Comparative analysis of 16S RNA nucleotide sequences of *Anaplasma phagocytophilum* detected in the blood of horses from various parts of Europe

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INTRODUCTION

Granulocytic anaplasmosis (GA) is an infectious multi-organ human and animal disease accompanied by thrombocytopenia. The aetiological agents of the disease are micro-organisms previously classified within the family *Rickettsiaceae*, genus *Ehrlichia*. Currently, they are classified within the order *Rickettsiales*, family *Anaplasmataceae*. 16S rRNA gene sequence studies have made it possible to classify three pathogens considered until recently as separate species in the aetiology of ehrlichiosis/anaplasmosis in companion animals (*Ehrlichia phagocytophilum*), horses (*Ehrlichia phagocytophilum*, *Ehrlichia equi*) and people (human granulocytic ehrlichiosis agent) to the taxon *Anaplasma phagocytophilum* (Dumler et al., 1995; Dumler et al., 2001).

The main vector of *A. phagocytophilum* in Europe is *Ixodes ricinus*, and less often *Dermacentor marginatus*.

Abbreviation: EGA, equine granulocytic anaplasmosis.

Haemophysalis punctata, *Hyalomma m. marginatum*, *Rhipicephalus bursa* and *Rhipicephalus sanguineus* (Merino et al., 2005; Naranjo et al., 2006). In Asia, rickettsia are transmitted by *Ixodes persulcatus* (Strle, 2004; Ohashi et al., 2005; Chan et al., 2010; Portillo et al., 2011), in North America by *Ixodes scapularis* and *Ixodes pacificus* (Richter et al., 1996; Daniels et al., 1997; Chen et al. 1994; Pusterla et al., 2002) and in Africa by *I. ricinus* and *Hyalomma detritum* (M’ghirbi et al., 2012).

The first case of equine granulocytic anaplasmosis (EGA) was noted in 1969 in California, USA (Gribble, 1969; Stannard et al., 1969). In Europe, the disease has been recognized for many years: in 1984 equine anaplasmosis was diagnosed in Germany (Büscher et al., 1984), in 1985 in Switzerland (Hermmann et al., 1985) and in 1990 in Sweden (Björsdorff et al., 1990). The disease has also been reported in: Great Britain (Korbutiak & Schneiders, 1994), Denmark (Eriksen et al., 1997), Austria (Fröhlich &
The research was carried out on 234 horses, aged from 8 months to 10 years, from Spain (150 cases), Poland (36 cases), Italy (32 cases), Ukraine (10 cases) and Germany (six cases). All animals had contact with ticks, after which thrombocytopenia was recorded in haematological examinations. Some animals exhibited clinical signs such as fever, joint oedema and ataxia, which raised suspicion of anaplasmosis (Table 1). Blood collected from all the horses that qualified for the study was examined microscopically and screened for piroplasmosis and anaplasmosis using PCR as described below.

Blood smear tests. Blood smears were made on degreased microscope slides, stained with the Diff-Quick (POCH S. A.) method, and viewed with an Olympus CH 20 (Olympus Optical) microscope when dry. Blood smears were screened for the presence of Babesia and Anaplasma.

DNA isolation. DNA for analysis was extracted from 100 μl fresh blood. DNA isolation was carried out with a DNA Blood Mini kit (DNA Gdańsk).

PCR. PCR amplification was performed using a programmable thermal cycler (Biometra). The PCR for Babesia spp. was carried out using a pair of primers, RIB 19 (5′-CGGGATCCACCTGTGCTGATCCTGAC-3′) and RIB 20 (5′-CCGAATTCCTGTAGTATGGCACCTGTC-3′), which amplified a 1712 bp fragment of the 18S rRNA conserved gene (Adaszek & Winiarczyk, 2008). The PCR for Anaplasma Ehrlichia spp. was carried out according to a method described by Inokuma et al. (2000) and modified by the authors (Adaszek et al., 2009) with the use of the following primers: EHR 521 (5′-TGTAGGCGGTGCCTACATGGC-3′) and EHR 747 (5′-GCCTACATGGCTTACAGGGT-3′). This limited the amplified DNA section to a length of 247 bp of the conserved part of the 16S rRNA gene. The positive control was A. phagocytophilum DNA from human blood (National Reference Center for Borreliae, Max von Pettenkofer Institute, Ludwig Maximilian University, Munich), and the negative control was DNA from the blood of a healthy horse. Each reaction was composed of 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 45 s.

Electrophoresis. The products obtained from the PCR were analysed by electrophoresis method in 1 % agarose gels and Tris/borate/EDTA buffer at a voltage of 10 V cm⁻¹ for 50 min.

Sequencing. PCR products were purified with a QIAquick PCR Purification kit (Qiagen) and sequenced at the DNA Sequencing and Synthesis Service of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Nucleotide sequence analysis and dendrogram analysis were performed using DNASTAR software. The 16S rRNA gene sequences of A. phagocytophilum obtained from isolates were compared with previously published 16S rRNA Anaplasma sequences of global isolates available in GenBank (Table 2).

RESULTS

Microscopic examination and PCR revealed that Babesia/Theileria protozoa were not present in the blood of any of the examined horses, whereas the DNA of A. phagocytophilum was detected in blood samples obtained from 42 of the 234 horses used in the study. In most cases, the presence of rickettsial DNA in the blood was accompanied by clinical signs of anaplasmosis (Table 1). The exception was Spanish horses, where the clinical signs of the disease were observed in 18 of 159 examined animals, while positive PCR results were obtained for 30 blood samples.

Fever and joint oedema were observed in all animals with clinical symptoms and anaplasmosis confirmed by molecular tests. These horses did not show any nervous symptoms (ataxia) or haemorrhages on mucous membranes observed in the group of animals selected for study; in these, PCR revealed no presence of rickettsial genetic material.

Microscopic analysis of the blood smears showed the presence of A. phagocytophilum morulae in neutrophils in 17 of 42 blood samples in which the presence of the rickettsial genetic material was confirmed by PCR. No
They differed in five substitutions at positions 74, 83, 12, 32, 50, 68, 69, 83, 158, 248 and 249 (Table 3) and were placed in a separate branch of the phylogenetic tree (Fig. 1).

By comparing the sequences of the 16S rRNA gene fragment of *A. phagocytophilum* obtained in our study with the sequences of other *Anaplasma* species available in GenBank, the highest degree of similarity (98.8 %) was found between our isolates and *A. platys* (AY837736), which differed only in three substitutions at positions 74, 248 and 249. In the case of *Anaplasma centrale* (EF520690), *Anaplasma marginale* (AF414876) and *Anaplasma ovis* (AY837736), the differences involved nine nucleotides at positions 12, 32, 50, 68, 69, 83, 158, 248 and 249 of the tested 16S rRNA gene fragment (Table 3).

**DISCUSSION**

*A. phagocytophilum* has been recognized as an animal pathogen and is an emerging human pathogen of public health relevance. Human *A. phagocytophilum* infection was first reported in 1994 in residents of Minnesota and Wisconsin (Chen et al., 1994). The clinical disease is indistinguishable from that seen with *Ehrlichia chaffeensis* and *Ehrlichia ewingii*. The most frequent clinical signs are fever, malaise, headaches, myalgia and arthralgia (Chen et al., 1994; Strle, 2004). In addition to humans, many domestic animals, such as dogs, cats, horses, sheep, goats and cattle, can become infected with *A. phagocytophilum* and show clinical signs (Yang et al., 2012). The potential for dogs, horses, sheep and other domestic animals to be zoonotic risks for human *A. phagocytophilum* infection is
The results of the study by Bakken et al. (1996) indicate that the disease in people may develop not only as a consequence of contact with an infected tick but also as a consequence of direct contact with the blood of animals infected with *A. phagocytophilum*. In this study, *A. phagocytophilum* was found in 42 of 234 horses that had contact with ticks. PCR proved to be a more sensitive test to diagnose infection with the discussed micro-organisms than microscopic examination of blood smears, which detected the presence of the rickettsial morulae in neutrophils in only 17 of 42 infected animals.

Although some authors suggest that serological tests are helpful in the diagnosis of anaplasmosis (Torina et al., 2007), we did not perform them in this study. The results of PCR and sequencing allow a more detailed characterization of the examined pathogens than the results of serology and allow the infection to be recognized in a shorter time (de la Fuente et al., 2005).

The comparison of the 16S rRNA gene fragment sequences of the micro-organisms obtained in our study with the sequences of *A. centrale, A. marginale, A. ovis*, *A. phagocytophilum* and *A. platys* allowed us to confirm that the examined horses had contracted *A. phagocytophilum*. Although the sequences of other rickettsial species (except for *A. platys* EU439948) showed a strong similarity to the sequences of our isolates (96.4 %), they formed a monophyletic group on the dendrogram, clearly separated from the group comprising *A. phagocytophilum* strains (Fig. 1).

## Table 3. Nucleotide substitutions in the conservative fragment of the 16S rRNA gene of *Anaplasma* spp.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Country</th>
<th>GenBank accession no.</th>
<th>Position</th>
<th>Nt change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>A. phagocytophilum</em></td>
<td>Spain, Poland, Italy, Ukraine, Germany</td>
<td>Own studies</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td><em>A. phagocytophilum</em></td>
<td>Poland</td>
<td>GU183908</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td><em>A. phagocytophilum</em></td>
<td>South Africa</td>
<td>AY570538, AY570539, AY570540</td>
<td>74, 83, 232</td>
<td>A→C, G→A, T→C</td>
</tr>
<tr>
<td>4</td>
<td><em>A. phagocytophilum</em></td>
<td>China</td>
<td>JN558811</td>
<td>78</td>
<td>T→A</td>
</tr>
<tr>
<td>5</td>
<td><em>A. phagocytophilum</em></td>
<td>China</td>
<td>JX914659</td>
<td>74</td>
<td>A→T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>83</td>
<td>G→A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>232</td>
<td>T→C</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>248</td>
<td>C→T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>249</td>
<td>T→G</td>
</tr>
<tr>
<td>6</td>
<td><em>A. phagocytophilum</em></td>
<td>USA</td>
<td>AF172164, AF172166, AY144728, AY527213, AY527214</td>
<td>248</td>
<td>C→T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>249</td>
<td>T→G</td>
</tr>
<tr>
<td>7</td>
<td><em>A. centrale, A. marginale, A. ovis</em></td>
<td>Italy</td>
<td>EF520690, AF414876, AY837736</td>
<td>12, 32, 50</td>
<td>C→T, G→A, A→G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Israel</td>
<td></td>
<td>68</td>
<td>C→A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kenya</td>
<td></td>
<td>69, 83</td>
<td>A→G, G→A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>158</td>
<td>A→G</td>
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<td></td>
<td></td>
<td>248</td>
<td>C→T</td>
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<td></td>
<td></td>
<td></td>
<td>249</td>
<td>T→G</td>
</tr>
<tr>
<td>8</td>
<td><em>A. platys</em></td>
<td>Italy</td>
<td>EU439948</td>
<td>74</td>
<td>A→C</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>248</td>
<td>C→T</td>
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<td></td>
<td></td>
<td></td>
<td>249</td>
<td>T→G</td>
</tr>
</tbody>
</table>
The bacteria found in the blood of the horses from Spain, Italy, Germany, Poland and Ukraine had a sequence identical to the tested 16S rRNA gene fragment, which might suggest the incidence of a new rickettsial genotype that is the cause of EGA in southern and eastern Europe. The comparison of the nucleotide sequences of our isolates with the sequences of A. phagocytophilum isolates available from GenBank allowed the presence of nucleotide substitutions at positions 248 and 249 in the tested strains to be demonstrated. In the sequences of the bacteria from Asia, Africa, North America and northern Europe, and in the sequences of other Anaplasma species (A. centrale, A. marginale, A. ovis and A. platys), these positions are occupied by T and G, which were replaced by C and T in our isolates. In the case of the strains that were the most closely related to our isolates (AF172164, AF172166, AF470700, AY144728, AY527213, AY527214, DQ458807, HQ872465, JN990105, JN990106, NC007797 and NR044762), the discussed differences were the only ones observed in the entire tested sequence. Due to the fact that the 16S rRNA gene is a conserved gene, within which mutations occur relatively infrequently (Portillo et al., 2011), the observed nucleotide substitutions at positions 248 and 249 of the gene’s tested sequence that were demonstrated in our isolates might be evidence of a new genotype of A. phagocytophilum.

A. phagocytophilum strains with the 16S rRNA gene fragment sequence identical to that analysed in our study were also found in wild ruminants in eastern Europe (Adaszek et al., 2012). It cannot be ruled out that these animals may serve as a source of infection for arthropods that transmit diseases to humans, farm animals and other domestic animals.

Although anaplasmosis is reported relatively rarely in horses in Europe, it should be presumed that, with changing climatic conditions, the threat of this disease in the horse population on our continent will increase. For this reason, it seems advisable to introduce continuous monitoring of this infection among domestic, farm and wild animals. In comparison with various other test methods, molecular biology techniques based on analysis of the 16S rRNA gene sequence of rickettsia seem to be the most credible tests for this purpose.

### REFERENCES


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**Fig. 1.** Phylogenetic tree (cladogram) based on the 16S rRNA nucleotide sequences of A. phagocytophilum strains showing the degree of similarity between fragments of the 16S rRNA gene of Anaplasma spp. from GenBank and the corresponding isolates obtained in our own studies. Bar, nucleotide substitutions per site.


