

Phylogentic analysis of strains isolated from sheep and goats using and genes

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Authors: Anna Psaroulaki, Dimosthenis Chochlakis, Vassilios Sandalakis, Iosif Vranakis, Ioannis Ioannou, Tselentis Yannis

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1	Title
2	Phylogentic analysis of Anaplasma ovis strains isolated from sheep and goats using groEL
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4	
5	Anna Psaroulaki ¹ *, Dimosthenis Chochlakis ¹ , Vassilios Sandalakis ¹ , Iosif Vranakis ¹ , Ioannis
6	Ioannou ² , Tselentis Yannis ¹
7	
8	¹ Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine
9	(WHO collaborating center), Medical School, University of Crete, Greece
10	² Veterinary Services, Nicosia, Cyprus
11	
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15	
16	* Corresponding author: Dr Anna Psaroulaki, PhD: Laboratory of Clinical Bacteriology,
17	Parasitology, Zoonoses and Geographical Medicine. Voutes, Heraklion - Crete, 71100,
18	Greece. e-mail: <u>annapsa@med.uoc.gr</u> , tel: 00302810394743, fax: 00302810394740
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21 Abstract

Evidence of *Anaplasma* spp. in goats and sheep in Cyprus has been demonstrated by previous research. Herein, further research was performed for the identification of the exact *Anaplasma* spp. resulting in the identification of *Anaplasma ovis* strains in all samples examined. We used a bioinformatics as well as a molecular approach (study of *groEl* and *msp4* genes) in order to verify the validity of the results. All samples depicted the presence of *A. ovis* regardless of the host (goat or sheep).

28

29 Introduction

Anaplasmosis is a disease caused by pathogens belonging to the genus of Anaplasma 30 31 (Rickettsiales: Anaplasmataceae). A. marginale, A. centrale and A. ovis are obligate intracellular bacteria, specifically located within membrane-attached cytoplasmic vacuoles of 32 33 vertebrate and invertebrate host cells (Dumler et al., 2001) and they use ruminants as their 34 main host (Kocan et al., 2003; Lew et al., 2003). The pathogens are biologically transmitted 35 through ticks; vertebrates and ticks with persistent bacteremia can be considered as natural pathogen reservoirs (Dumler et al., 2001; Kocan et al., 2003; Rikihisa, 2003). Ovine 36 37 anaplasmosis is most commonly related with haemolytic anaemia in goats. However, A. ovis infections in sheep have also been reported (Friedhoff, 1997; Splitter et al., 1956). 38

The interaction of *Anaplasma* spp. and their hosts is mediated by the major surface proteins (MSPs). Thus, rapid evolution of these genes is likely to occur due to the natural selective pressure exerted by the immune system of the hosts (Brayton et al., 2006; de la Fuente et al., 2005a; Hotopp et al., 2006; Kocan et al., 2004). Former surveys on *A. ovis* revealed a variation of the *msp4* gene which lead to the identification of three different genotypes (de la

Fuente et al., 2006; de la Fuente et al., 2002a; de la Fuente et al., 2005c; Yabsley et al.,
2005). Moreover, heat shock operon *groESL* has been used for identification of *Anaplasmataceae* (Sumner et al., 1997) as well as partial *groEL* genes, which encode a 60kDa heat shock protein, GroEL (Park et al., 2005).

In a study conducted in Cyprus (Chochlakis et al., 2008) blood samples collected from sheep and goats were tested by PCR for the presence of *Anaplasma* spp. and *A. phagocytophilum*. Of the genotypes revealed, four corresponded to *Anaplasma* spp. In the current study, samples which corresponded to these genotypes were processed by PCR amplification of *groEL* and *msp4* genes, Restriction Fragment Length Polymorphism (RFLP) and sequencing analysis in order to identify the *Anaplasma* spp. to which they corresponded.

54

55 Materials and methods

56 Sampling

Six hundred and eighty nine (689) blood samples (343 from sheep and 346 from goats) were drawn, by trained Veterinarians, from March 2005 – March 2006, from 38 different regions of the 5 prefectures of Cyprus (Nicosia, Limassol, Famagusta, Larnaka, Pafos). Blood samples were tested for the presence of Anaplasma DNA using 16S rRNA gene primers. Four *Anaplasma* spp. genotypes were revealed (EU090181, EU090183, EU090184 and EU090185) (Chochlakis et al., 2008).

All tests were carried out at the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses
and Geographical Medicine (WHO Collaborating Centre) in Crete (Greece).

65

66 Amplification of groEL and msp4 genes

67 Primers HSPB (TTA(GA)AA(GATC)CC(GATC)CCCAT(GATC)CC(GATC)CCCATG CC-68 3') and HSPC (5'-AAATGGCGAATGTTGT(TA)GT(TC)AC-3') (brackets indicate mixed 69 bases) were used to amplify an approximately 1650 bp fragment of the groEL gene (Lew et al., 2003). Primers msp43 (5'-CCGGATCCTTAGCTGAACAGGAATCTTGC-3') and 70 71 msp45 (5'-GGGAGCTCCTATGAATTACAGAGAATTGTTTAC-3') were used to amplify 72 an approximately 850 bp fragment of the *msp4* gene (de la Fuente *et al.*, 2007). Negative 73 controls (distilled water) were used every 10 samples to ensure the accuracy of the method. 74 The first sample that was tested positive by PCR and sequence analysis was further used as 75 the positive control. PCR products were electrophoresed on 1% agarose gel and visualized 76 following 30-minute incubation in GelStar Nucleic Acid Gel Stain (Lonza, USA) according 77 to the manufacturer's instructions. Positive PCR products were purified (PCR product purification kit, Qiagen, Germany) and positive PCR products were re-amplified in order to 78 79 multiply the DNA and gain more appropriate results in sequence analysis.

80

81 Prediction of RFLP patterns using Bioinformatics tools – RFLP analysis

DNA sequences for Anaplasma *groEL* and *msp4* genes were retrieved from the National
Center for Biotechnology Information (NCBI) (<u>http://www.ncbi.nlm.nih.gov/BLAST</u>)
database. MegAlign module of the *Lasergene* Ver.7.1 software (*DNASTAR* Inc., Madison,
WI, USA) and Seqbuilder module of the *Lasergene* Ver.7.1 software (*DNASTAR* Inc.,
Madison, WI, USA) were used; *HaeIII* (Fermentas) and *ApoI* (New England Biolabs)
restriction enzymes corresponding to *groEL* and *msp4* genes were used (Figure 1) as follows:
16µl nuclease-free water, 2µl 10X Buffer (Buffer R for *HaeIII* and NEBuffer 3 for *ApoI*), 1µl

of purified PCR product and 0.5µl of the corresponding enzyme where gently mixed,
incubated for 1 hour at 37°C, electrophoresed at 1.5% agarose gel and visualised as above.

91

92 Sequencing analysis

Purified PCR products were directly sequenced with the above described primers using the
sequencer CEQ 8000 Beckman Coulter (Bioanalytica – Genotype, Athens). All PCR
products were sequenced twice to verify the revealed sequences. The sequences revealed
were processed using nucleotide BLASTn, Chromas v1.49 and *Lasergene* Ver.7.1 software.

97

98 Revealed sequence accession numbers

As regards *groEL*, the accession numbers submitted at GenBank database were FJ460434,
FJ460435, FJ460436, FJ460437, FJ460438, FJ460439, FJ460440, FJ460441, FJ460442,
while for *msp4* the accession numbers were FJ460444, FJ460445, FJ460446, FJ460447,
FJ460448, FJ460449, FJ460450, FJ460451, FJ460452, FJ460453, FJ460454 and FJ460455.

104 **Phylogenetic analysis**

105 Clustalx 2.0 was used to align sequences and MEGA software v. 4.0.2 to construct the 106 phylogenetic trees (Figures 2, 3). The Neighbor-Joining (NJ) algorithm (Saitou and Nei, 107 1987) with Kimura 2-parameters correction (Kimura, 1980) were used with bootstrap 108 analysis of 1000 iterations.

109

110 **Results**

111 An algorithm was created (Figure 1) to distinguish the species of *Anaplasma* spp. detected in 112 ruminants can be distinguished. Digesting the groEL gene with HaeIII reveals a band near 113 900bp narrowing the selection to A. centrale and A. marginale, where discrimination 114 between is conferred by the absence (A. centrale) or presence (A. marginale) of a 92bp band. 115 Absence of the band around 900bp denotes that the species is A. ovis. Digestion of the msp4 116 gene with Apol may reveal a single 849bp band (A. marginale) or two bands (765bp and 87bp) (A. centrale) (537bp and 315bp) (A. ovis). Digestion of our msp4 and groEL PCR 117 118 products with ApoI and HaeIII restriction enzymes respectively, revealed profiles denoting 119 the presence of A. ovis (pattern not shown).

In the case of *groEL* we retrieved parts of the genomic sequence the length of which, varied between 698bp and 1649bp. Consequently, comparison of the sequences was performed in the sequence region that could be cross-checked among all sequencing products. In the case of *msp4* we obtained the whole length sequence. Nine *groEL* sequences (4 detected in goats and 5 in sheep) and 12 *msp4* sequences (5 detected in goat and 7 in sheep) were revealed.

As regards *msp4*, a 99% identity, among *A. ovis* strain AM5 (EF067341), *A. ovis* strain HungaryV8 (EF190511), *A. ovis* strain Italy20 (AY702923) and *A. ovis* strain Italy147 (AY702924) was revealed (Figure 2). In the case of *groEL*, one high similarity (99%) candidate, *A. ovis* isolate OVI (AF441131) was revealed (Figure 3).

The *groEL* sequences alignment revealed a high degree of similarity since several of the nucleotide base substitution mutations were silent mutations (Table 1). We found 54 mutation sites hosting 45 point mutations and 3 triplet insertions. The mutation level observed in strain goat 9 (FJ460435) was higher than expected and higher compared to the rest of the sequences (Table 1). As regards *msp4* sequences, we detected 53 mutation sites,

all of which were point mutations (Table 2). Eleven of these were silent mutations. In most
silent mutations the initial amino acids were replaced by others with similar properties on
charge or hydrophobicity (e.g. replacement of L by P); in less occasions a non polar amino
acid was replaced by a polar amino acid, negatively or positively charged and *vice versa*.
Eleven mutation regions were parsimonious in both genes. The percentage of transitions and
transversions was almost the same, 45.3% and 54.7% respectively for *msp4*; 44.2% and
55.8% respectively for *groEL*.

141

142 **Discussion**

Genetic characterization of Anaplasma spp. pathogens in ruminant and non-ruminant hosts 143 144 can be differentiated by examining the msp4 gene and protein sequence as already described 145 (de la Fuente et al., 2007; de la Fuente et al., 2005a; de la Fuente et al., 2005b; de la Fuente et al., 2002b). Phylogenetic studies have used *groEL* genes to distinguish among the species of 146 147 Anaplasma spp. (Lew et al., 2003). In previous surveys it has also been stated that msp4 genotypes could exhibit heterogeneity among geographic regions, hosts and perhaps among 148 149 individuals of the same flock (de la Fuente et al., 2007; Hornok et al., 2007). Similar 150 conclusions have been drawn for the A. ovis groEL gene as it has been found to exhibit a much higher heterogeneity compared to A. marginale and A. centrale groEL genes. 151 152 Furthermore, past surveys on isolates of A. marginale proved that analysis of gene and 153 protein sequences (MSP1a and MSP4) could provide phylogeographic information (de la 154 Fuente et al., 2001).

155 Herein, even though we can comment on the heterogeneity of the sequences, we can not 156 extract any phylogeographic information. The reason could be the relatively small

157 geographic area the island of Cyprus and the often trading of animals among farmers. Several 158 different strains can exist in Cyprus being dispersed throughout the island. This also might be 159 the answer why more similar strains were detected in farms separated by greater distances 160 while more dissimilar ones were found to be present in farms with close proximity (data not 161 shown).

Phylogenetic analysis clusters our sequences along with other *A. ovis* strains, clearly distinguishing them from *A. marginale* and *A. centrale*, also supported by the strong bootstrap values both for *msp4* and *groEL* phylogenetic analysis, suggesting that *A. ovis groEL* may vary among hosts as has already been supported for *msp4* (de la Fuente et al., 2007).

167 Investigation of individual organisms in a flock in order to determine whether different 168 strains exist in the same flock or even co-exist in single individual could lead to a more 169 sufficient view on the phylogeny of *Anaplasma* spp. Further investigation is of great 170 importance as ovine anaplasmosis although benign, can manifest due to possibly 171 predisposing factors (Friedhoff, 1997) and turn into a persistent subclinical infection (Kieser 172 et al., 1990; Palmer et al., 1998).

In the current survey we identified *A. ovis* strains, in sheep and goats of the island of Cyprus, through bioinformatics and sequencing analysis approach of *groEL* and *mps4* genes. We also proposed a model, based on *groEL* and *msp4* genes, for simple and accurate discrimination among the 3 *Anaplasma* spp. In any case, attention should be given in the dispersal of the pathogen through its vectors and the need to recognize the mechanisms of evolution and genetic diversity in this pathogen becomes obvious.

180 Conflict of interest statement: Authors clearly state that no financial and personal 181 relationships with other people or organisations that could inappropriately influence (bias) 182 their work exist.

183

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188

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274

275 **Tables and Figures**

Table 1: Positions in the *groEL* sequences presenting point mutations or triplet insertions.
The number of the corresponding amino acid for each mutation is shown directly below each
case. Silent mutations are shown in light green. Reference sequences are in light grey.

Table 2: Positions in the *msp4* sequences presenting point mutations. The number of the corresponding amino acid for each mutation is shown directly below each case. Silent mutations are shown in light green. Reference sequences are in light grey.

Figure 1: Schematic representation of the expected patterns using restriction enzyme *HaeIII* on *groEL* gene and *ApoI* on *msp4* gene. An actual 50bp Ladder is shown as a guide.

Figure 2: Neighbor-joining tree with bootstrap analysis (1000 iterations) showing the clustering of *groEL* sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches.

287

Figure 3: Neighbor-joining tree with bootstrap analysis (1000 iterations) showing the clustering of *msp4* sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches.

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		ID						Ŧ	Ŧ	X 7		7	Б	X 7	D	Б	TN	NT	X 7
AF441131 A. ovis OV1	S			A	A			I	I	V		5	E	V	P	F	IN	N	V
FJ460441 A. ovis goat /6	S	IK	C X	A	Α		~ •	I	I	V		£	E	V	S	L	NH	N	V
FJ460435 A. ovis goat 9	S	IR	GK	C	P	PG	GI	I	M	V	1		Q	V	P	Y	I D	N	V
FJ460434 <i>A. ovis</i> sheep 8	S	IR		Α	Α			1	I	V	ł	£	E	V	Р	С	IT	N	V
FJ460436 A. ovis sheep 37	S	IR		Α	Α			I	I	V	I	£	E	Ι	Р	F	IN	Ν	V
FJ460442 A. ovis goat 77	S	I R		Α	Α			Ι	Ι	V	I	£	Ε	V	Р	С	IH	Ι	V
FJ460439 A. ovis sheep 64	Y	IE		Α	Α			Ι	Ι	V	I	£	Е	V	Р	F	IT	Ι	V
FJ460737 A. ovis sheep 40	Y	IE		Α	Α			Ι	Ι	V	I	£	Ε	V	Р	F	I N	Ν	V
FJ460440 A. ovis goat 71	Y	K R		Α	Α			Ι	Ι	V	I	£	Ε	V	Р	S	IT	Ν	V
FJ460438 A. ovis sheep 54	Y	K R		Α	Α			Ι	Ι	V	I	E	Е	Ι	Р	F	I N	Ν	V
EF520695 A. centrale strain 16	S	I R		Α	Α			Ι	Ι	V	I	E	E	V	Р	F	IN	Ν	V

AF414862 A. marginale Israel	S	I R	Α	Α		Ι	Ι	V	Е	Е	V	Р	F	I N	Ν	V

Table 1: Positions of the *groEL* sample sequences presenting point mutations or triplet insertions. The number of the corresponding amino acid for each mutation is shown directly below each case. Light green is used to show the amino acids that remained unchanged due to silent mutation. The sequences coloured in light grey correspond to reference sequences for Anaplasma *groEL*.

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	53bp	75bp	119bp	126bp	470bp	546bp	660bp	695bp	736bp	767bp	770bp	789bp	801bp	817bp	-824bp	-828bp
															819	827
EF067341 A. ovis SpainAM5	С	G	G	G	Т	Т	Т	Т	Т	Α	Т	С	Α	G	TTTTAA	TT
AY702923 A. ovis Italy20	С	G	G	G	Т	Т	Т	Т	Т	Α	Т	С	Α	G	TTTTAA	TT
EF190511 A. ovis HungaryV8	С	G	G	G	Т	Т	Т	Т	Т	Α	Т	С	Α	G	TTTTAA	TT
FJ460454 A. ovis goat 73	С	G	G	G	Т	Т	Т	Т	Т	Α	Т	С	Α	G	TTTTAA	CT
FJ460447 A. ovis goat 23	С	G	G	G	Т	Т	Т	Т	Т	С	С	С	Α	G	GTTTAA	CT
FJ460448 A. ovis sheep 35	С	G	G	G	Т	T	Т	С	Α	С	Т	С	Α	G	TTGTGA	CT
FJ460455 A. ovis goat 77	С	G	G	G	Т	Т	Α	Т	Т	С	Т	С	С	G	TGTTGA	CT
FJ460445 A. ovis goat 9	С	G	G	G	Т	Т	Α	Т	Т	С	Т	С	Α	G	TTTTGA	CT
FJ460455 A. ovis goat 71	С	G	G	G	Т	С	Т	Т	Т	С	Т	С	G	G	TTTAAA	CT
AY702924 A. ovis Italy147	С	G	G	G	С	Т	Т	Т	Т	Α	Т	С	Α	G	TTTTAA	TT
FJ460452 A. ovis sheep 64	С	G	G	G	С	Т	Т	Т	Т	С	Т	С	Α	G	TTTGAA	CT
FJ460451 A. ovis sheep 40	С	G	G	G	С	Т	Т	Т	Т	С	Т	С	Α	G	TTTGAA	CT
FJ460450 A. ovis sheep 37	Α	T	G	G	С	Т	Т	Т	Т	Α	Т	G	G	Α	TGTTAC	TT
FJ460449 A. ovis sheep 36	C	Т	Т	Т	С	Т	Т	Т	Т	С	Т	С	Α	G	TTTTAA	CT
FJ460446 <i>A. ovis</i> sheep 10	С	G	G	G	С	Т	Т	Т	Т	Α	Т	С	Α	G	TTTTAC	TG
FJ460444 A. ovis sheep 8	С	G	G	G	С	Т	Α	Т	Т	Α	Т	С	Α	G	TTTTAA	TT
	aa	aa	aa	aa	7aa	2aa)aa	2aa	Saa	Saa	7aa	3aa	7aa			
	18	25	40	42	157	182	22(232	240	25(257	263	267		273-276aa	
EF067341 A. ovis SpainAM5	Α	G	G	G	V	Y	V	L	Y	K	F	Α	Α		GFNL	
AY702923 A. ovis Italy20	Α	G	G	G	V	Y	V	L	Y	K	F	Α	Α		GFNL	
EF190511 A. ovis HungaryV8	Α	G	G	G	V	Y	V	L	Y	K	F	Α	Α		GFNL	
FJ460454 A. ovis goat 73	Α	G	G	G	V	Y	V	L	Y	K	F	Α	Α		G F N P	
FJ460447 A. ovis goat 23	Α	G	G	G	V	Y	V	L	Y	Т	S	Α	Α		G F N P	
FJ460448 A. ovis sheep 35	Α	G	G	G	V	Y	V	S	Ν	Т	F	Α	Α		GCDP	
FJ460455 A. ovis goat 77	Α	G	G	G	V	Y	V	L	Y	Т	F	Α	Α		G V D P	
FJ460445 A. ovis goat 9	Α	G	G	G	V	Y	V	L	Y	Т	F	Α	Α		G F D P	
FJ460455 A. ovis goat 71	Α	G	G	G	V	Y	V	L	Y	Т	F	Α	Α		GLNP	
AY702924 A. ovis Italy147	Α	G	G	G	Α	Y	V	L	Y	K	F	Α	Α		GFNL	

FJ460452 A. ovis sheep 64	Α	G	G	G	Α	Y	V	L	Y	Т	F	Α	Α	G L N P
FJ460451 A. ovis sheep 40	Α	G	G	G	Α	Y	V	L	Y	Т	F	Α	Α	GLNP
FJ460450 A. ovis sheep 37	D	G	G	G	Α	Y	V	L	Y	K	F	Α	Α	SVTL
FJ460449 A. ovis sheep 36	Α	G	V	G	Α	Y	V	L	Y	Т	F	Α	Α	G F N P
FJ460446 A. ovis sheep 10	Α	G	G	G	Α	Y	V	L	Y	K	F	Α	Α	G F T L
FJ460444 A. ovis sheep 8	Α	G	G	G	Α	Ŷ	V	L	Y	K	F	Α	Α	GFNL

Table 2: Positions in the *msp4* sequences presenting point mutations. The number of the corresponding amino acid for each mutation is shown directly below each case. Light green is used to show amino acids that remained unchanged due to silent mutation. The sequences coloured light grey, correspond to reference sequences.



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