Short Communication

Variability within the *msp2* Gene in Populations of *Anaplasma phagocythopilum*

(*Anaplasma phagocytophilum* / *msp2* gene / polymorphism / strains of *A. phagocytophilum* in Europe)

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Abstract. *Anaplasma phagocytophilum* is an obligate intracellular bacterial parasite of verterbrate granulocytes. This bacterium is the aetiologic agent of human granulocytic anaplasmosis. The *msp2* gene encoding major surface protein 2 is unique for *Anaplasma* and displays high antigenic variation. A fragment of the *msp2* gene (334 bp) of *A. phagocytophilum,* amplified with DNA isolated from *Ixodes ricinus*, *Syringophilidae*, *Capreolus capreolus* and *Canis lupus familiaris*, was used to determine polymorphisms of *Anaplasma* within Polish populations. Sequence analysis of this fragment was used for observation of five different genetic variants of the bacterium within Polish sequences. The average genetic distance in Polish sequences was 0.7 % and the majority of observed substitutions had a synonymous character. High intraspecific variability observed in the *msp2* gene of *A. phagocytophilum* is a strong proof of the high evolutionary plasticity, adaptation abilities, and abilities for fast distribution of this parasite in various environments.

Introduction

Anaplasma phagocytophilum is an obligate intracellular bacterial parasite that in its life cycle uses, among other cells, vertebrate granulocytes. This bacterium is transmitted by ticks, particularly *Ixodes* ticks, in Europe and USA, and causes febrile disease human granulocytic anaplasmosis (HGA). The first infections in the world were recorded in the United States of America in 1994 (Chen et al., 1994). In Europe, HGA was diagnosed in patients from Slovenia in 1996 (Petrovec et al., 1997). However, in Poland, the first patients infected by *A. phagocytophilum* after tick bites were diagnosed in 1999 (Tylewska-Wierzbanowska et al., 2001). So far, no vaccine has been developed for this pathogen. Treatment is commonly based on antibiotics therapy, in which the most effective are doxycycline or rifampin (Maurin et al., 2003).

In terms of epidemiology, the identification of pathogens in material from animals relies on molecular techniques. PCR analysis is a tool that allows quick and accurate detection of *Anaplasma* in tick, human, and other animals. Commonly used methods such as PCR, RT-PCR, and PCR-RFLP or sequencing allow not only identification of bacteria in the host, but namely recognition of genetic information and understanding of processes that are related with keeping pathogens in variable environment.

The small-sized genome of *A. phagocytophilum* consists of a single circular chromosome (Dunning-Hotoop et al., 2006). The sequence of the entire bacterial genome belonging to HZ strain submitted to GenBank database counts 1,471,282 nt (NC 007797). In total 1,411 genes have been identified, including 1,264 protein-coding genes (www.ncbi.nlm.nih.gov). Among other genes, three *omp1,* one *msp2*, two *msp2* homologues, one *msp4*, and 113 loci belonging to the OMP1/MSP2/P44 superfamily, characteristic for Anaplasmataceae, were distinguished in the genome of *A. phagocytophilum* (Dunning-Hotoop et al., 2006). The *msp2* gene-encoded immunodominant protein, major surface protein 2 (MSP2), is detected in the *Anaplasma* such as *A. phagocytophium* or *A. marginale*; although the *msp2* gene of *A. marginale* is distinct from the same gene belonging to *A. phagocytophium* (Barbet et al., 2003; Dunning-Hotoop et al., 2006). In addition, the *msp2* gene has high intraspecific variability. The variability of the *msp2* gene, and in consequence the phenotypic variability of the protein and antigenic variation, helps keeping bacteria in different hosts. It was also observed that the occurrence of various variants of the MSP2 protein depends on geographic distribution (Barbet et al., 2003; Dumler et al., 2003; Lin et al., 2004).

The aim of the presented study was to demonstrate the genetic variability in the *msp2* gene fragment of *A. phagocytophilum*, amplified with DNA isolated from animal material (ticks, deer, dogs, *Syringophilidae*) originating from two regions of Poland (West Pomera-

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Abbreviations: HGA – human granulocytic anaplasmosis, MSP2 – major surface protein 2.

nia and Wielkopolska). The results indicate bacterial diversity occurring in the natural environment. In addition, these results demonstrate bacterial capability of infecting humans and livestock.

Material and Methods

Study material consisted of DNA isolated from *I. ricinus* ticks collected from vegetation occurring in the Dabie Forest Park (six specimens), dog blood with confirmed borreliosis (one specimen), and deer blood of *Capreolus capreolus* (four specimens). These specimens originated from West Pomerania, and *Syringophilidae* – quill mite obtained from birds (one specimen) – from the National Park of Wielkopolska (Wielkopolska, Poland). DNA was isolated with MasterPure™ DNA Purification Kit (Epicentre, Madison, WI, USA) and stored at -20 °C until analysed. A fragment of *msp2* gene with 334 bp length was used for amplification of DNA *A. phagocytophilum*. This fragment was determined with two primers, msp2-3F and msp2-3R (Levin et al., 2002). For PCR reaction, all conditions described in Levin et al. (2002) were applied.

All specimens were sequenced using the same set of primers for amplification as in the initial PCR reaction. Sequencing was performed at the Institute of Biochemistry and Biophysics, Polish Academy of Science (Warsaw, Poland). Multiple alignment analysis, distance matrix calculation, and construction of a homology tree were performed using MEGA4 and DNAMAN software (Lynnon BioSoft, Pointe-Claire, Canada). Distance matrices for the aligned sequences were calculated using the Kimura two-parameter method (Kimura, 1980), and the observed divergence method was used for constructing a homology tree.

Nucleotide sequence accession number used in this experimental work

Sequences of *A. phagocytophilum* stored in GenBank databases (http://www.ncbi.nlm.nih.gov/genbank/) (Table 1) were used for sequence comparisons and construction of homology tree based on the *msp2* gene fragment.

Nucleotide sequence accession number from Poland

DNA sequences obtained from West Pomerania and Wielkopolska (Poland) were deposited at GenBank database under accession numbers AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673. Detailed description of these DNA sequences is presented in Table 1.

Results and Discussion

Sequences from Poland were compared with analogical sequences from the USA (Table 1). Sequence with accession number AY166490 (3,801 bp) was the longest sequence used in our analysis. Positions of polymorphisms for all compared sequences were given against this sequence. The remaining American sequences were 2,861 bp long (position 521–3,381 bp for AY166490).

Table 1. Sequences of the msp2 gene fragment used in the analysis of polymorphism in A. phagocytophilum

Accession number	Country	Host	References
AY642115	Poland/WNP	*Syringophilidae*	Rymaszewska, this study
DQ105669	Poland /Pomerania	dog	Rymaszewska, this study
DQ105670	Poland /Pomerania	*C. capreolus*, strain ST91	Rymaszewska, this study
DQ105671	Poland /Pomerania	*C. capreolus*, strain ST128	Rymaszewska, this study
DQ105672	Poland /Pomerania	*I. ricinus*, strain KDR-1	Rymaszewska, this study
DQ105673	Poland /Pomerania	*I. ricinus*, strain KDR-2	Rymaszewska, this study
AY164490	USA	strain NY18 cultured in HL-60	Barbet et al., 2003
AY164491	USA	strain Webster cultured in HL-60	Barbet et al., 2003
AY164492	USA	strain HGE2 cultured in ISE6	Barbet et al., 2003
AY164493	USA	sample from patient-2 blood collected on day 3	Barbet et al., 2003
AY164494	USA	sample from patient-2 blood collected on day 27	Barbet et al., 2003

Table 2. Sequence polymorphism of the msp2 gene fragment. Parsimony informative sites were marked with asterisk.

	2035*	2041*	2042*	2062*	2113	2122	2125*	2140	2154	2182	2186	2215	2269*	GENOTYPE
AY642115	A	A	C	T	C	T	T	T	A	G	A	A	G	GENOTYPE 1
DQ105669	T	T	A	T	C	T	T	C	A	G	A	A	G	GENOTYPE 2
DQ105670	T	T	A	T	C	T	T	C	A	G	A	A	G	GENOTYPE 2
DQ105671	A	T	C	A	C	T	T	C	A	G	A	A	G	GENOTYPE 3
DQ105672	A	T	C	T	C	T	C	C	A	G	A	A	G	GENOTYPE 4
DQ105673	A	T	A	T	C	T	T	C	A	G	A	A	G	GENOTYPE 5
AY164490	A	A	C	A	C	G	C	C	A	G	A	A	A	GENOTYPE 6
AY164491	A	T	A	A	C	T	T	C	A	G	A	A	A	GENOTYPE 7
AY164492	A	A	C	T	T	T	T	C	G	G	A	A	G	GENOTYPE 8
AY164493	T	G	C	A	C	T	T	C	A	A	A	T	G	GENOTYPE 9
AY164494	A	A	C	T	C	T	T	C	A	G	G	A	G	GENOTYPE 10

The shortest sequences were represented by Polish sequences of 334 bp (position 1,937–2,270 for AY166490).

In sequences originating from Poland, five genotypes were differentiated based on six nucleotide substitutions (Table 2). The phenotype of *A. phagocytophilum* marked as 2 occurred in the isolate obtained from two *C. capreolus* (DQ105670) and *Canis lupus familiaris* (DQ105669), genotype 3 – from two deer (DQ105671), genotype 4 was obtained from four *I. ricinus* ticks (DQ105672), and genotype 5 from two ticks (DQ105673). Transversion of A↔T type and rarely of A↔C type made as much as 66.67 % of the observed changes within the Polish sequences. In addition, seven polymorphic sites (Table 2), from which 71.4 % are transitions, one T↔C and the rest of A↔G type, were observed by comparing Polish sequences to the American ones. Regardless of the type of mutation, 76.9 % of them had a character of synonymic changes, which did not result in amino acid substitutions in the protein.

Only four substitutions at positions 2,042, 2,154, 2,186 and 2,215 (positions determined in reference to the longest analysed sequence AY164490) were detected as missense mutation. The consequences of these mutations were determined based on deduced translation that was carried out. At position 2,042, the first codon base was substituted (A↔C) and resulted in the change of asparagine (N) into histidine (H) in the MSP2 protein; both amino acids are polar and have basic properties (Table 3). Histidine-coding sequences occurred in four specimens from patients or cell tissues originating

Table 3. Character of changes in DNA sequence and its consequences in the translation process. Position of a changed nucleotide in DNA was given according to the sequence with accession number AY164490 (3,801 nt).

ACCESSION NUMBER (GenBank)	SITE IN DNA*	CODON	AA	MUTATION
AY642115, DQ105671, DQ105672, DQ105673, AY164490, AY164491, AY164492, AY164494	2035	CT**A**	L	Synonymous substitution
DQ105669, DQ105670, AY164493		CT**T**	L	
AY642115, AY164490, AY164492, AY164494	2041	TC**A**	S	Synonymous substitution
DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164491		TC**T**	S	
AY164493		TC**G**	S	
AY642115, DQ105671, DQ105672, AY164490, AY164492, AY164493, AY164494	2042	**C**AC	H	Non-synonymous substitution
DQ105669, DQ105670, DQ105673, AY164491		**A**AC	N	
AY642115, DQ105669, DQ105672, DQ105673, AY164492, AY164494	2062	AC**T**	T	Synonymous substitution
DQ105670, DQ105671, AY164490, AY164491, AY164493		AC**A**	T	
AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164490, AY164491, AY164493, AY164494	2113	GG**C**	G	Synonymous substitution
AY164492		GG**T**	G	
AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164491, AY164492, AY164493, AY164494	2122	GG**T**	G	Synonymous substitution
AY164490		GG**G**	G	
AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164491, AY164492, AY164493, AY164494	2125	TA**T**	Y	Synonymous substitution
DQ105672, AY164490		TA**C**	Y	
DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164490, AY164491, AY164492, AY164493, AY164494	2140	GC**C**	A	Synonymous substitution
AY642115		GC**T**	A	
AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164490, AY164491, AY164493, AY164494	2154	G**A**G	E	Non-synonymous substitution
AY164492		G**G**G	G	
AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164490, AY164491, AY164492, AY164494	2182	AA**G**	K	Synonymous substitution
AY164493		AA**A**	K	
AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164490, AY164491, AY164492, AY164493	2186	**A**TT	I	Non-synonymous substitution
AY164494		**G**TT	V	
AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164490, AY164491, AY164492, AY164494	2215	GA**A**	E	Non-synonymous substitution
AY164493		GA**T**	D	
AY642115, DQ105669, DQ105670, DQ105671, DQ105672, DQ105673, AY164492, AY164493, AY164494	2269	GG**C**	G	Synonymous substitution
AY164490, AY164491		GG**A**	G	

Table 4. Pair-wise distance calculation of 11 sequences of the msp2 gene fragment of A. phagocytophilum (Model: Nucleotide: Kimura 2-parameter)

	AY642115	DQ105669	DQ105670	DQ105671	DQ105672	DQ105673	AY164490	AY164491	AY164492	AY164493	AY164494
AY642115											
DQ105669	0.012										
DQ105670	0.015	0.003									
DQ105671	0.009	0.009	0.006								
DQ105672	0.009	0.009	0.012	0.006							
DQ105673	0.009	0.003	0.006	0.006	0.006						
AY164490	0.015	0.021	0.018	0.012	0.012	0.018					
AY164491	0.015	0.009	0.006	0.006	0.012	0.006	0.012				
AY164492	0.009	0.015	0.018	0.012	0.012	0.012	0.018	0.018			
AY164493	0.018	0.015	0.012	0.012	0.018	0.018	0.021	0.018	0.021		
AY164494	0.006	0.012	0.015	0.009	0.009	0.009	0.015	0.015	0.009	0.018	

from the USA and in three Polish specimens obtained from our own studies. The asparagine-coding codon occured in all three sequences from Poland (isolates from dog, deer and tick). At position 2,154 (the second base in the codon), transition A↔G makes a codon that results in the change of polar glutamic acid (E) into non-polar, hydrophobic glycine (G). The presence of GGG codon determining glycine was detected in one sequence (AY164494) which originated from the isolate of one patient (USA, collected on 27th day of the disease). The third nucleotide change of missense type concerns position 2,186, where substitution of the first nucleotide in the codon (transition A↔G) results in the change of isoleucine into valine in the protein. Both amino acids are non-polar and hydrophobic regarding their physicochemical properties. The consequence of the fourth mutation, transversion (A↔T) at position 2,215 (third base in the codon) was the change of amino acid in the codon determining glutamic acid (polar). The sequence registered under accession number AY166493 (isolate from blood of an American patient collected on the third day of the disease) is the result of the mutation where glutamic acid was replaced by the codon for aspartic acid (polar) (Table 3).

The frequency of individual bases in the fragment containing 334 bp was comparable in all sequences. The average value for G and C bases was 29.7 % and 13.1 %, respectively. Ratio substitutions of transition type to substitutions of transversion type (R) were 0.7 on average in the fragment containing 334 nucleotides, which is common for all 11 compared sequences. The lowest genetic distance (0.3 %) was observed in specimens coming from Poland, such as DQ105669 (isolate from dog), DQ105670 (isolate from deer, strain ST91), DQ105669 and DQ105673 (isolate from tick, strain KDR-2). The longest genetic distance (2.1 %) was recorded for specimens originating from the USA, such as AY166493 (isolate from patient), AY166490 (isolate from cell culture), and between sequences AY166490 (USA) and DQ105669 (isolate from dog, Poland) (Table 4). The mean genetic distance for all studied specimens was 1.2 %.

In the fragment that was 3,801 bp long, 124 parsimony informative sites were determined. On the other hand, in the fragment 334 bp long, where 11 comparative sequences were overlapping, only six informative places were observed at positions 2,035, 2,041, 2,042, 2,062, 2,125, 2,269 (Table 2). Five informative places were represented by synonymic mutations. However, weak selection could also occur between them, i.e. some codons could be preferential at the expense of others. Homology tree based on the model of observed divergency indicates similarity of the compared sequences (Fig. 1). Sequences AY164490 and AY164493 are on separate branches, although the remaining sequences make a common clad.

Isolates of *A. phagocytophilum* studied by various authors show high diversity in pathogenicity in relation to the species of vertebrates in which they are parasites. These differences are also related with the geographic region where they are observed and the interaction between bacteria and vectors, reservoir and/or host occurring in the given territory (Lin et al., 2004). One of the genes that had an important function in the adaptation of *Anaplasma* to the environment is the *msp2* gene, which encodes the antigenic protein MSP2 (Barbet et al., 2003; Lin et al., 2004; Scorpio et al., 2004). According to Scorpio et al. (2004), MSP2 diversity could be an important adaptation factor to alternate niches, such as small mammal or cervid reservoir of neutrophils or tick cells.

In our study, it was demonstrated that bacterium *A. phagocytophilum*, representing some different genotypes that were established based on a fragment of the *msp2* gene, occurs in neutral environment in West Pomerania (Poland). Five different variants, which were determined during analysis of nucleotide sequences, are unique in relation to the compared sequences originating from the USA. All variants are results of substitution-type point mutations, and do not result in changes in the length of the encoded protein. If we take under consideration only missense-type mutations, which could have impact on protein properties, then only two variants containing histidine or asparagine at position 681 could be determined within Polish sequences. Because both amino acids have similar physicochemical properties, it seems highly improbable that they could in some way differentiate the Polish *Anaplasma* strains.

The three remaining missense substitutions were only observed in sequences of the *msp2* gene stored in GenBank (specimens from the USA). Within these three

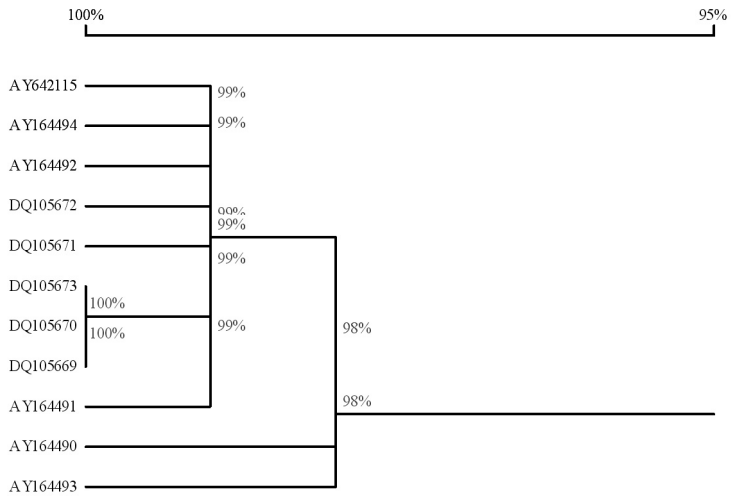

Fig. 1. Tree similarity determined by the analysis of sequences of the *msp2* gene fragment of *A. phagocytophilum* is based on the model of observed divergence.

missense substitutions, only transitions at position 2,154 could have impact on the change of physicochemical properties of the protein. Non-polar, hydrophobic glycine was observed only in one specimen coming from cell culture of strain HGE2 cultured in ISE6 (tick cell line of *Ixodes scapularis*). Similarly to analysed fragments of the *msp2* gene from Poland, also isolates from the USA displayed a high degree of variability. The aim of the comparison of Polish and American nucleotide sequences was to determine the conformity between genotypes of bacteria discovered in patients from the USA and animals recognized as vectors or reservoirs of *Anaplasmas* in Europe. No conformity was observed between the genotypes. High intraspecific variability between populations of *A. phagocytophilum* can serve as a proof of their adaptation properties. Variability within different genes for European and American strains has been described many times. However, the search for interrelation between genotypes, pathogenicity and adaptation to the environment (vectors and reservoirs) is still going on (Massung et al. 2002, 2007; von Loewenich et al., 2003; Poitout et al., 2005; Ladbury et al., 2008; Rymaszewska, 2008; Gaowa et al., 2009; Zeman and Jahn, 2009).

Can our observations of variability serve as a proof of bacterial adaptation to the new environments and reservoirs? Do more benign bacterial strains occur in Poland and in the entire Europe?

The occurrence of *Anaplasma* in *I. ricinus* ticks of wild ruminants (*C. capreolus* and *C. elaphus*) and the evidence of detected bacterial DNA in dogs susceptible to tickborne diseases were recorded many times in Poland (Skotarczak et al., 2003; Adamska and Skotarczak, 2007; Skotarczak et al., 2008; Wodecka et al., 2009). There is lack of information about infections within humans and livestock in West Pomerania, where for many years a programme of monitoring the occurrence of *A. phagocytophilum* DNA has been running. Maybe the fact that these tickborne infections are too benign, undiagnosed, or very rarely diagnosed lies behind the reason why it is impossible to get the real statistics of the infections. However, similar tendencies are observed in the neighbouring countries. No cases of HGA infections were confirmed in Germany, Czech Republic, Russia or Baltic countries, although there are data about detection of *A. phagocytophilum* DNA in ticks or wild or home animals (Hulínská et al., 2002; Shpynov et al., 2006; Zeman and Jahn, 2009). In Germany, Hunfeld and Brade (1999) demonstrated the presence of anti-HGA (formerly anti-HGE) antibodies in patients with recognized Lyme borreliosis or suspicion of this disease (positive Lyme serology but lack of the clinical symptoms of borreliosis). These authors encountered seroactivity against the HGA agent in 5.5 % individuals tested, while these cases were not diagnosed as human granulocytic anaplasmosis. Cross-reaction between HGA and *Borrelia burgdorferi* could not be excluded. Hulínská et al. (2002) observed the presence of DNA of *Anaplasma* in 17.5 % studied patients, and these patients consisted of individuals with different manifestations of Lyme borreliosis or were represented by huntsmen. Most cases of anaplasmosis were detected in a system of co-infection with *B. burgdorferi*. Based on the described cases, it could be stated that double infection markedly deteriorates the patient's condition. Contrary to these data, information from the USA indicates 600–800 cases of anaplasmosis that are reported to CDC every year (www.cdc.gov/ticks/diseases/anaplasmosis/faq.html). The course of HGA is more serious in patients from the USA than in the European cases. Approximately one-half of patients required hospitalization (56 %) and mortality was recorded on 0.5% level. In Europe, no decease caused by HGA was recorded (Blanco and Oteo, 2002; Dumler, 2005).

It may be suggested that the strains of *A. phagocytophilum* occurring in Europe have lower virulence than the strains from the USA. High variability observed among others within the *msp2* gene could serve as a proof of higher evolutionary plasticity of the bacterium, its adaptation ability and its ability of fast distribution in the environment.

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