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Genotyping of *Anaplasma phagocytophilum* Strains from Poland for Selected Genes

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Anaplasma phagocytophilum is a veterinary and medical tick-borne pathogen of vertebrates which invades granulocytes. The potential reservoirs of *Anaplasma* include game animals and small mammals. The aim of this study was to gain insight into the variability of nucleotide sequences of ribosomal and selected protein-coding genes (*ankA, msp4* and the *groESL* heat-shock protein operon) present in isolates of *A. phagocytophilum* collected from wild ruminants (*Cervus elaphus* and *Capreolus capreolus*) in Poland. Fragments of the genes were amplified using PCR and sequenced. High DNA variability was found in fragments of the *msp4* and *groESL* genes (15 and 8 alleles, respectively), whereas *ankA* and 16S rRNA demonstrated very little or no variability (2 and 1 alleles, respectively). Unique allelic profiles were determined for the examined groups and allowed distinguishing 6 *A. phagocytophilum* genotypes, 2 of which were reported only in the roe deer (*C. capreolus*), and 1 in the red deer (*C. elaphus*).

Key words: *Anaplasma phagocytophilum* variants, potential reservoir of *Anaplasma phagocytophilum*, pathogenic strains of *A. phagocytophilum,* wild ruminants as potential reservoirs of *Anaplasma*.

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Gram-negative bacteria of the *Anaplasma* genus are obligatory intracellular pathogens of both humans and domestic animals. *A. phagocytophilum* are transmitted by ticks, in Europe mainly by those belonging to the *Ixodes* genus (PETROVEC *et al.* 1999; RYMASZEWSKA & SKOTARCZAK 2005; SANTOS *et al.* 2004; SKOTARCZAK *et al.* 2003). *A. phagocytophilum* infects the blood of vertebrates, demonstrating tropism for neutrophils in which it forms membrane-bound cytoplasmic vacuoles (DUMLER *et al.* 2001). In the wild, *Anaplasma* has been detected in ruminants such as the roe deer, *Capreolus capreolus,* or the red deer, *Cervus elaphus*, as well as in small mammals (ADAMSKA 2006; LIZ *et al.* 2002; PETROVEC *et al.* 2002), all of which represent potential reservoirs of the bacterium. In humans and farm animals such as sheep, calves and goats, *A. phagocytophilum* causes a disease known as granulocytic anaplasmosis (GA) (DUMLER *et al.* 2001; PETROVEC *et al.* 1999).

There are numerous reports on *A. phagocytophilum* infecting ticks or some domestic and wild animal species, and there is an increasing incidence of human anaplasmosis in Europe, America and Asia (JIN *et al*. 2012). The symptoms of human granulocytic anaplasmosis (HGA) are markedly less severe in Europe than in North America. In Europe, infection incidence varies depending on geographic location, with the disease being more common, for example, in the Mediterranean and in Central rather than Eastern Europe (BLANCO & OTEO 2002; CHOCHLAKIS *et al.* 2009; PETROVEC *et al.* 1999). Consequently, it appears that some *A. phagocytophilum* strains may be more virulent than others.

Knowledge of the variability of genes coding for proteins critical for the functioning of bacterial cells may lead to a better understanding of the way in which *Anaplasma* circulates and spreads in the environment.

The aim of the study was to examine the nucleotide variability of ribosomal and selected protein-coding genes in sequences from West Pomerania (Poland) and to compare them to homologous sequences deposited in GenBank with respect to the origin of *A. phagocytophilum* (host and area).

Material and Methods

In order to genotype *A. phagocytophilum*, DNA isolates obtained from the blood of wild game species including the roe deer, *Capreolus capreolus* (12 samples), and the red deer, *Cervus elaphus* (3 samples), were used. The animals had been hunted in West Pomerania (Poland) in 2004-2005 (biological material was provided by hunters during seasonal hunting, no special permission required). Blood samples of 1 ml, collected from the animals, were placed in Eppendorf tubes. DNA was isolated using the phenol-chloroform extraction procedure and stored at -70°C until analysis (HALOS *et al.* 2004). Only those isolates in which *Anaplasma* DNA was detected as a result of screening were selected (15 samples).

For genotyping, sequences of *msp4, ankA*, 16S rRNA, and a fragment of the *groESL* operon, amplified using specific primers (Table 1) were used. Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland) at a concentration of 0.5 U/20 μl of mixture was used for PCR. Final reagent concentrations were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM for each deoxynucleoside triphosphate, 10 pM of each primer and 2 μl DNA. The PCR regime was adjusted following the requirements of Phusion High-Fidelity DNA Polymerase, according to the manufacturer's recommendations. Annealing temperatures for each primer pair are given in Table 1. PCR was performed using additional negative and positive control samples. As a template for nested PCR, one microliter of the primary PCR mixture was used, previously diluted 10×. The results of PCR amplification were visualized by electrophoresis of 2-5 μl of each sample on 1.5% agarose gels with ethidium bromide.

All samples that produced positive results were selected for sequencing, which was performed at Macrogen Inc. (Seoul, South Korea). Amplification was conducted with a set of primers identical to those used in the nested PCR and the results were compared with each other and with corresponding sequences deposited in GenBank. Table 2 presents accession numbers of the bacterial gene sequences used for the homology analysis. Multiple alignment analysis and distance matrix calculation were performed and a homology tree was constructed using the DNAMAN software (Lynnon BioSoft, 1994-2001, Canada) and MEGA4 (TAMURA *et al.* 2007). Estimates of genetic divergence (K) were calculated with the Kimura two-parameter model (KIMURA 1980). Divergences in the protein-coding genes both at synonymous (K_s) and non-synonymous (K_a) sites were calculated using the modified Nei-Gojobori method (NEI 1986) with Jukes-Cantor correction. All diversity indicators were calculated in MEGA4.

Results

Analysis of the *ankA* gene fragment

Nucleotide sequences obtained from Polish *A. phagocytophilum* isolates were compared and two characteristic variants of the *ankA* gene were found. One variant, marked as PZA-1, was found in 12 isolates from roe deer blood, *C. capreolus*, and 2 isolates from *C. elaphus* blood. The other variant, marked as PZA-2, occurred in only one sample (from the red deer, *C. elaphus*). Both se-

Table 1

The primers used to amplify DNA of *Anaplasma phagocytophilum*

Gene	Primer's sequence	Annealing Temperature	PCR product	Reference
msp4	**MSP4AP5**: 5'– ATGAATTACAGAGAATTGCTTGTAGG **MSP4AP3**: 5'– TTAATTGAAAGCAAATCTTGCTCCTATG	58 °C	849 bp	de la Fuente *et al*. 2005, JCM 43: 1309, Bown *et al.* 2007, JCM 45: 1771
	MSP4F: 5'– CTATTGGYGGNGCYAGAGT **MSP4R**: 5'– GTTCATCGAAAATTCCGTGGTA	58 °C	452 bp	
ankA	**ANK-F1**: 5'– GAAGAAATTACAACTCCTGAAG **ANK-R1**: 5' – CAGCCAGATGCAGTAACGTG	55°C	721 bp	Massung *et al*. 2007, JCM 45: 2138
	ANK-F2: 5'– TTGACCGCTGAAGCACTAAC **ANK-R2**: 5'– ACCATTTGCTTCTTGAGGAG	55°C	667 bp	
16S rRNA	**PER 1**: 5'– TTTATCGCTATTAGATGAGCCTATG **PER 2**: 5'– CTCTACACTAGGAATTCCGCTAT	45°C	452 bp	Goodman *et al*. 1996, NEJM 334:209
groESL	**HS1**: 5'– TGGGCTGGTAMTGAAAT **HS6**: 5'– CCICCIGGIACIAYACCTTC	48°C	1350 bp	Sumner *et al*. 1997, JCM 35:2087
	HSVR: 5'– CTCAACAGCAGCTCTAGTAGC **HS43**: 5'– ATWGCWAARGAAGCATAGTC	52°C	1297 bp	Lotric-Furlan *et al*. 1998, CID, 27:424

Table 2

The sequences used for comparative analysis obtained from GenBank

Gene	Accesion number	Country	Samples (strain)	Reference (Gen Bank)
msp4	EU857674	USA	*Homo sapiens* (Webster)	Laloy *et al*., unpublished
	AY530194	USA	*Homo sapiens* (NY)	de la Fuente *et al*., 2005
	AY530195	USA	*Homo sapiens* (W1-H1)	de la Fuente *et al*., 2005
	AY530196	USA	*Equus caballus* (MRK)	de la Fuente *et al*., 2005
	AY530197	USA	*Ovis aries* (Ap-V1)	de la Fuente *et al*., 2005
	HM028679	Germany	*Bos taurus*	Silaghi *et al*., unpublished
	HM028678	Germany	*Bos taurus*	Silaghi *et al*., unpublished
	HM028677	Germany	*Bos taurus*	Silaghi *et al*., unpublished
	AY530198	Switzerland	*Bos taurus* (Eliza)	de la Fuente *et al*., 2005
	HM028680	Switzerland	*Bos taurus*	Silaghi *et al*., unpublished
	HM028674	Switzerland	*Capra aegagrus hircus*	Silaghi *et al*., unpublished
	HM028675	Switzerland	*Capra aegagrus hircus*	Silaghi *et al*., unpublished
	EU857673	France	*Ovis aries*	Laloy *et al*., unpublished
	EU857672	France	*Ovis aries*	Laloy *et al*., unpublished
	EU008082	China	*Niviventer fulvescens*	Zhan *et al*., 2008
ankA	GU236806	USA	*Homo sapiens*	Scharf *et al*., 2011
	GU236807	USA	*Homo sapiens*	Scharf *et al*., 2011
	GU236809	USA	*Homo sapiens*	Scharf *et al*., 2011
	GU236811	USA	*Homo sapiens*	Scharf *et al*., 2011
	GU236861	Germany	*Equus caballus*	Scharf *et al*., 2011
	GU236855	Germany	*Equus caballus*	Scharf *et al*., 2011
	GU236862	Switzerland	*Equus caballus*	Scharf *et al*., 2011
	GU391597	France	*Canis lupus familiaris*	Domingos *et al*., 2011
	GU391598	France	*Canis lupus familiaris*	Domingos *et al*., 2011
	GU236815	Slovenia	*Canis lupus familiaris*	Scharf *et al*., 2011
	GU236819	Germany	*Canis lupus familiaris*	Scharf *et al*., 2011
	GU236731	Norway	*Bos taurus*	Scharf *et al*., 2011
	GU236755	Norway	*Ovis aries*	Scharf *et al*., 2011
	GU236757	Norway	*Ovis aries*	Scharf *et al*., 2011
	GU236758	Norway	*Ovis aries*	Scharf *et al*., 2011
	GU236799	Norway	*Ovis aries*	Scharf *et al*., 2011
	GU236718	Slovenia	*Cervus elaphus*	Scharf *et al*., 2011
	GU236864	Austria	*Felis catus*	Scharf *et al*., 2011
groESL	AF033101	Slovenia	*Homo sapiens*	Petrovec *et al*., 1999
	AF172159	USA	*Homo sapiens*	Chae *et al*., 2000
	U96728	USA	*Homo sapiens*	Sumner *et al*., 1997
	EU157921	Poland	*Capreolus capreolus*	Rymaszewska, 2008
	AF478556	Slovenia	*Capreolus capreolus*	Petrovec *et al*., 2002
	AF478559	Slovenia	*Capreolus capreolus*	Petrovec *et al*., 2002
	AF478554	Slovenia	*Capreolus capreolus*	Petrovec *et al*., 2002
	AF478551	Slovenia	*Capreolus capreolus*	Petrovec *et al*., 2002
	AF478560	Slovenia	*Capreolus capreolus*	Petrovec *et al*., 2002
	AF478564	Slovenia	*Capreolus capreolus*	Petrovec *et al*., 2002
	AF383226	Switzerland	*Capreolus capreolus*	Liz *et al*., 2002
	AF383227	Switzerland	*Capreolus capreolus*	Liz *et al*., 2002
	DQ779567	Poland	*Cervus elaphus*	Rymaszewska, 2008
	AF478552	Slovenia	*Cervus elaphus*	Petrovec *et al*., 2002
	AF478553	Slovenia	*Cervus elaphus*	Petrovec *et al*., 2002
	AF478557	Slovenia	*Cervus elaphus*	Petrovec *et al*., 2002
	AF478562	Slovenia	*Cervus elaphus*	Petrovec *et al*., 2002
	AF478563	Slovenia	*Cervus elaphus*	Petrovec *et al*., 2002
	AY529490	Sweden	*Equus caballus*	Franzen *et al*., unpublished
	AF548385	Norway	*Ovis aries*	Petrovec *et al*., 2002
	AF548386	Norway	*Ovis aries*	Petrovec *et al*., 2002
	AY281848	Germany	*Ixodes ricinus*	von Loewenich *et al*., 2003
	AY219849	USA	*Canis lupus familiaris*	Liddell *et al*., 2003
16S rRNA	GU236654	Slovenia	*Homo sapiens*	Scharf *et al*., 2011
	GU236655	Slovenia	*Homo sapiens*	Scharf *et al*., 2011
	U02521	USA	*Homo sapiens*	Chen *et al*., 1994
	AF481852	Slovenia	*Capreolus capreolus*	Petrovec *et al*., 2002
	AF481851	Slovenia	*Capreolus capreolus*	Petrovec *et al*., 2002
	HM480385	Germany	*Capreolus capreolus*	Heyl *et al*., unpublished
	HM480384	Germany	*Capreolus capreolus*	Heyl *et al*., unpublished
	HM480382	Germany	*Capreolus capreolus*	Heyl *et al*., unpublished
	GU236574	Norway	*Capreolus capreolus*	Scharf *et al*., 2011
	GU236637	Norway	*Ovis aries*	Scharf *et al*., 2011
	GU236639	Norway	*Ovis aries*	Scharf *et al*., 2011
	GU236585	Norway	*Bos taurus*	Scharf *et al*., 2011
	GU236668	Slovenia	*Canis lupus familiaris*	Scharf *et al*., 2011
	GU236669	Slovenia	*Canis lupus familiaris*	Scharf *et al*., 2011
	HM138366	Czech Republic	*Felis catus*	Hulinska, unpublished
	HM366581	Russia	*Ixodes persulcatus*	Rar *et al*., 2011
	HM366585	Russia	*Ixodes persulcatus*	Rar *et al*., 2011
	HM366586	Russia	*Ixodes persulcatus*	Rar *et al*., 2011

Table 3

Characteristics of nucleotide sequences of selected fragments of genes

Gene	Sequence lenght (bp)	Variable sites n (%)	Trans-version	Transi-tion	Codon number	Nucleotide position change in codon (position)			Character substitution	
						1	2	3	sense	missense
*groESL**	1 210	28 (2.3)	7	22	402	4	0	24	27	1
ankA	654	33 (5.0)	10	23	217	9	14	10	3	30
msp4	861	75 (9.2)	17	62	287	14	5	56	49	16
16S rRNA	1 143	11 (0.9)	2	9	–	–	–	–	–	–

* – statistics only for sequences Polish

Table 4

Sequence divergence in the protein-coding genes both in synonymous (K_s) and non-synonymous (K_a) sites

Distance / *Genes*	*K*	K_s	K_a
ankA	0.03	0.02	0.04
groESL	0.01	0.04	0.01
msp4	0.06	0.18	0.01

quences were deposited in GenBank under accession numbers JN005740 and JN005741, respectively. Thereafter, variability analysis of all 20 nucleotide sequences was carried out using these sequences and those downloaded from GenBank (Table 2). The longest sequences were 3726 bp, and the shortest were 654 bp. Within the entire gene, 231 substitutions were found. Detailed analysis was conducted on a 654 bp fragment of the gene, whose length corresponded to that of the sequences obtained from the Polish isolates, and 33 nucleotide substitutions were found. In a single case, the substitutions pertained to three positions in codon 746, and in five cases, the substitutions pertained to two positions (in codons 683, 713, 775, 778, 817). The remaining substitutions involved single bases per codon. The second nucleotide in a codon was substituted most often; 14 such substitutions were found, i.e. 42.4% of all substitutions observed in this fragment. The degree of changes in the first and the third position was comparable, 27.3% and 30.3%, respectively (Table 3). The index of non-synonymous substitutions (K_a= 0.04) was higher for the analyzed fragment, as compared to synonymous ones (K_s= 0.02) (Table 4).

Protein sequences revealed six alterations in the following codons: 683 (Val/Ser), 690 (Leu/Pro), 692 (Glu/Ala), 776 (Ala/Pro), 778 (Leu/Ser), and 813 (Asn/Asp), which divided the samples into two groups. The gene variant containing, respectively, triplets for Val, Leu, Glu, Ala, Leu, and Asn at these codons was found in human pathogenic *A. phagocytophilum* strains. Moreover, this version of the gene occurred in nucleotide sequences derived from a roe deer from Poland (PZA-1) and ruminants from Norway.

The phylogenetic tree in Figure 1 shows relationships among the samples from Poland and those from other European countries and the USA. In order to construct the tree, nucleotide sequences derived from roe deer from Germany (GU236865, GU236877, GU236878) and Slovenia (GU236906), as well as from red deer and a bison from Poland (GU236724, GU236725, GU236742) were additionally used. The sequences were characterized by a relatively very high variability. The analyzed sequences form four main clades, one of which contains 5 sequences, including 4 from humans from the USA and one from Poland, PZA-1. The second clade contains European sequences of various origin, e.g., from ruminants, dogs and cats. In this clade, one subgroup is formed by the Polish sequences PZA-2 and GU236725 and a sequence from Slovenia, all isolated from red deer. Two separate clades are formed by sequences isolated from *C. capreolus* from Germany and Slovenia and *C. elaphus* from Poland.

Analysis of the *msp4* fragment

A total of 30 *msp4* gene sequences of *A. phagocytophilum* were compared, including 15 described herein and homologous sequences from Europe and the USA deposited in GenBank (Table 2). All nucleotide sequences were deposited in GenBank un-

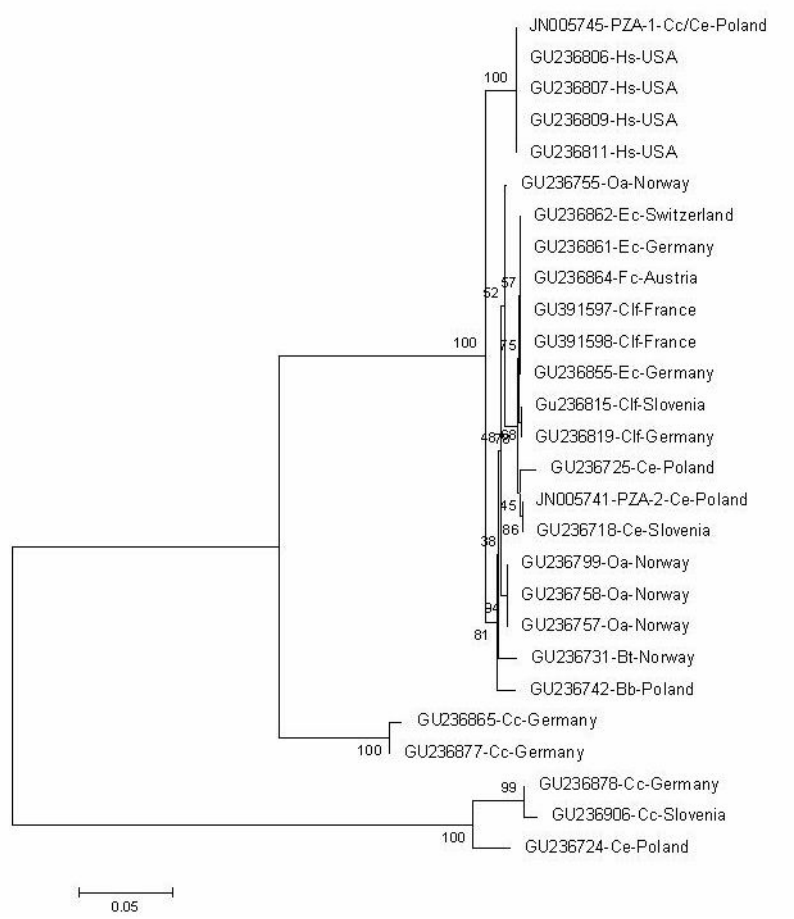

Fig. 1. Phylogenetic analysis of *Anaplasma phagocytophilum* strains based on the *ankA* sequences using the Neighbor Joining method with Kimura two-parameter correction. Strains of *A. phagocytophilum* included in the analysis are described in Table 2. Abbreviations: Bb – *Bison bonasus*, Bt – *Bos taurus*, Cc – *Capreolus capreolus*, Ce – *cervus elaphus*, Clf – *Canis lupus familiaris*, Ec – *Equus caballus*, Fc – *Felis catus*, Hs – *Homo sapiens*, Oa – *Ovis aries.*

der accession numbers: JN005725, JN005726, JN005727, JN005728, JN005729, JN005730, JN005731, JN005732, JN005733, JN005734, JN005735, JN005736, JN005737, JN005738, JN005739. Five genotypes found in this study were identical to the sequences previously deposited in GenBank. The length of the compared sequences ranged from 318 to 849 bp, which corresponds to 106 to 283 codons. Sequences were highly variable. Within the analyzed fragment, 75 substitutions were found (throughout the compared fragment), including 42 in the deer sequences from Poland described by the author. In four of them, two substitutions were observed in positions 360, 405, 411, and 464. Transitions were the prevailing type of the observed substitutions (72.5% of all cases).

Parsimony-informative sites represented 78.7% (59 substitutions) of all changes and 50.7% (38 substitutions) of the Polish sequences. In eleven cases, substitutions of two bases per codon were found, whereas the remaining ones were single-base substitutions, most often concerning the third base in a codon (90.6%, Table 3). Consequently, substitutions in the DNA produced 15 amino-acid substitutions in the studied fragment (K_a = 0.01, Table 4). The mean genetic distance for all analyzed *A. phagocytophilum* samples was 0.066 (taking into account sequence EU008082 from China – 0.099). On the phylogenetic tree (Fig. 2), the PZM-14 sequence (JN005738, red deer) from Poland is closely related to the sequences from small ruminants (*O. aries,* France and *Capra aegagrus hircus,* Switzerland). The sequence described as PZM-15 (JN005739, red deer) is closely related to a sequence derived from *Bos taurus* from Germany. The remaining sequences from Poland group separately, whereas one group is more closely related to the other strains in the database. The nucleotide sequences of the *msp4* gene derived from patients form a common clade containing sequences isolated from horses and sheep, all of them originating from the USA.

Analysis of the *groESL* operon fragment

Three of the analyzed nucleotide sequences in the studied populations were the same as previ-

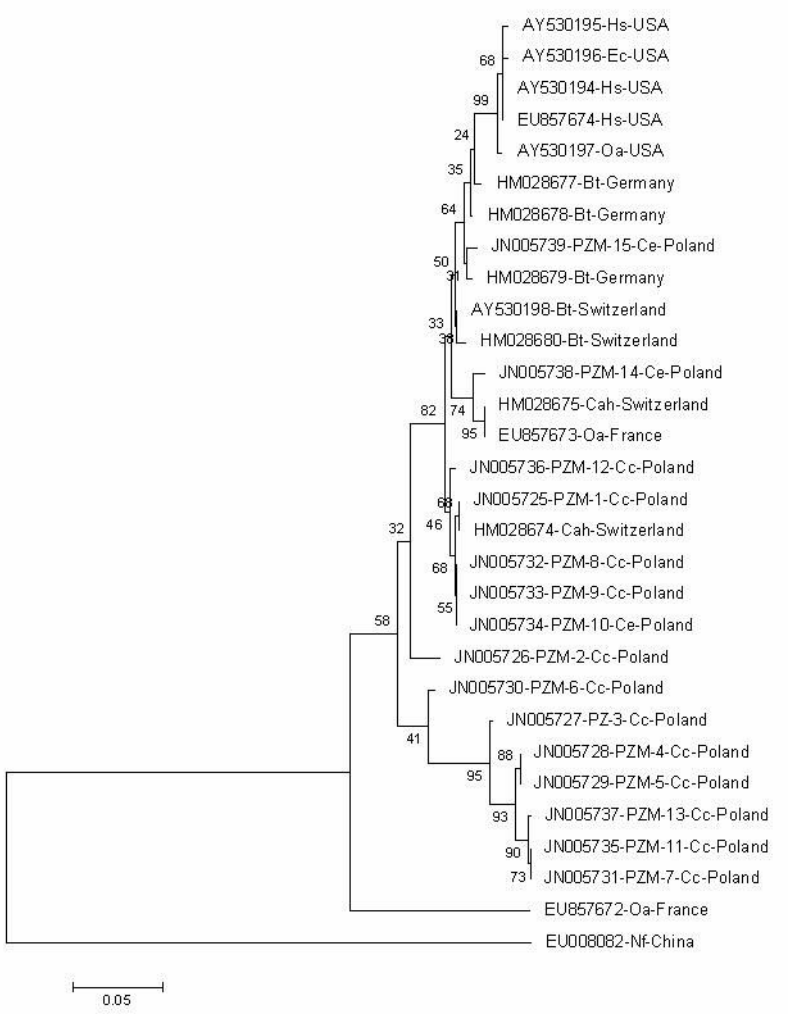

Fig. 2. Phylogenetic analysis of *Anaplasma phagocytophilum* strains based on the *msp4* sequences using the Neighbor Joining method with Kimura two-parameter correction. Strains of *A. phagocytophilum* included in the analysis are described in Table 2. Abbreviations: Bt – *Bos taurus*, Cah – *Capra aegagrus hircus*, Cc – *Capreolus capreolus*, Ce – *cervus elaphus*, Ec – *Equus caballus*, Fc – *Felis catus*, Hs – *Homo sapiens*, Nf – *Niviventer fulvescens*, Oa – *Ovis aries.*

ously submitted to GenBank (DQ779567 and EU157921) and described in a paper by RYMASZEWSKA (2008). For the remaining 12 samples, sequencing of the *groESL* fragment was performed. New variants of the operon fragment were deposited in GenBank under accession numbers JN005743, JN005744, JN005745, JN005746, JN005747, JN005748.

All 15 nucleotide sequences obtained from the Polish isolates were compared and 8 characteristic variants of the analyzed *groESL* fragment in *A. phagocytophilum* were found. Genotypes marked as PZG-3 and PZG-5 (3 isolates each), PZG-4, PZG-7, and PZG-8 (2 isolates each) were the most common, whereas the remaining groups were represented by single isolates.

The sequences from West Pomerania were compared with each other over the length of 1171 bp, corresponding to 390 codons. A total of 28 variable sites were observed, the majority of which (78.6%) were transitions. Third nucleotides in a codon were the most frequently substituted; only in three cases was the change found in position 1. In two out of 390 codons, two nucleotides were substituted simultaneously. Aligning the Polish *groESL* operon sequences to those from other parts of the world showed that only one nucleotide substitution caused an amino-acid substitution in the protein. This pertains to codon 282 where serine occurs interchangeably with alanine, however, alanine was observed more frequently (66.7% of analyzed samples, K_a= 0.01, Table 4). The average genetic distance for the analyzed population was 0.007. The variant with the alanine-determining codon was found in five Polish genotypes (in a total of 10 *A. phagocytophilum* strains, mostly isolated from *C. capreolus*) and marked Vgro-A (Table 5). The variant with the serine codon (Vgro-S, Table 5) was present in three genotypes

Table 5

Combination of variant genes in different populations of *Anaplasma phagocytophilum* from *Capreolus capreolus* and *Cervus elaphus*

Number of isolates (origin)	*16S rRNA*	*ankA*	*GroESL* genotyp/variant AA	*Msp4* genotyp/variant AA
1 – C.c.	PZR-1	PZA-1	PZG-1/Vgro-A	PZM-1/Vmsp4-1
2 – C.c.	PZR-1	PZA-1	PZG-2/Vgro-A	PZM-2/Vmsp4-2
3 – C.c.	PZR-1	PZA-1	PZG-3/Vgro-A	PZM-3/Vmsp4-3
4 – C.c.	PZR-1	PZA-1	PZG-3/Vgro-A	PZM-4/Vmsp4-4
5 – C.c.	PZR-1	PZA-1	PZG-3/Vgro-A	PZM-5/Vmsp4-4
6 – C.c.	PZR-1	PZA-1	PZG-4/Vgro-A	PZM-6/Vmsp4-5
7 – C.c.	PZR-1	PZA-1	PZG-4/Vgro-A	PZM-7/Vmsp4-6
8 – C.c.	PZR-1	PZA-1	PZG-5/Vgro-A	PZM-8/Vmsp4-7
9 – C.c.	PZR-1	PZA-1	PZG-5/Vgro-A	PZM-9/Vmsp4-8
10 – C.e.	PZR-1	PZA-1	PZG-5/Vgro-A	PZM-10/Vmsp4-8
11 – C.c.	PZR-1	PZA-1	PZG-6/Vgro-S	PZM-11/Vmsp4-9
12 – C.c.	PZR-1	PZA-1	PZG-7/Vgro-S	PZM-12/Vmsp4-10
13 – C.c.	PZR-1	PZA-1	PZG-7/Vgro-S	PZM-13/Vmsp4-11
14 – C.e.	PZR-1	PZA-2	PZG-8/Vgro-S	PZM-14/Vmsp4-12
15 – C.e.	PZR-1	PZA-1	PZG-8/Vgro-S	PZM-15/Vmsp4-13

C.c. – *Capreolus capreolus,* C.e. – *Cervus elaphus;* accesion number for gene: *16S rRNA*: PZR-1-JN005742 –; *ankA*: PZA-1- JN005740, PZA-2- JN005741; *groESL*: PZG-1- JN005743, PZG-2- JN005744, PZG-3- JN005745, PZG-4- JN005746, PZG-5- JN005747, PZG-6- JN005748, PZG-7- EU157921; PZG-8-DQ779567; *msp4:* PZM-1- JN005725, PZM-2- JN005726, PZM-3- JN005727, PZM-4- JN005728, PZM-5- JN005729, PZM-6- JN005730, PZM-7- JN005731, PZM-8- JN005732, PZM-9- JN005733, PZM-10- JN005734, PZM-11- JN005735, PZM-12- JN005736, PZM-13- JN005737, PZM-14- JN005738, PZM-15- JN005739. Amino acid sequences for *msp4* gene of *A. phagocytophilum* isolates number 4-5, and 9-10 are identical.

(in all 5 *A. phagocytophilum* strains, two of which were isolated from red deer *C. elaphus*). On the phylogenetic tree (Fig. 3), this variant was also characteristic for the *A. phagocytophilum* strains isolated from human patients diagnosed with anaplasmosis as well as livestock, such as *Equus caballus, Canis lupus familiaris, O. aries.* Variants of alanine-coding sequences formed a separate clade from the serine-coding sequences, and they were mainly isolated from roe deer (*C. capreolus*).

Analysis of the *16S rRNA fragment*

All nucleotide sequences of the ribosomal small-subunit gene fragment obtained from the West Pomerania samples were identical and the genotype was marked as PZR-1 for further analysis (submitted to GenBank, accession number JN005742). They were compared with 18 other sequences deposited in GenBank and derived from various European and American species (e.g. *C. capreolus, O. aries, B. taurus* or *I. persulcatus*) including humans (U02521, *H. sapiens*). The length of the compared sequences ranged from 412 to 1433 bp, within the analyzed sequences, 22 variable sites were found, including 4 differences unique to the Polish sequences. Transitions represented 9 out of 11 substitutions. Mean genetic distance between the analyzed populations was 0.002. On the phylogenetic tree, the Polish sequence forms a common clade with the *A. phagocytophilum* sequences from other European countries and the USA (Fig. 4). These are DNA sequences extracted from the blood of both wild and domestic animals and from the Russian tick *I. persulcatus*, a vector of *Anaplasma*.

Multilocus sequence typing

By concatenating the 16S rRNA, *groESL*, *ankA* and *msp4* genes, a multilocus sequence typing analysis was conducted to create a unique sequence profile for the *A. phagocytophilum* strains inhabiting Poland (Fig. 5). Six groups were revealed, with groups 4 and 6 represented by single strains, group 1 represented by 4 strains and the remaining groups represented by 3 strains each. Groups 1 and 5 are characteristic of the *A. phagocytophilum* strains isolated only from *C. capreolus.* Groups 2 and 3 occur in both *C. capreolus* and *C. elaphus*. Group 4 differs the most from the other groups in having a unique sequence of the *ankA* gene and a serine in position 282 of the *groESL* operon. *A. phagocytophilum* strains sharing this group were found only in the blood of *C. elaphus*, whose samples originated from Poland and Slovenia.

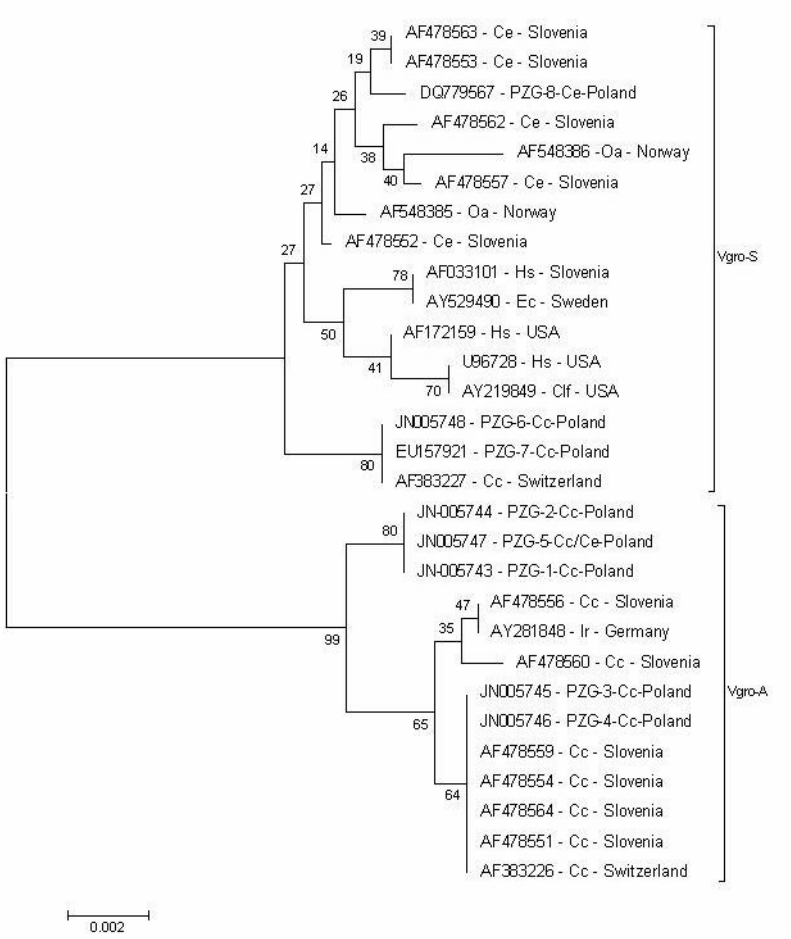

Fig. 3. Phylogenetic analysis of *Anaplasma phagocytophilum* strains based on the *groESL* sequences using the Neighbor Joining method with Kimura two-parameter correction. Strains of *A. phagocytophilum* included in the analysis are described in Table 2. Abbreviations: Vgro-S – the variant with the serine codon, Vgro-A – the variant with the alanine codon. Cc – *Capreolus capreolus*, Ce – *cervus elaphus*, Clf – *Canis lupus familiaris*, Ec – *Equus caballus*, Hs – *Homo sapiens*, Ir – *Ixodes ricinus*, Oa – *Ovis aries.*

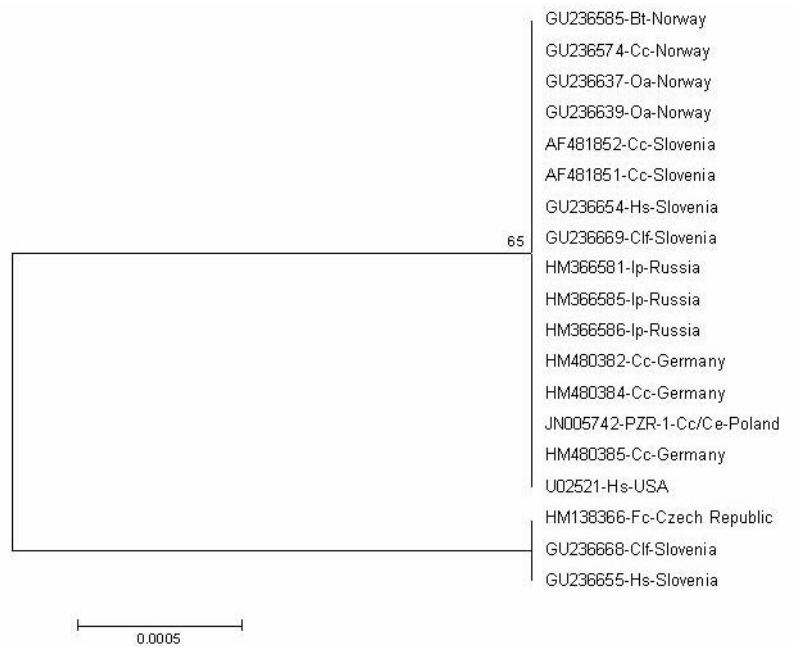

Fig. 4. Phylogenetic analysis of *Anaplasma phagocytophilum* strains based on the 16S rRNA sequences with the Neighbor Joining method with Kimura two-parameter correction. Strains of *A. phagocytophilum* used in the analysis are described in Table 2. Abbreviations: Bt – *Bos taurus*, Cc – *Capreolus capreolus*, Ce – *Cervus elaphus*, Clf – *Canis lupus familiaris*, Fc – *Felis catus*, Hs – *Homo sapiens*, Ip – *Ixodes persulcatus*, Oa – *Ovis aries.*

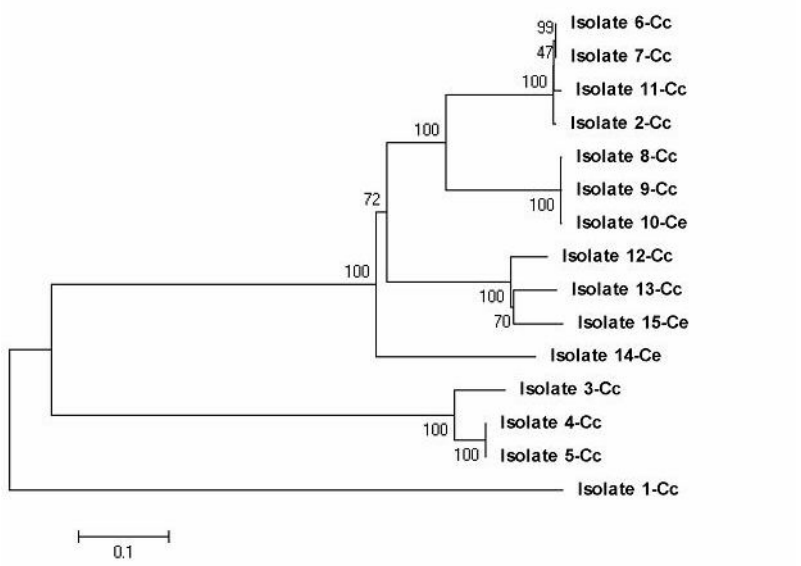

Fig. 5. Phylogenetic trees derived from the comparison of concatenated sequences of the *ankA, msp4, groESL* and 16S rRNA genes using the Neighbor Joining method. The *A. phagocytophilum* isolates included in the analysis are described in Table 5. Group 1: isolates 2, 6, 7, 11; group 2: isolates 8, 9, 10; group 3: isolates 12, 13, 15; group 4: isolate 14; group 5: isolates 3, 4, 5; group 6: isolate 1.

Discussion

Ribosomal and selected protein-coding genes were used to perform genotyping and to establish phylogenetic relationships between various *A. phagocytophilum* populations from Western Poland and other regions of Europe, as well as the USA. The genes are essential for bacterial cell functioning in the host organism.

The *ankA* gene codes for a 160-kDa cytoplasmic protein antigen AnkA, which is a unique protein found in the host cells near the granulocyte nucleus (CATUREGLI *et al.* 2000). The role of AnkA is to control the transcription of critical genes in the host, and thus to change neutrophil functions and biological response to infection. This, in consequence, enhances the infection of host cells and the protection of bacterial cells (PARK *et al.* 2004).

The *msp4* gene codes for a major protein antigen which is homologous to immunodominant major surface protein found on the outer membrane of *Anaplasma marginale* (MSP4) and *Ehrlicha ruminantium* (MAP1). The biological function of MSP4 in *A. marginale* is unknown, however, it is hypothesized that the protein is responsible for the host-pathogen interaction, hence the observed more rapid evolution of the gene (DE LA FUENTE *et al.* 2005). DE LA FUENTE *et al*. (2007) found higher variability in the nucleotide sequence of the *msp4* gene in *A. phagocytophilum*, depending on the origin of their host organism, as compared to *A. marginale.*

The heat shock operon contains two genes coding for the GroES (M.W. 20 kDa) and GroEL (M.W. 58 to 65 kDa) proteins. GroEL is similar to the HSP60 family of eukaryotic heat shock proteins. The variability in the *groEL* gene, resulting in changes in the amino acid sequence of the protein, may affect the interaction with the vector and the host or affect the virulence of *A. phagocytophilum* (SUMNER *et al.* 1997).

The analysis of nucleotide sequence fragments of the four genes, including three protein-coding genes, did not enable the establishment of uniform genetic variants of *A. phagocytophilum* due to large variability within the studied fragments of the two protein-coding *msp4* and *groESL* genes. Hence, the study focused on the amino-acid sequences that were deduced from the nucleotide sequences of the amplified genes.

For the *ankA* gene, two genotypes (PZA-1 and PZA-2) which translate into two different amino-acid sequences, were found. Some of the changes, especially in codons 683, 778, or 813, apparently lead to substitutions in amino acids demonstrating different properties, which may affect protein activity. One of the genotypes (PZA-2) was rare in West Pomerania (Poland). The analysis of phylogenetic trees shows that PZA-2 groups together with the sequences that were detected in the majority of livestock (Fig. 1). The phylogenetic trees determined from the DNA sequence of the *ankA* gene and the amino acid sequence of the protein (data not shown) reveal the same relationship.

By conducting a phylogenetic analysis based on the *ankA* gene SCHARF *et al.* (2011) obtained a dendrogram in which two clusters were composed almost exclusively of roe deer sequences. Samples of various origin were grouped in the other two clus-

ters, including those from patients and domestic animals (cluster I), whereas in cluster IV, 5 sequences of *A. phagocytophilum* isolated from roe deer grouped together with sequences of different origin. The Polish *ankA* sequences (PZA-1 and PZA-2) didn't form a separate cluster. This configuration could be a sign of different adaptation of the bacteria to the environment (geographic origin, vectors, hosts).

The analysis of the *groESL* operon suggests that the substitution in the codon determining the amino acid in position 242 of the protein, in which alanine is interchangeable with serine, is of high significance. PETROVEC *et al*. (1999) suggest that the serine-containing protein always occurs in bacteria isolated from patients' blood, and therefore this *A. phagocytophilum* strain might have pathogenic properties. The strain has been detected previously in wild ruminants in West Pomerania (RYMASZEWSKA 2008).

In the light of the most recent analyses, the hypothesis regarding the pathogenicity of *A. phagocytophilum* depending on the amino-acid variant of a heat shock protein seems dubious. Clinical studies which may verify the hypothesis demonstrate that *A. phagocytophilum* carrying a *groEL* variant encoding alanine at its critical site may also occur in patients. In two patients with suspected tick-borne diseases, HASCHKE-BECHER *et al*. (2010) reported the presence of *Anaplasma* DNA and, based on the *groEL* gene nucleotide sequence, determined its genotype as *groEL-A* (encoding alanine, not serine, as previously detected in most patients). The authors indicated that this genotype is most often carried by bacteria found in ticks in Austria (86%). This genotype is also commonly detected in *A. phagocytophilum* isolated from its vectors and organisms such as red deer or roe deer in Europe, which may be considered as potential reservoirs of the bacteria (LIZ *et al.* 2002; PETROVEC *et al.* 2002; RYMASZEWSKA 2008).

All authors that have analyzed the *msp4* gene indicate its high variability. First, 10 variants of the *msp4* gene were studied in *A. phagocytophilum* by DE LA FUENTE *et al.* (2005) by examining DNA from eight mammalian species in five European countries. LADBURY *et al.* (2008) analyzed 85 *msp4* gene sequences, 21 of which had not been previously described. Analyses of the 16S rRNA gene using the same samples revealed only three variants, two of which had already been detected in Norwegian sheep. Moreover, high variability (11 genotypes) was reported by BOWN *et al.* (2007), who compared partial *msp4* sequences derived from 20 *A. phagocytophilum* strains found in different hosts from several European countries and the USA. By comparing partial 16S rRNA gene sequences, the authors distinguished five variants within the same group. In the case of Polish sequences presented in this study, high variability at the DNA level was observed for the gene *msp4*; at the level of amino-acid sequence, only two variants were detected.

SHUKLA *et al.* (2007), by analyzing genetic diversity of *A. phagocytophilum* populations isolated from ill dogs, also within the *ankA* and 16S rRNA genes, as well as the *groESL* operon, distinguished 2, 5 and 3 variants of the nucleotide sequences for individual genes, respectively. Similar results were obtained by KATARGINA *et al.* (2012), who analyzed *A. phagocytophilum* DNA isolated from the *I. ricinus* ticks and identified four 16S rRNA alleles and two variants of the *groESL* operon, encoding serine or alanine in position 242 of the protein. Far greater diversity was found by VON LOEWENICH *et al.* (2003) in a population of *A. phagocytophilum* isolated from *I. ricinus* ticks. They described as many as 9 genotypes of the gene encoding the small subunit of the ribosome. Within the *groESL* operon, as in the present study, they distinguished versions of the gene either coding for serine or alanine, while the number of *A. phagocytophilum* populations for both variants was similar. VON LOEWENICH *et al.* (2003) observed the highest diversity in the nucleotide sequence of the gene *ankA,* but this may be due to the fact that they used primers amplifying the complete open reading frame, which included the ankyrin repeat elements.

Drawing conclusions on the *Anaplasma* virulence in humans and domestic animals based on nucleotide sequences and, therefore, amino-acid sequences of proteins is only an indirect method. Data on this issue found in the global scientific literature are still insufficient, especially regarding clinical studies. Nonetheless, it seems interesting to seek relationships between *A. phagocytophilum* strains carrying unique allelic profiles and their host species. However, analysis of single genes is not fully informative. In the presented study, a broad analysis was attempted and multilocus sequence typing was performed. Based on the results, *A. phagocytophilum* strains invading only *C. capreolus* and strains without tropism for particular host species, present in both roe deer and red deer (*C. elaphus*), were identified.

The Polish populations of *A. phagocytophilum* and other *Anaplasma* populations compared using the analyzed protein-coding genes *(ankA, msp4*, and *groESL)* are polymorphic. The assessment of genetic variability within different conserved and species-specific genes is a common trend in today's research of bacterial pathogens. Analysis of variability based on multiple independent studies may shed light on migration and evolution of bacteria. It also allows the assessment of adaptation of pathogens, including *A. phagocytophilum*, to their new environments.

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