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Identification of *Anaplasma phagocytophilum* on the Basis of a Fragment of the 16S rDNA Gene*

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Anaplasma phagocytophilum is a bacterial parasite of human and animal granulocytes. Instances of granulocytic anaplasmosis (formerely HGE – human granulocytic ehrlichiosis) have been noted in Europe, including northeastern Poland. Here the results of studies on the occurrence of *Anaplasma* pathogens in ticks *Ixodes ricinus* in Western Pomerania are presented. 4,6% of the tick population was infected. Sequence analysis of the 16S rDNA gene, necessary for the classification of the bacteria, revealed that the pathogens were *A. phagocytophilum,* causing disease in people and domestic animals. The DNA sequences were identical to the sequence considered as a prototype for human granulocytic anaplasmosis (U02521, USA). A neighbor-joining tree was constructed using the *A. phagocytophilum* sequence (AY303572) from Western Pomerania and 10 sequences from the *Ehrlichiae* and *Anaplasmataceae* families downloaded from GenBank. The AY303572 sequence clusters with other pathogenic species found in Europe and the USA.

Key words: *Anaplasma phagocytophilum*, *Ixodes ricinus,* phylogenetic relationships of *Anaplasma phagocytophilum*.

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Anaplasma phagocytophilum is an obligate intracellular bacterial parasite in human and animal granulocytes. It causes a disease formerly known as granulocytic ehrlichiosis, now, after changes in the systematics of the families *Ehrlichiae* and *Anaplasmataceae* (DUMLER *et al*. 2001), it is known as granulocytic anaplasmosis.

The first cases of infection in Europe were registered in Slovenia in 1997 (PETROVEC *et al*. 1997), while in Poland the first *A. phagocytophilum* infected patients by tick bites were diagnosed in 2001 (TYLEWSKA-WIERZBANOWSKA *et al.* 2001).

The vectors of *A. phagocytophilum* in Europe are ticks from the *Ixodes* genus, in Poland it is *Ixodes ricinus*. The identification of pathogens in ticks is one of the stages of "epidemiological procedure" that increasingly relies on molecular techniques. A basic tool is the polymerase chain reaction with species-specific primers that allows the quick and precise detection of pathogens in ticks. The 16S rDNA gene is most commonly used for the identification of microorganisms. This gene is conservative in *Prokaryota*, therefore appropriately designed primers allow for the detection of a few closely related species, and afterwards nested PCR is used for species-specific identification (CHEN *et al.* 1994).

The 16S rDNA gene belongs to a group of ribosomal RNA coding genes. Typical expression takes place in a single operon in the following order: *rrs – rrl – rrf* (16S – 23S – 5S, respectively). Several versions of this order are known, characteristic for each species, e.g. 15 copies of this operon exist in *Clostridium paradoxum* (MASSUNG *et al.* 2002). In the genome of *A. phagocytophilum*, as in *Rickettsia*, a single copy of the *rrs* and *rrl* genes can be found. Additionally, MASSUNG *et al*. (2002) have shown that these genes are not contiguous in *A. phagocytophilum* but are divided by about 495 bp. Genes *rrl* and *rrf* are linked closer in this species, the intergenic region amounts to 72 bp. The 16S rDNA gene, because of its conservative nature, is commonly used in phylogenetic studies and for the identification of microorganisms, including *A. phagocytophilum*.

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The aim of this study is to describe the state of infection of *I. ricinus* ticks by *Anaplasma* bacteria in the Western Pomeranian region of Poland, and also to describe the identification of these pathogens based on a complete molecular analysis of a fragment of the 16S rDNA gene, i.e. through PCR and sequencing of bacteria isolated from ticks.

Material and Methods

The study was based on DNA isolated from *Ixodes ricinus* ticks captured in Dabie Forest Park. This area is situated close to a large housing estate, therefore it is often used by people as a sports-recreational facility. Dabie Forest Park is characterized by mixed forest with a predominance of oak and beech with a rich understory. Vegetation in this area is potential *I. ricinus* habitat. Ticks were collected by dragging a flannel flag across vegetation up to a meter in height. Developmental stage and sex in adults were determined during collection. Ticks were captured during spring and autumn of 2002.

DNA was isolated from single, homogenized ticks according to GUY and STANEK (1991) and was stored at –70°C until analysis.

A fragment of the conservative 16S rDNA gene was used to detect DNA of *A. phagocytophilum* amplified by primers EHR 521 and EHR 747 (PANCHOLI *et al*. 1995). PCR reactions were carried out in 20 μl containing 1x reaction buffer, 0.2 mM dNTP's, 1.5 mM $MgCl_2$, 0.5 U polymerase (QIAGEN, Niemcy) and 2 μl of genomic DNA isolated from ticks. Reactions were carried out with positive (*E. phagocytophila*, Y. Rikihisa, Department of Vetrerinary Biosciences, USA) and negative (without DNA) controls in a T-gradient thermocycler (Biometra, Niemcy). PCR products were eletrophoresed in 2% agarose (ICN, USA) and stained with ethidium bromide.

A 247 bp fragment of the 16S rDNA gene from randomly chosen positive isolates of *I. ricinus* (male, female and nymph) were sequenced with primers EHR 521 and EHR 747.

Sequencing was carried out in the Department of Tropical Parasitology, Inter-Faculty Institute of Maritime and Tropical Medicine, Medical University of Gdańsk, Gdynia. The obtained sequences were compared with analogous ones downloaded from GenBank. Sequences of the 16S rDNA gene used in comparisons were from species of *Anaplasmataceae* (AF084907, U02521, AF093788, M73223, M73220, M82801, AF283007, M60313), and a related family, *Ehrlichiaceae* (AF104680, M73222).

Table 1

Frequency of *A. phagocytophilum* DNA in ticks *I. ricinus* from Dabie Forest Park

	Female		Male		Nimph		Total	
	PCR+/N	%	PCR+/N	%	PCR+/N	%		
Spring	4/36	11.1	1/23	4.35	1/45	2.2	6/104	5.8
Autumn	1/11	9.1	0/9	0	1/50	2	2/70	2.9
Total in year	5/139	3.6	1/32	3.1	2/95	2.1	8/174	4.6

Table 2

Homology matrix of 11 sequences *A. phagocytophilum*

	U02521	AF093788	AF104680	AF283007	AY303572*	M60313	M73220	M73222	M73232	M82801	AF084907
U02521	100.0%										
AF093788	99.9%	100.0%									
AF104680	92.5%	92.4%	100.0%								
AF283007	96.0%	95.8%	91.8%	100.0%							
AY303572*	100.0%	100%	93,90%	97.7%	100.0%						
M60313	96.4%	96.2%	91,70%	98.2	97.7%	100.0%					
M73220	99.9%	100.0%	92,50%	95.9%	100.0%	96.3%	100.0%				
M73222	92.9%	92.7%	93.4%	91.7%	94.0%	92.1%	92.9%	100.0%			
M73223	99.9%	100.0%	92.4%	95.8%	100.0%	96.2%	99.9%	92.8%	100.0%		
M82801	98.5%	98.5%	92.1%	95.4%	99,10%	96.1%	98.5%	91.9%	98.5%	100.0%	
AF084907	100.0%	92.5%	92.5%	96.0%	100.0%	96.4%	99.9%	92.9%	99.9%	98.5%	100.0%

* 16S rDNA gene of *A. phagocytophilum* from West Pomerania (Poland).

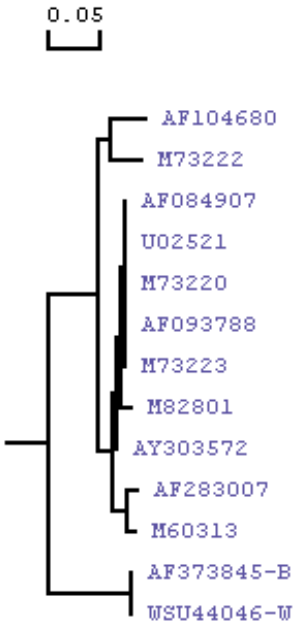

Fig. 1. Phylogenetic tree – 16S rDNA (outgroup: *Bartonella* sp., *Wolbachia* sp.).

Analysis of genetic similarity and construction of a phylogenetic tree by means of neighbor-joining were carried out using the software DNAMAN 5.2.9 (Lynnon Biosoft, Canada). Sequences of 16S rDNA of *Bartonella* sp. (AF373845) and *Wolbachia* sp. (WSU44046) from GenBank were used as outgroups in the phylogenetic reconstruction. The sequence of a fragment of the 16S rDNA gene of *A. phagocytophilum* from Western Pomerania was deposited in GenBank under accession number AY303572.

Results and Discussion

A total of 174 individuals of *I. ricinus* were collected in Dabie Forest Park, 104 and 70 of these were captured during the spring and autumn seasons, respectively. Females, males and nymphs composed 27%, 18.4% and 54.6% of the collection, respectively.

Preliminary molecular analysis in this work was based on the primers EHR 521/EHR 747, used by many authors for the detection and identification of *A. phagocytophilum* DNA in ticks (PANCHOLI *et al.* 1995; GUY *et al.* 1998; JENKINS *et al.* 2001; STAŃCZAK *et al.* 2002). The presence of *A. phagocytophilum* DNA was detected in 4,6% of *I. ricinus* ticks. This is a relatively high degree of infection as compared to other sites in Western Pomerania (SKOTARCZAK & RYMASZEWSKA 2001; RYMASZEWSKA & SKOTARCZAK 2005). A detailed report of anaplasma infection in females, males and nymphs of *I. ricinus* from Dabie Forest Park is given in Table 1.

The level of *A. phagocytophilum* infection in ticks from Western Pomerania (Poland) is similar to that reported in other European regions.

FINGERLE *et al*. (1999) and BAUMGARTEN *et al.* (1999) established infection in ticks from southern Germany at 1.6% do 2.2%. PETROVEC *et al*. (1999) have shown that the level of infection in *I. ricinus* in central Slovenia, a region endemic for Lyme borreliosis, is 3.2%. Similar results (3.1%) were obtained by von STENDINGK *et al.* (1997) in ticks from eastern Sweden. The lowest levels of infection detected so far in Europe have been in Switzerland – 0.8% (PUSTRLA *et al.* 1998) and Scotland – 0.25-2% (ALBERDI *et al*. 1998).

There are several instances of high levels of infection in Europe. CINCO *et al.* (1997) reported HGE infection at 24.4% in *I. ricinus* from central Italy, this being the highest reported level in Europe. In Bulgaria, CHRISTOVA *et al.* (2001) also documented a relatively high 19.8%. In northeastern Poland, GRZESZCZUK *et al.* (2002) and STAŃCZAK *et al.* (2002) found levels at 19.2% and 16%, respectively.

Analysis of the 16 S rDNA gene is commonly used in identification and phylogenetic studies, however, its conservative sequence makes it necessary to conduct additional treatments in order not to misclassify the microorganism involved. Several cases of PCR amplification of closely related species may be found in the literature (JENKINS *et al.* 2001; MASSUNG *et al.* 2003; SCHOULS *et al*. 1999). Therefore, correct identification is only possible through sequencing or the utilization of supplemental, species-specific markers. This allows classification of the pathogen and an assessment of genetic similarity to other species or intraspecific strains. The PCR method and sequencing were used in this study in order to identify pathogens in *I. ricinus* ticks in Western Pomerania. The results of comparisons are given in Table 2.

Sequences of a fragment of the pathogen 16S rDNA gene amplified in a female, male and nymph *I. ricinus* collected in Dabie Forest Park were compared and found to be identical. The sequences were deposited in GenBank under the accession number AY303572. The sequence was further compared to other sequences from *E. phagocytophila, E. equi* and the HGE factor, isolated from human and animal blood. Since 2001, these three species names have been considered synonymous and are considered as the polymorphic species *A. phagocytophilum* (DUMLER *et al*. 2001).

The sequence presented in this study is fully homologous to sequences isolated from human blood in the USA, including the sequence considered standard for factor HGE (now known as the factor of human granulocytic anaplasmosis – U 02521, AF 093788). The sequence was also identical to sequences isolated from *I. ricinus* ticks from Switzerland (AF 084907, Table 2), canine blood in the USA (M73223), and sheep blood from Great Britain (M73220). For three anaplasma species that are not human pathogens, the similarity of the sequence from Western Pomerania was 97.7% for *A. marginale* (M 60313) and *A. centrale* (AF 283007), and also 99.1% for *A. platys* (M 82801, Table 2).

Similarity to 16S rDNA in species of monocytic ehrlichia was determined at 94 % and 93.9 % in *E. chaffeensis* (M73222) and *Ehrlichia* – like (AF104680) (Table 2).

In the phylogenetic tree constructed on the basis of the conservative 16S rDNA gene (Fig.1), the sequence from Western Pomerania clustered with granulocytic species pathogenic towards humans and animals that occur in Europe and the USA (U02521, AF084907, AF093788, M73223, M73220, M82801). Monocytic species (AF104680, M73222), on the other hand, were clearly separated. The genetic distance between the Polish anaplasma and other species in the cluster, i.e. *A. marginale* and *A. centrale*, is greater than 0.05, which means that there are over 5 different nucleotides in each 100.

In conclusion, a PCR-mediated molecular analysis, based on detection of the conservative 16S rDNA gene, can be used for recognition of *A. phagocytophilum* DNA in *I. ricinus* ticks, although it has to be supplemented by additional sequencing of the product. Only in this way is it possible to identify and classify the pathogens inhabiting ticks.

Analysis of a fragment of the 16S rDNA gene has revealed the presence of *A. phagocytophilum,* a pathogen in humans and animals, in Western Pomerania. This pathogen shows 100% similarity to granulocytic species known as pathogens in Europe and the USA, classified in the genus *Anaplasma.*

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