 39 sequence types identified was found in more than one group (Table 1 and Fig. 2), even when groups Europe1 and Europe2, which overlapped geographically and spatially, were considered.

We successfully used a molecular approach to investigate the presence of *A. phagocytophilum* in an area characterized by a high rate of tick bite-related disease in humans and animal species. Our approach also allowed us to avoid the potential pitfalls caused by the high degree of genetic similarity between *A. platys* and *A. phagocytophilum*. Hancock and coworkers (6) addressed this problem when they discussed their *A. phagocytophilum*-specific 16S rRNA gene-based PCR that resulted in coamplification of *A. platys* DNA. Indeed, one of the dog buffy coats isolated during this study resulted in *A. platys* DNA amplification.

Two main *groEL* sequence types have been reported in Europe by von Loewenich and coworkers (18), in agreement with

the findings of Petrovec and coworkers (11), who identified two genetic lineages of *groEL* sequences, one more closely related to strains isolated from humans and infecting mainly red deer and one infecting roe deer.

The phylogenetic analyses conducted during this study (Table 1 and Fig. 2) confirmed the presence of two distinct European lineages of *A. phagocytophilum*. Unexpectedly, both Sardinian equine and canine *A phagocytophilum* isolates do not fall in either of the two European lineages but are phylogenetically closely related to the American strains. The latter observation has implications for risk assessment, since *A. phagocytophilum* American strains seem to be associated with higher mortality rates in humans and HGA cases in Europe are less severe (1, 4). Statistical evaluation of the branches and group analysis strongly support the morphology of the trees. These observations could suggest that there was recent introduction via mammalian hosts and tick vectors that moved from the United States to Sardinia.

Alternatively, we cannot exclude the possibility of introduction from other regions of the Mediterranean area or from North Africa (for which information on the *groEL* gene type sequences are not yet available) through ticks transported by migratory birds. Indeed, 8 of 57 ticks (14%) collected on passerine migratory birds in the Baltic region of Russia were found to host *A. phagocytophilum* (3), thus demonstrating that *Anaplasma* exchange could occur between ticks cofeeding on animals without systemic infections. In conclusion, molecular analyses are required in order to characterize *A. phagocytophilum* strains circulating in hosts (especially humans) and vectors in the Mediterranean area and to confirm our observations.

Nucleotide sequence accession numbers. The nucleotide sequences of *A. phagocytophilum* have been deposited in the GenBank database under the accession numbers shown in Table 1, and the accession number for the *A. platys* nucleotide sequence is AY848753.

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