Short communication

Novel hemotrophic mycoplasma identified in naturally infected California sea lions (*Zalophus californianus*)

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1. Introduction

The hemoplasmas (the common name for hemotrophic *Mycoplasma* species) are facultative intracellular, cell wall-less, erythrocytic parasites comprising the group of non-cultivable in vitro bacteria of the genus *Mycoplasma* that have been described in several mammalian hosts worldwide. This study is the first report of hemoplasmas in marine mammals. EDTA anticoagulated whole blood samples from 137 California sea lions (*Zalophus californianus*) and 20 northern elephant seals (*Mirounga angustirostris*) admitted to the Marine Mammal Center (Sausalito, CA; www.marinemammalcenter.org) or live captured in Oregon were collected during 2008. Hemoplasma-specific genomic DNA was detected in blood samples from 12.4% California sea lions tested using PCR. Hemoplasma PCR positive blood specimens also were tested in reverse transcription polymerase chain reaction (RT-PCR) using the hemoplasma-specific primers for the 16S and 23S rRNA genes. The RT-PCR showed the presence the hemoplasmal rRNA, strongly suggesting the presence of potentially viable hemoplasmas in the bloodstream of the animals. BLAST search and phylogenetic analysis of the 16S rRNA sequences of the hemoplasma from California sea lions revealed that the organism is a novel hemoplasma species with only 92.1% of its nucleotide similarity to the 16S rRNA gene of the previously described hemoplasma species of alpacas, Candidatus *Mycoplasma haemolamae*. Thus, due to low level of genetic similarity of the hemoplasma to other described hemoplasmas and the mammalian host in which the hemoplasma was detected we propose that this novel hemoplasma species has been given the provisional name Candidatus *Mycoplasma haemozalophi* sp. nov.

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animals is mild or severe anemia (Messick, 2004; Stoffregen et al., 2006), but infections can occur with or without hematological signs of anemia (Almy et al., 2006; Groebel et al., 2009; Hoelzle et al., 2009; Messick, 2004; Ritzmann et al., 2009; Stoffregen et al., 2006; Willi et al., 2007). The anemic illness can be more severe in young, splenectomized, and immunocompromised animals (Messick, 2004). Hemotrophic mycoplasmas can be found as free-floating bacterial cells or attached to the surface of host red blood cells and they are able to invade erythrocytes under some clinical circumstances (Groebel et al., 2009; Willi et al., 2007). The intracellular invasion of host erythrocytes by the hemoplasmas is proposed as a mechanism to escape detection and killing by innate and immune-mediated host defenses as well as killing by antibiotics that have less membrane penetrability (Groebel et al., 2009; Vogl et al., 2008). The intracellular life cycle of some hemotrophic mycoplasmas also may explain the chronic nature of hemotrophic mycoplasma infections (Groebel et al., 2009).

The goal of the present study was to determine the existence of hemotrophic mycoplasmas in two wild caught marine mammalian species, California sea lions (CSL; Zalophus californianus) and northern elephant seals (Mirounga angustirostris), in California and Oregon.

2. Materials and methods

2.1. Sample collection and medical records

EDTA-anticoagulated blood samples were collected from 137 California sea lions and 20 northern elephant seals that were admitted to the Marine Mammal Center facilities in California or captured for sampling in Oregon, USA. The samples were collected and frozen at −80 °C until their analysis. The following information was collected for all the tested animals: species, age, gender, clinical status, and diagnosis. Blood samples were collected from each animal for a complete blood count (CBC; Vet ABC hematology analyzer, Heska Corporation, Fort Collins, CO, USA), a manual 200-cell differential count, and a clinical chemistry profile (Olympus AU5200 Chemistry Immuno Analyzer, Olympus America Inc., Melville, NY, USA) within 48 h of admission.

2.2. Nucleic acid preparations

Total DNA and RNA were extracted from 200 μl of EDTA-anticoagulated blood using DNAeasy blood and tissue kit and RNeasy Protect Mini Kit, respectively (QIAgen, Chatsworth, CA) according to the manufacturer's protocols. DNA and RNA were stored at −80 °C until use.

2.3. PCR amplification

The fragments of the 16S and 23S RNA genes were amplified from blood DNA samples using the PCR primers designed for this study (Table S1). Briefly, the standard PCR mixture (50 μl) contained 1.5 U of HotStar Taq DNA polymerase, 1x reaction buffer supplemented with 2.5 mM MgCl₂ (Qiagen, Chatsworth, CA), 500 nM of each forward and reverse primer, a 200 μM concentration of each deoxyribonucleoside triphosphosphate (dATP, dGTP, dCTP, and dTTP), and 5–7 μl of DNA template (ca. 0.1–0.2 μg of total DNA). The PCR was performed using a GeneAmp PCR system model 9700 thermocycler (PE Applied Biosystems, Foster City, CA), with the following cycle conditions: initial activation at 95 °C for 15 min; 40 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C (extension) for 2 min; and a final extension at 72 °C for 7 min. The synthesis of PCR products with the expected molecular weights was confirmed by electrophoresis using a 1% SeaKem Gold TAE-agarose gel with ethidium bromide (Lonza, Allendale, NJ) followed by UV visualization. The sizes of the 16S and 23S RNA amplicons were 1438 bp and 1795 bp, respectively. The amplicons obtained were directly sequenced. The absence of PCR inhibitors in isolated blood DNAs was monitored by PCR amplification of the mammalian mitochondrial 16S rRNA gene as described elsewhere (Volokhov et al., 2008).

2.4. Detection of the 16S rRNA in blood samples using RT-PCR amplification

The presence of the hemoplasmal 16S and 23S rRNA in the total RNA samples isolated from the blood specimens was confirmed by using one-step reverse transcription polymerase chain reaction (RT-PCR) with the PCR primers specifically designed for this novel hemotrophic Mycoplasma species (Table S1). To remove any traces of hemoplasmal DNA in the RNA samples the latter were treated with TURBO DNase (Ambion, CA) in accordance with the manufacturer's protocol. After the treatment, the TURBO DNase in the samples was inactivated by heating at 75 °C for 15 min. The blood RNA samples shown to be free of hemoplasmal genomic DNA by PCR with the 16S and 23S rRNA gene-specific primers were used for the detection of rRNA transcripts of these genes by using RT-PCR. Briefly, QIAGEN OneStep RT-PCR Kit, 500 nM of each forward and reverse primer, and 7 μl of extracted RNA template were used in this reaction. The RT-PCR was performed using a GeneAmp PCR system model 9700 thermocycler (PE Applied Biosystems, Foster City, CA) with the following cycle conditions: RT-step at 57 °C for 60 min; initial activation at 95 °C for 15 min; 40 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C (extension) for 1 min; and a final extension at 72 °C for 7 min. The synthesis of PCR products with the expected molecular weights was confirmed by electrophoresis using a 1% SeaKem Gold TAE-agarose gel with ethidium bromide (Lonza, Allendale, NJ) followed by UV visualization. The sizes of the 16S and 23S rRNA-specific amplicons were 636 bp and 1004 bp, respectively.

2.5. Phylogenetic analysis of sequences and construction of phylogenetic trees

The obtained sequences of the hemoplasmal 16S and 23S rRNA genes were compared to those available in the GenBank nucleotide database using procedures and algorithms as described elsewhere (Volokhov et al., 2007). The 23S rRNA gene sequences of several Mycoplasma and Ureaplasma species that were unavailable in the GenBank were determined during the study. The 23S
rRNA gene was amplified with the universal primers (Table S1) under the PCR conditions described above.

2.6. Nucleotide sequence accession numbers

DNA sequences from this study were deposited in GenBank under accession numbers GU124600–GU124614, GU904996–GU905012, GU905033, HM135458–HM138663, HM135465–HM135467, HM197715, HM197716, and HM241735.

3. Results

3.1. Blood sample characteristics

Overall, 17/137 (12.4%) CSL and 0/20 northern elephant seals had evidence of hemoplasma infection. A total of 149 of the 157 tested animals were diseased and their individual diagnoses included either one pathological condition (i.e., malnutrition, local or systemic infections, traumas, leptospirosis, domoic acid intoxication, and lungworm infestation) or their combinations. Most animals were males (70%) with yearlings and juveniles as predominant age groups. There were no significant deviations from normal hematological ranges in the hematological parameters for either species. Also, no characteristic hemoplasma-like organisms were observed on the May–Gruenwald–Giemsa stained blood smears. The presence or absence of hemoplasmas in all tested animals were confirmed by PCR and sequencing. A summary of the hemoplasma-positive animals is presented in Table 1. Most of the CSLs (132/137; 96.3%) tested and all animals with hemoplasmosis also were infected with a filarial nematode, Acanthocheilonema species, of the family Onchocercidae. Microfilariae were detected on May–Gruenwald–Giemsa stained blood smears and were genetically identified to the genus level by combination of PCR amplification and sequencing of the mitochondrial and genomic regions suitable for the identification (Casiraghi et al., 2004). Based on the sequence similarity and phylogenetic analysis of the 18S small subunit rRNA, coI (cytochrome oxidase I), and sod1 (putative copper/zinc superoxide dismutase) genes, the filarial nematode species was found to be closely related to Acanthocheilonema vitaeae with 99%, 89% and 91% nucleotide similarity, respectively.

3.2. PCR primers design and PCR amplification of hemoplasma-specific sequences

To assess the prevalence of hemoplasma bacteremia in the blood samples of the marine mammals, special PCR primers were designed based on the conserved sequences found within the 16S rRNA gene sequences of all known hemotrophic mycoplasmas and phylogenetically closely related Mycoplasma and Ureaplasma species (Fig. S1). The 16S rRNA phylogenetic tree had two well divided clusters with bootstrap value of 98% for each cluster. The cluster I included all known hemotrophic Mycoplasma species together with three non-hemotrophic species, M. caviae, M. fastidiosum and M. inos, while the cluster II comprised other non-hemotrophic Mycoplasma and Ureaplasma species. Based on the phylogenetic analysis and the 16S rRNA gene sequences of Mycoplasma species within the cluster I, we designed the PCR primers for screening of all blood specimens that matched most conserved regions of all hemotrophic Mycoplasma and non-hemotrophic, closely related Mycoplasma species (Table S1). We also used other PCR primers, 8F and 1492R, which were previously demonstrated to be suitable for the PCR-based detection of hemotrophic Mycoplasma ovis (Neimark et al., 2004). The evaluation of the newly designed, broadly specific primers, as well as the previously published primers, showed that the primers were able to amplify the 16S rRNA sequences of all non-hemotrophic closely related Mycoplasma and Ureaplasma species. However, these primers also amplified the 16S rRNA gene sequences of Streptococcus phocae, Staphylococcus warneri and Salmonella enterica subsp. enterica, which also were found in two hemoplasma-positive and several hemoplasma-negative blood samples. Only one hemoplasma-specific sequence

Table 1

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Age group</th>
<th>Sex</th>
<th>Strand locality</th>
<th>Diagnosis</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSL-7666</td>
<td>Adult</td>
<td>Female</td>
<td>Oceano Dunes, San Luis Obispo Co.</td>
<td>Chronic domoic acid toxicity</td>
<td>Euthanized</td>
</tr>
<tr>
<td>CSL-7750</td>
<td>Adult</td>
<td>Female</td>
<td>San Tomas Aquinas Creek, Santa Clara Co.</td>
<td>Chronic domoic acid toxicity</td>
<td>Euthanized</td>
</tr>
<tr>
<td>CSL-7755</td>
<td>Adult</td>
<td>Female</td>
<td>Oceano Dunes State Vehicular Recreation Area, San Luis Obispo Co.</td>
<td>Trauma, carcinoma</td>
<td>Euthanized</td>
</tr>
<tr>
<td>CSL-7758</td>
<td>Adult</td>
<td>Female</td>
<td>Pismo State Beach, San Luis Obispo Co.</td>
<td>Leptospirosis</td>
<td>Released</td>
</tr>
<tr>
<td>CSL-7801</td>
<td>Juvenile</td>
<td>Male</td>
<td>Coast Guard Jetty, Monterey Co.</td>
<td>Leptospirosis, pneumonia</td>
<td>Euthanized</td>
</tr>
<tr>
<td>CSL-7783</td>
<td>Yearling</td>
<td>Male</td>
<td>Rio Del Mar Beach, Santa Cruz Co.</td>
<td>Malnutrition, lungworms</td>
<td>Released</td>
</tr>
<tr>
<td>CSL-7822</td>
<td>Juvenile</td>
<td>Male</td>
<td>Pier 41, San Francisco Co.</td>
<td>Leptospirosis</td>
<td>Euthanized</td>
</tr>
<tr>
<td>CSL-7860</td>
<td>Juvenile</td>
<td>Male</td>
<td>Middle Harbor Shoreline Park, Alameda Co.</td>
<td>Leptospirosis</td>
<td>Released</td>
</tr>
<tr>
<td>CSL-7871</td>
<td>Adult</td>
<td>Female</td>
<td>Asilomar State Beach, Monterey Co.</td>
<td>Chronic domoic acid toxicity</td>
<td>Euthanized</td>
</tr>
<tr>
<td>CSL-7873</td>
<td>Juvenile</td>
<td>Male</td>
<td>Breakwater Cove Marina, Monterey Co.</td>
<td>Leptospirosis, septicemia</td>
<td>Euthanized</td>
</tr>
<tr>
<td>CSL-7881</td>
<td>Juvenile</td>
<td>Male</td>
<td>Ocean Beach, San Francisco Co.</td>
<td>Leptospirosis</td>
<td>Died in treatment</td>
</tr>
<tr>
<td>CSL-7897</td>
<td>Juvenile</td>
<td>Male</td>
<td>Dunes Beach, San Mateo Co.</td>
<td>Leptospirosis</td>
<td>Released</td>
</tr>
<tr>
<td>C606</td>
<td>Juvenile</td>
<td>Male</td>
<td>Bonneville Dam, Oregon</td>
<td>Healthy</td>
<td>Released</td>
</tr>
<tr>
<td>C795</td>
<td>Juvenile</td>
<td>Male</td>
<td>Bonneville Dam, Oregon</td>
<td>Healthy</td>
<td>Released</td>
</tr>
<tr>
<td>C796</td>
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<td>Bonneville Dam, Oregon</td>
<td>Healthy</td>
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</tr>
<tr>
<td>C739</td>
<td>Juvenile</td>
<td>Male</td>
<td>Bonneville Dam, Oregon</td>
<td>Healthy</td>
<td>Released</td>
</tr>
</tbody>
</table>

Note: All these hemoplasma-positive California sea lions also were infected with a filarial nematode, Acanthocheilonema species, of the family Onchocercidae.
with minor single point mutations (≤1%) was detected among the sequences obtained from CSL hemoplasma-positive blood samples. To enable the specific PCR-based detection of novel hemoplasma species, new sets of PCR primers were designed using unique sequences found within the 16S rRNA and 23S rRNA genes of the CSL hemoplasma (Table S1). All blood samples were retested with these new primers. The new primers specifically amplified only the novel hemoplasma species but not any other bacterial microorganisms previously detected in the samples using the broadly specific primers. We suggest employing these specific primers for diagnostic PCR screening of California sea lions blood samples for the presence of this particular hemoplasma species.

To determine whether the hemoplasma were viable organisms in the blood of the sea lions, we tested the blood specimens of PCR positive animals by RT-PCR using the hemoplasma-specific 16S and 23S rRNA primers (Table S1). The presence of the hemoplasma rRNA detected in the CSL blood strongly indicated the viability of the microorganisms.

To amplify the 23S rRNA gene of this novel hemoplasma species from the blood samples, which were the hemoplasma-positive in the 16S rRNA-based PCR, we designed a set of universal primers (Table S1). At the time this study was initiated, there were no 23S rRNA sequences available for all known hemotrophic Mycoplasma species. Thus, the universal primers were designed

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Fig. 1. Dendrogram showing phylogenetic relationships based on nucleotide sequence data for the 16S rRNA gene among the novel hemoplasma species (the provisional name Candidatus *M. haemozalophi* sp. nov.) with other hemotrophic *Mycoplasma* spp., and three non-hemotrophic phylogenetically closely related *Mycoplasma* spp. (*M. insonis*, *M. fastidiosum*, and *M. cavipharyngis*). The trees were constructed by the minimum evolution method in the MEGA 4 package. The images are representing mammalian and insect species in which those hemoplasma species were detected.
based on the 23S rRNA sequences available for non-hemotrophic Mycoplasma and Ureaplasma species (M. pneumoniae, M. genitalium, M. gallisepticum, M. penetrans, and U. urealyticum) known to be phylogenetically closely related to known hemoplasmas (Fig. S1). PCR products from all hemoplasma-positive blood samples were amplified using the designed 23S rRNA universal primers and sequenced. No PCR amplification was observed when hemoplasma-negative blood samples were tested. Negligible intragenic variations (≤1%) among the 23S rRNA gene sequences of this novel hemotrophic mycoplasma detected in the different animals were observed. All our attempts to amplify the 16S–23S internal transcribed spacer region (ITS) from all the hemoplasma-positive blood specimens were unsuccessful. The separate localization of the 16S and 23S rRNA genes in the genome of M. suis, which is phylogenetically closely related to the CSL hemoplasma, has been noted by Dr. Messick (personal communication).

3.3. Phylogenetic analysis of the 16S rRNA and the 23S rRNA genes

We used the sequences of the 16S and 23S rRNA genes to determine the phylogenetic relatedness of the novel hemoplasma with other hemotrophic Mycoplasma and non-hemotrophic, closely related, Mycoplasma and Ureaplasma species (Figs. 1 and 2). The dendrograms in Figs. 1 and 2 showed the inferred phylogenetic position of our new hemoplasma species among known hemotrophic Mycoplasma spp. and three non-hemotrophic but closely related mycoplasmas (M. insons, M. fastidiosum, and M. cavipharyngis). The interspecies similarity of the 16S rRNA and the 23S rRNA genes among species was assessed (Tables S1 and S2). The 16S rRNA-based phylogenetic analysis demonstrated that the novel hemoplasma species from California sea lions was phylogenetically most closely related to known hemoplasma species, Candidatus Mycoplasma haemolamae, which was previously described in alpacas (Lama pacos) (Almy et al., 2006; Lascola et al., 2009; Messick et al., 2002). The 23S rRNA-based phylogenetic analysis demonstrated that the novel hemoplasma species from California sea lions formed a separate branch with two other hemoplasmas, M. haemofelis and M. suis, with M. suis being most closely related to the CSL hemoplasma. There was no 23S rRNA gene sequence available for the alpacas’ hemoplasma, M. haemolamae, and therefore the similarity between the 23S rRNA gene sequences of the CSL hemoplasma and the alpacas’ hemoplasma cannot be assessed. Based on the position on the phylogenetic tree (Fig. 2) and nucleotide similarity data for the 23S rRNA gene (Table S2), M. suis was found to be the genetically closest species to the CSL hemoplasma.

![Fig. 2. Dendrogram showing phylogenetic relationships based on nucleotide sequence data for the 23S rRNA gene among the novel hemoplasma species (the provisional name Candidatus M. haemozalophi sp. nov.) with other hemotrophic Mycoplasma spp., and three non-hemotrophic phylogenetically closely related Mycoplasma spp. (M. insons, M. fastidiosum, and M. cavipharyngis). The trees were constructed by the minimum evolution method in the MEGA 4 package.](image-url)
4. Discussion

The genus *Mycoplasma* comprises several hemotropic mycoplasma species which infect different hosts and attach to, and sometime invade, their erythrocytes (Groebel et al., 2009; Willi et al., 2007). Hemoplasmas have not been cultured *in vitro* and detection of hemotropic mycoplasmas using Romanowsky–Giemsa type stained blood smears in combination with specific PCR remains the gold standard for detection of these infections in animals and humans (Almy et al., 2006; dos Santos et al., 2008; Willi et al., 2007). Remarkably, the prevalence of hemoplasma-infected animals in host populations has been reported to be around 0.5–15.4% (Hackett et al., 2006; Hoelzle et al., 2009; Kenny et al., 2004; Roura et al., 2010; Sykes et al., 2008). The infection of animals with hemotropic mycoplasmas is usually self-limited and the establishment of inapparent chronic bacteremia is possible in immunosuppressed or immunocompromised individuals (dos Santos et al., 2008; Yuan et al., 2007).

This study represents the first report of a hemotropic *Mycoplasma* species in marine mammals. Although about 13% of CSL were infected with hemoplasma in this study, the majority of the studied animals were diseased (149/154; 96.7%) and thus no estimate of prevalence of the infection in clinically normal animals can be made. All blood samples from 20 northern elephant seals tested were hemoplasma-negative, but since only a few animals were examined, hemoplasma prevalence data cannot be determined for this species. Assuming that the lowest prevalence of hemoplasma infection in marine mammals is similar to the 0.2–0.5% reported for some other host species (Roura et al., 2010; Willi et al., 2006), a minimum as 100–200 animals are required to confidently detect the existence of the infection.

The sequencing analysis of the hemoplasma-specific 16S and 23S amplicons from the CSL blood samples revealed only minor single nucleotide variations (<1%). Thus, the obtained sequence data indicate the existence of one hemoplasma species in CSL populations inhabiting California and Oregon.

The infections of animals with hemotropic *Mycoplasma* species are often associated with different types of anemias and positive Coombs tests (Messick, 2004; Tagawa et al., 2010; Willi et al., 2007). However, the laboratory or clinical manifestations of anemia and alteration of other hematological parameters do not always accompany the hemoplasma infection. In our cases, the presence of the hemotropic mycoplasma organisms in CSLs was not accompanied by anemia, even though all hemoplasma-positive animals also were simultaneously infected with filarial nematodes of the genus *Acanthocheilonema*.

Others have stated that subjective evaluations of hemoplasma infections may lead one to the conclusion that the numbers of hemoplasma present in the blood seemed to be correlated with the level of anemia in hosts (Willi et al., 2007). Our observation on the absence of anemia in hemoplasma-positive CSLs may reflect a low pathogenicity of this hemoplasma species for the host or adaptation in a host-parasite system (Schulte et al., 2010). Because we have detected hemoplasmas in CSLs of different age groups (adults, yearlings, and juveniles), the affect of hemoplosmosis on the health of different individuals should be studied in the future.

Transmission of hemotropic mycoplasmas among animals other than CSLs has been shown to be accomplished by a number of blood-feeding arthropod vectors, including mosquitoes and stable flies, as well as direct transmission via infected blood (e.g. aggressive interaction, blood transfusion, and by an iatrogenic route) (Willi et al., 2007). Potentially, all these routes of transmission may work for hemoplasma spreading in CSL populations. Pinnipeds are known to have sucking lice, *Echinophthirius horridus* (Leidenberger et al., 2007). The seal louse is suggested to play an important role as an intermediate host transmitting the heartworm *Acanthocheilonema spirucauda* among seals (Leidenberger et al., 2007). The potential role of the seal louse in the transmission of hemoplasma infection within CSL populations is speculative, but warrants additional investigation. Additional routes of transmission for hemoplasmas appear to exist as well. For example, transplacental and transmammary (through colostrum) transmissions of *M. haemolamae*, which is closest phylogenetically related to our novel hemoplasma, have been suggested in alpacas (Almy et al., 2006). Clearly, additional study of the natural history of hemoplasma infections in many species is warranted.

Finally, we propose that the newly discovered hemotropic *Mycoplasma* isolates from California sea lions (*Z. californianus*) to be given the taxonomic status *Candidatus Mycoplasma haemozalohiphi*. The *Candidatus* designation is appropriate because it is specifically reserved for newly described, non-cultivable and incompletely characterized species in order to give them provisional status (Brown et al., 2007; Murray and Schliefer, 1994; Murray and Stackebrandt, 1995).

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2010.10.026.

References


