



FIRST MOLECULAR DETECTION AND PHYLOGENETIC ANALYSIS OF ANAPLASMA PHAGOCYTOPHILUM IN HORSES IN MUŞ PROVINCE OF TURKEY

TÜRKİYE 'NİN MUŞ İLİNDE ATLARDA ANAPLASMA PHAGOCYTOPHILUM'UN İLK MOLEKÜLER TESPİTİ VE FİLOGENETİK ANALİZİ

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Abstract

Objective: Equine granulocytic anaplasmosis (EGA) caused by *Anaplasma phagocytophilum* is a disease that generally develop characteristic high fever and a number of changes in blood such as thrombocytopenia. The main transmission of *A. phagocytophilum* is hard ticks through intrastadially. The aim of this study was to determine the presence of *A. phagocytophilum* in horses in Mus province of Turkey by using molecular methods.

Methods: In the present survey, blood samples were collected in Mus province from 93 horse. Commercial ELISA kit (IgG) and nested-PCR were performed. The DNA was extracted from blood and analysed by nested-PCR technique targeting 16S rRNA gene of *A. phagocytophilum* and then positive PCR products were sequenced.

Results: The seroprevalence against *A. phagocytophilum* was 8.6% and 6 horses (6.4%) showed positive nested-PCR results. The sequences had 99.5 and 100% homology with the BLAST published *A. phagocytophilum* amplicons.

Conclusion: To the best of our knowledge, this is the first molecular data of *A. phagocytophilum* in horses in Mus province of Turkey. This study also reveals that *A. phagocytophilum* isolates isolated from a small region are closely related to *A. phagocytophilum* isolated from a wide variety of hosts.

Keywords: *Anaplasma phagocytophilum*, horses, phylogeny, Turkey, PCR, Mus

Öz

Amaç: *Anaplasma phagocytophilum*'un neden olduğu equine granülositik anaplasmosis (EGA), genellikle karakteristik yüksek ateş ve trombositopeni gibi kanda bir dizi değişiklik geliştiren bir hastalıktır. *A. phagocytophilum*' un biyolojik bulaşması intrastadial yolla sert keneler tarafından yapılmaktadır. Bu çalışmanın amacı, Türkiye'nin Muş ilinde atlarda *A. phagocytophilum* varlığının moleküler yöntemlerle belirlenmesidir.

Yöntem: Bu çalışma için Muş ilinde bakılan 93 attan kan örnekleri alındı. Ticari ELISA kiti (IgG) ve nested PCR yapıldı. DNA'lar kandan ekstrakte edildi ve *A. phagocytophilum*'un 16S rRNA genini hedef alan nested PCR tekniği ile analiz edildi. Daha sonra pozitif PCR ürünleri sekanslandı.

Bulgular: *A. phagocytophilum*'a karşı seroprevalans % 8,6 bulunurken, 6 atın (% 6,4) nested PCR sonuçları pozitif bant gösterdi. Elde edilen sekanslar, BLAST tarafından yayınlanan *A. phagocytophilum* ampliconlar ile %99,5 ve %100 benzerlik gösterdi.

Sonuç: Yaptığımız literatür taramalarına göre bu çalışma, Türkiye'nin Muş ilinde atlarda *A. phagocytophilum*'un ilk moleküler verisidir. Aynı zamanda, küçük bir bölgeden izole edilen *A. phagocytophilum* izolatlarının, çok çeşitli konakçılardan izole edilen *A. phagocytophilum* ile yakından ilişkili olduğunu ortaya koymaktadır.

Anahtar Kelimeler: *Anaplasma phagocytophilum*, atlar, filogeni, Türkiye, PZR, Muş

Introduction

Tick-borne diseases (TBDs) are an increasingly outstanding health problem in all over the world, including Turkey. Some TBDs, particularly babesiosis, theileriosis and anaplasmosis are known to be prevalent in several geographic range of Turkey.¹ Among these, anaplasmosis is caused by six species (*Anaplasma bovis*, *A. ovis*, *A. marginale*, *A. centrale*, *A. phagocytophilum* and *A. platys*).² *Anaplasma phagocytophilum* is found infected eukaryotic host cells such as neutrophils and eosinophilic granulocytes. Its is the main causative agent for equine granulocytic anaplasmosis (EGA). This pathogen also infects cats, dogs, wild carnivores, wild and domestic ruminants, rodents, and humans. The occurrence of *A. phagocytophilum* was found closely related to the distribution of the mainly vector *Ixodes* spp ticks.³ In Turkey, *Ixodes ricinus*, *Haemaphysalis sulcata*, and *Rhipicephalus bursa* seems to be the only three vector.⁴

PCR technology based on genetic information have proved extremely useful for the diagnosis of EGA.³ In recent years, *msp4*, *ankA*, 16S rRNA, *groESL*, *msp2* (P44) and *gltA* genes are used to detect *A. phagocytophilum* infections.^{5-8,29} On account of the fact that the 16S rRNA is a limited and preserved function gene has been more widely used in defining this agent.¹⁰ Recently, a 16S rRNA nucleotide sequences investigation reported a new rickettsial genotype in horses in southern and eastern Europe.¹¹ Moreover, this gene (EC9/EC12a and SSAP2f/SSAP2r primer set) is quite accurate for the diagnosis of *A. phagocytophilum* DNA in ruminants and ticks.¹²

Some studies conducted in Turkey have been reported DNA positivity for *A. phagocytophilum* in dogs, cattle, sheep and tick. Its has been also detected by serologic methods in mice and human.¹ On the other hand, a report is available on the detection of *A. phagocytophilum* in horses from Nevşehir province using IFAT, but the study lacks sequence analysis.¹³ Therefore, this study was aimed at the molecular identification and phylogenetic analysis of *A. phagocytophilum* in horses in Mus province of Turkey.

Methods

Sample collection

The study materials were blood samples collected from 93 healthy horses ranging in Mus province (39°29'28.29"N 41°06'41.47"E) of Turkey in June-August 2017 but was not found in any tick species.⁹ Whole blood samples were collected from the vena jugularis of the horses into EDTA tubes and serum tubes according to the technique, and the data on the animals were recorded with protocol numbers. The serum tubes were centrifugated at 3000 rpm for 15 min and sera were separated. Both sera and blood EDTA tubes were stored at -20°C until the time for serological and molecular analysis.

ELISA (Enzyme-Linked ImmunoSorbent Assay)

Samples were screened for IgG against *A. phagocytophilum* using commercially available ELISA assay from EUROIMMUN, Germany (Catalog no: EI 220m-9601 GE). This test detects antibodies from both active infection and previous exposures to the pathogen and has high sensitivity (100%) and specificity (90%).

DNA extraction and Nested-PCR amplification

Genomic DNA was obtained from the blood samples collected from the horses by using a commercial blood kit (EcoSpin Blood Genomic DNA Kit, Turkey). For the detection of *A. phagocytophilum*, primers targeting the 16S rRNA gene were selected from the literature. Nested PCRs (nPCR) were performed using the EC9/ EC12a primers, which amplify a 1462 bp fragment, and SSAP2f/ SSAP2r, which amplify a 641 bp fragment.¹⁴ PCR was carried out in a final volume of 25 µL, containing 7.5 µL DNase- and RNase-free sterile distilled water (Biobasic, Canada), 10 µL 5X MyTaq Reaction buffer (Bioline, USA), 1 µL of each primer (20 pmol), 5 µL of template DNA (100-200 ng), and 0.5 µL of Taq DNA polymerase recombinant (5 U/µL) (Thermo Scientific, USA).

A. phagocytophilum DNA from Genekam Biotechnology Company (Germany) validated by sequencing (Accession no. MW642479) was used as a positive control, and sterile water was used as the blank control for each run. The cycling conditions for the first and second round amplification involved 3 min of denaturation at 95 °C, 35 cycles at 95 °C for 30 s, annealing for 30 s at a temperature dependent on the primers applied (50 °C and 54°C, respectively), and 72 °C for 1 min, with a final extension step at 72 °C for 5 min. PCR products were electrophoresed (Biorad, USA) in 1.5% agarose in Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide (Sigma-Aldrich, St-Louis MO, USA) to visualize the amplified DNA fragments with gel documentation system (Avegene, Taiwan).

Sequence analysis

The PCR products and primers coding the 16S rRNA gene region were properly packaged and sent to the Sentebiolab Company (Ankara) for performing DNA sequence analysis. The sequence chromatograms were checked and arranged using Bioedit software.¹⁵ The final consensus sequences of our isolates were subjected to the "BLAST analysis" (<http://www.ncbi.nlm.nih.gov/BLAST>) in the GenBank Database, and their similarity rates were compared with the isolates reported from different countries and hosts. Genetic distances were calculated using the Kimura 2 parameter model in MEGA 7.0. The 16S rRNA phylogenetic analysis data set was formed from the nucleotide sequences of 35 isolates in total. *Babesia canis* was used as the "out-group." The phylogenetic analyses and tree creation were carried out using the "maximum likelihood" method on MEGA 7.0 software with 1000 bootstrap replicates.¹⁶ The nucleotide sequences obtained in the study were recorded in the GenBank with the accession numbers of MW642476-MW642478.

Statistical analysis

Statistical evaluation was carried out by the chi-squared and Fisher's exact test (SPSS 22 program) to analyze the results of seroprevalence and molecular in relationship to age, gender and breed of horses. *P*-values of less than 0.05 were considered significant.

Results

Eight (8.6%) of the 93 sera examined were seropositive in terms of equine granulocytic anaplasmosis, using ELISA method. There were no statistically significant differences

between age, gender and breed horses (for both molecular and serological results) (Table 1). The results of nested PCR of DNA showed 6.4% (6/93) of horses were positive to *A. phagocytophilum* (Figure 1). None of the 6 PCR positive horses were seropositive for *A. phagocytophilum*. We amplified three PCR products of the 16S rRNA for sequencing analysis. At the same time, our positive control was sequenced (MW642479). The three isolates (MW642476, MW642477 and MW642478) showed 100% similar nucleotid with each other. The our isolates MW642476, MW642477 and MW642478 most closely related to the isolate detected in red deer from Poland (GQ450278) and in dog from South Africa (MK814406) (according to BLASTN analysis).

Phylogenetic analysis based on the 16S rRNA sequences showed that *A. phagocytophilum* comprised of two clades have emerged that are very sharply separated from each

other (Figure 2). Clade 1 involved isolates from several hosts, including horses, sheeps, cats, human, dogs, goats, Brown rat,

cattle and ticks and this sequences identified with together the our three isolates and showed genetic affinity. Four sequences obtained in South Korea (cat, cattle and horse comprised for host, respectively) and Tunisia (horse) classified as clade 2 and were quite diverge from the isolates in our study. The current study sequences didn't form a separate clade. Hence, they showed similar homology among themselves. This configuration could be a sign of different adaptation of the patogen to the environment (geographic zones, ticks, hosts). If we compared our isolates and clade 2, there were distinct differences in numerous nucleotide sequences (Figure 3), pairwise distances were most 0.587.

Table 1. Epidemiological data according to serological and molecular results

	Study population N= 93	ELISA positive horses N=8 (%)*	nPCR positive horses N=6 (%)*
Age (years)			
	1-3	0	0
	4-9	7 (10.1)	5 (7.2)
	≥10	1 (7.7)	1 (7.6)
Gender			
	Female	3 (5.2)	4 (7)
	Male	5 (13.9)	2 (5.5)
Breed			
	Mixed-breed	3 (6)	5 (10)
	Arabian Horse	4 (10.3)	1 (2.5)
	British Horse	1 (25)	0
Total	93	8 (8.6)	6 (6.4)

* $p > 0.05$; statistically insignificant

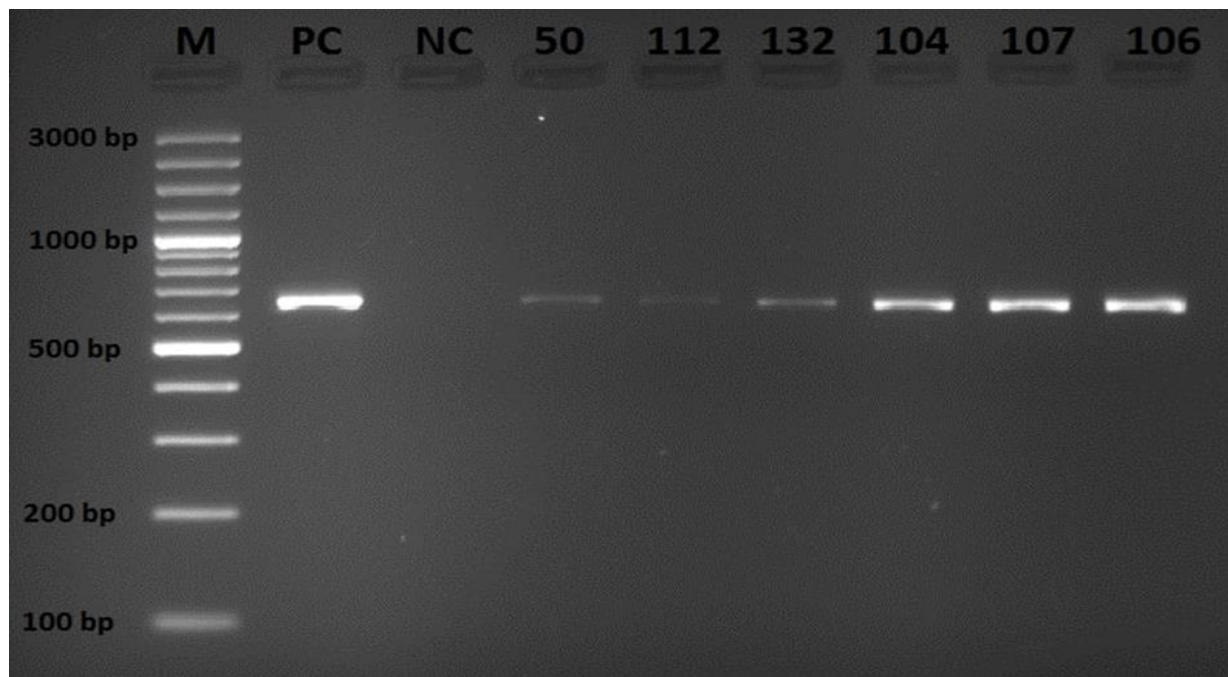


Figure 1. *Anaplasma phagocytophilum* positive samples on agarose gel at the 2nd step of the Nested PCR analyses. M: Marker (100bp), 50/112/132/104/107/106/: Positive samples, PC: Positive control, NC: Negative control

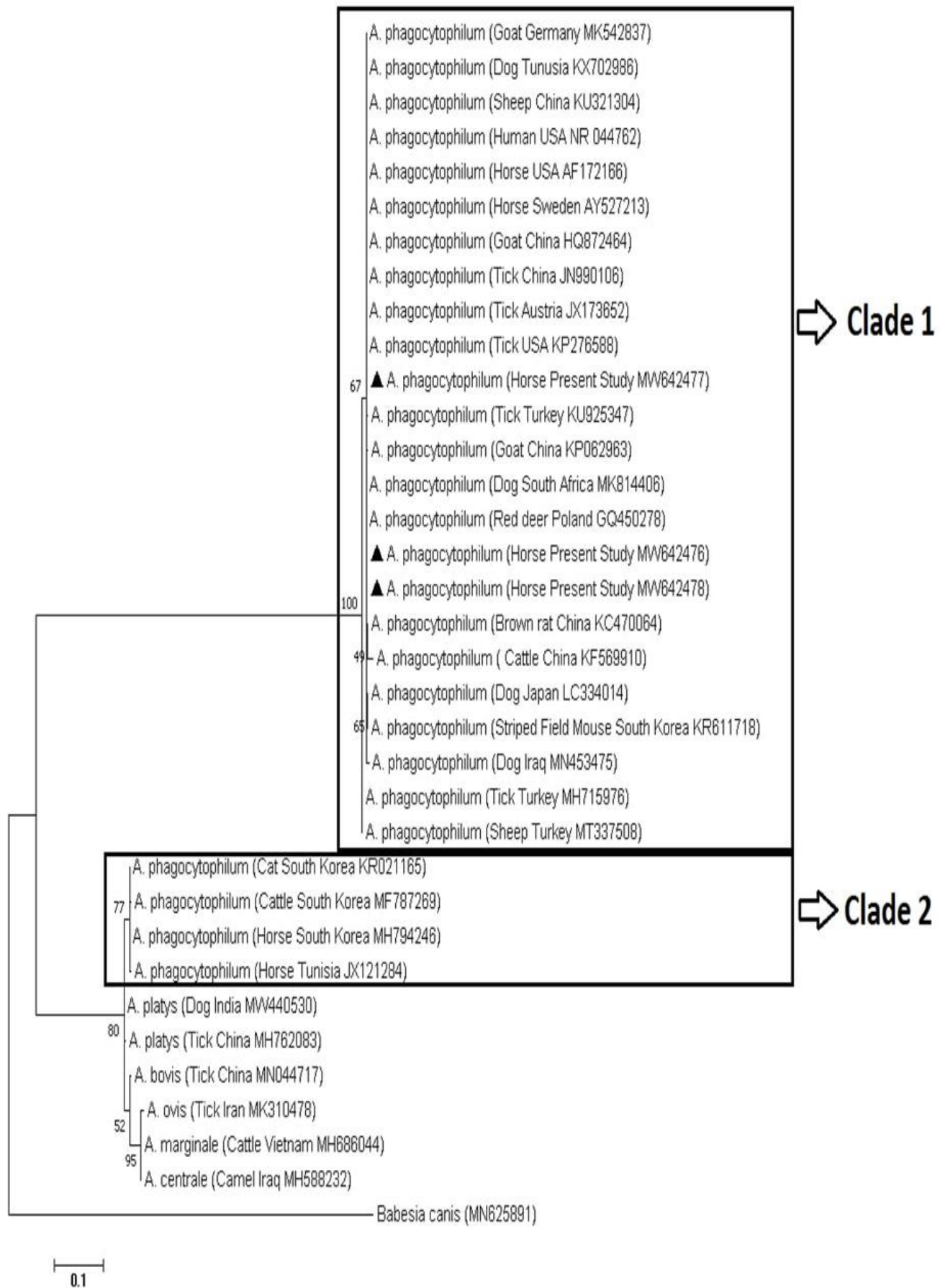


Figure 2. Maximum likelihood phylogenetic tree of *A. phagocytophilum* 16S rRNA gene sequences with 1,000 bootstrap replicates. *Babesia canis* isolate as outgroup (MN625891) used. Isolates from this study are indicated with a triangle round. Scale line shows the nucleotide change per site.

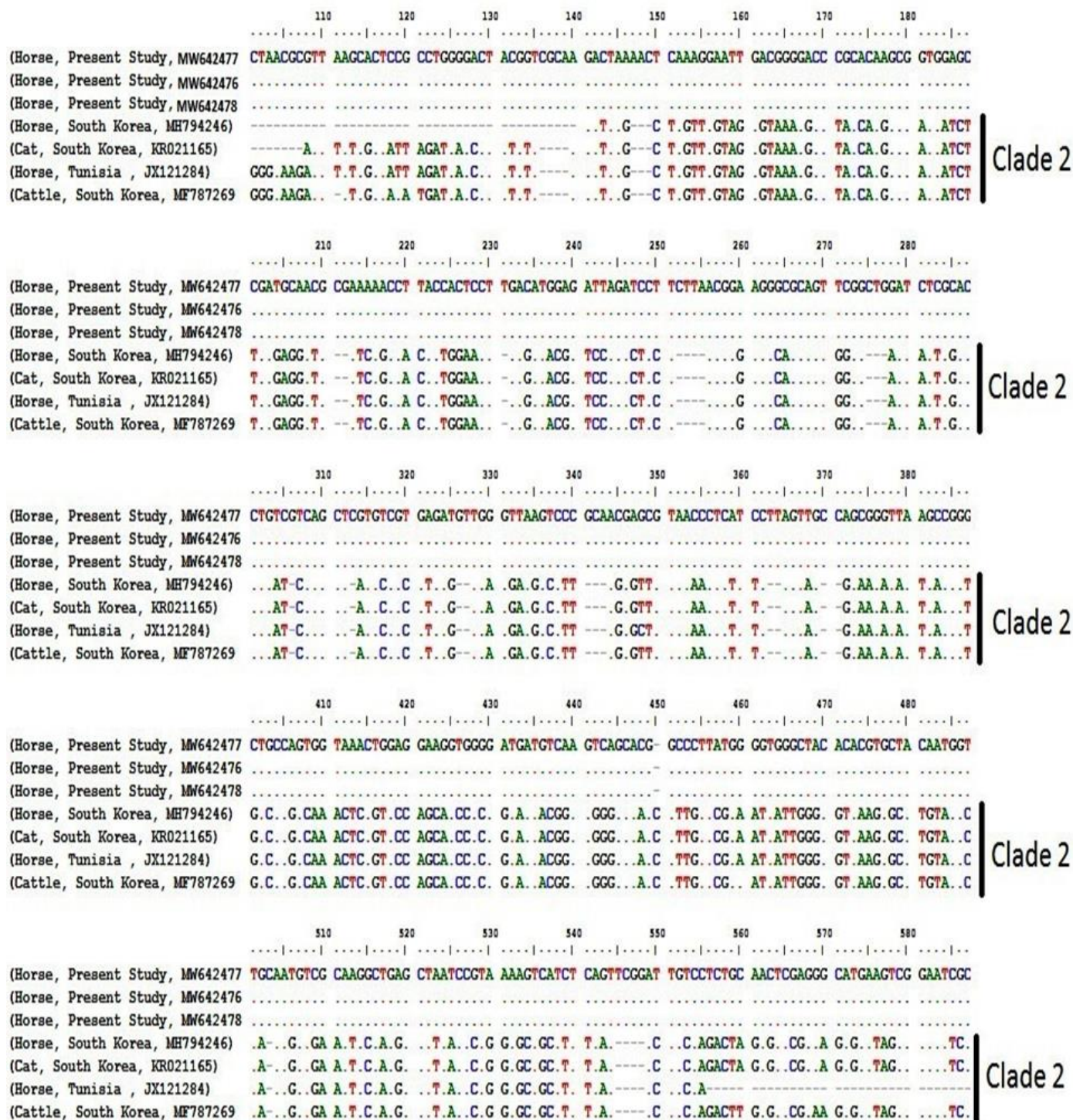


Figure 3. Multiple alignments of the primers designed from 16S rRNA gene region of our isolates *A. phagocytophilum* with the other *A. phagocytophilum* (especially Clade 2) sequences from GenBank.

Discussion

In this study, *A. phagocytophilum* was detected in horses for the first time in Mus province of Turkey by molecular methods. In addition, we detected a low prevalence of this pathogen. The incidence of *A. phagocytophilum* may be associated with predominantly tick distribution or asymptomatic animals. The information on the epidemiology of the potential tick vectors is unclear in the Turkey. It has been detected in three species of ticks (*I. ricinus*, *H. sulcata*, and *R. bursa*) so far.⁴ The present study, tick was not detected in any horse sample. Hence, the

investigation of other tick species transmitting *A. phagocytophilum* in Turkey should be further executed. *A. phagocytophilum* IgG antibodies have been detected in horses in Turkey (8.57%).¹³ Our result was found to be approximately equal with the above report (8.6%). There were no statistically significant differences between genders ($p>0.05$). Similarly, M’ghirbi et al.¹⁷ and Laamari et al.¹⁸ reported that no significant differences in seroprevalence according to gender. On the contrary, there are previous studies reporting that higher seroprevalence in females than in males.^{19,20} Therefore, we assume that seroprevalence may be related to the housing and lifestyle of horses. British Horse proved to be the breed with the highest

seroprevalence but we think that it does not provide an accurate epidemiological data for a total of four horses. The seroprevalence was significantly higher in 4-9 year-old horses in comparison to the other age groups. Though, the difference was not statistically significant ($p>0.05$). Surprisingly, there is also a study in which young animals were infected at a higher rate.¹⁸ It is in accordance with other studies indicating that older animals were more frequently infected than young animals.^{20,21} These horses likely have been exposed to ticks contaminated with *A. phagocytophilum* for a longer period of time.

Investigating whether IgG antibodies have been formed for *A. phagocytophilum* offers an excellent screening method to detect the seroprevalence of pathogens. Immunologic studies with *A. phagocytophilum* demonstrated that first antibody titers against this pathogen occur after 19 days. In this case, the importance of molecular methods is revealed once again for earlier diagnosis.^{22,23} *A. phagocytophilum* DNA was detected by nested PCR in 6 blood samples among 93 tested horses (6.4%). The molecular prevalence observed in the horse population in present study is substantially lower than the prevalence (19.6%) lately observed among small ruminants living in the same geographical region.²⁴ These small ruminants may have potentially played a role in distribution of the pathogen such as vectors.

It has been reported that the GroEL and msp2 genes could separately describe *A. phagocytophilum*.²⁵ Several studies have demonstrated that the pathogenicity or genotype of *A. phagocytophilum* would be determined especially by using the groEL gene region.^{8, 26-28} In the present study, we couldn't replicate horses DNA using groEL fragment, in this way, we couldn't classify *A. phagocytophilum* with regard to genotype. The isolates belonging to clade 1 were most closely related to the isolate detected in dog from South Africa (MK814406). Researchers identified the isolate obtained from the dog in South Africa as a new variant "Aph1/GltA".²⁹ These results demonstrate that *A. phagocytophilum* identified in horses in this study might be Aph1/GltA sequence variant. In this regard, there is a need to further study.

Conclusion

This is the first study to report molecular detection and phylogenetic analysis of *A. phagocytophilum* in horses in Mus province of Turkey. Our findings indicated that there was quite a change genetic mutability by comparison to the isolates of *A. phagocytophilum* circulating in the South Korea and Tunisia. Further studies are needed to research both the tick vectors and different host of this pathogen which has zoonotic potential.

Conflict of Interest

The author has no conflicts of interest.

Compliance with Ethical Statement

Ethics Committee approval for this research was obtained from the Local Ethics Committee for Animal Experiments of Van Yüzüncü Yıl University, (dated July 29, 2021, no. 2021/07-02).

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Author Contributions

B.O: Design; B.O: Project development; B.O Literature search; B.O: Analysis; B.O: Manuscript writing; B.O: Critical review

References

- Inci A, Yildirim A, Duzlu O, Doganay M, Aksoy S. Tick-Borne Diseases in Turkey: A Review Based on One Health Perspective. *PLoS Negl Trop Dis.* 2016; 10(12):e0005021. doi: 10.1371/journal.pntd.0005021.
- Oğuz B, Özdal N, Kılınç ÖO, Karakuş A, Çelik BA, Değer MS. Van, Muş, Siirt ve Diyarbakır İllerinde Sığırlarda Anaplasmosis'in Seroprevalansı. *Kocatepe Vet J.* 2018;11(3):208-214. doi.org/10.30607/kvj.397726.
- Pusterla N, Madigan JE. Equine Granulocytic Anaplasmosis. *J Equine Vet Sci.* 2013;33:493-496. doi.org/10.1016/j.jevs.2013.03.188.
- Ceylan O, Byamukama B, Ceylan C, et al. Tick-Borne Hemoparasites of Sheep: A Molecular Research in Turkey. *Pathogens.* 2021;10(2):162. doi: 10.3390/pathogens10020162.
- Fuente JDL, Massung RF, Wong SJ, et al. Sequence Analysis of the msp4 Gene of *Anaplasma phagocytophilum* Strains. *J Clin Microbiol.* 2005;43(3):1309-1317. doi: 10.1128/JCM.43.3.1309-1317.2005.
- Wang Y, Chen C, Zhang L. Molecular characterization of Msp2/P44 of *Anaplasma phagocytophilum* isolated from infected patients and *Haemaphysalis longicornis* in Laizhou Bay, Shandong Province, China. *PLoS One.* 2013;8:e78189. doi: 10.1371/journal.pone.0078189.
- Smrdel KS, von Loewenich FD, Petrovec M, Županc TA. Diversity of ankA and msp4 genes of *Anaplasma phagocytophilum* in Slovenia. *Ticks Tick Borne Dis.* 2015;6(2):164-166. doi.org/10.1016/j.ttbdis.2014.11.008.
- Chisu V, Zobba R, Lecis R, et al. GroEL typing and phylogeny of *Anaplasma* species in ticks from domestic and wild vertebrates. *Ticks Tick Borne Dis.* 2018; 9:31-36. doi: 10.1016/j.ttbdis.2017.10.012.
- Akkoyun Z, Oğuz B. Seroprevalence of Theileria equi and Babesia caballi in horses of Mus province, Turkey. *AJVS.* 2019;60:22-29. doi: 10.5455/ajvs.22338.
- Machado RZ, Teixeira MM, Rodrigues AC, et al. Molecular diagnosis and genetic diversity of tick-borne Anaplasmataceae agents infecting the African buffalo Syncerus caffer from Marromeu Reserve in Mozambique. *Parasit Vectors.* 2016;9:454. doi: 10.1186/s13071-016-1715-y.
- Dzięgiel B, Adaszek L, Carbonero A, et al. Comparative analysis of 16S RNA nucleotide sequences of *Anaplasma phagocytophilum* detected in the blood of horses from various parts of Europe. *J Med Microbiol.* 2013;62:1891-1896. doi: 10.1099/jmm.0.058636-0.
- Yang J, Liu Z, Niu Q, et al. Evaluation of different nested PCRs for detection of *Anaplasma phagocytophilum* in ruminants and ticks. *BMC Vet Res.* 2016;12(1):35. doi: 10.1186/s12917-016-0663-2.
- Günaydın E, Pekaya S, Kuzugüden F, Zeybek M, Gökmen TG, Ütük AE. The First Detection of anti-*Anaplasma phagocytophilum* Antibodies in Horses in Turkey. *Kafkas Univ Vet Fak Derg.* 2018;24(6):867-871. doi: 10.9775/kvfd.2018.20171.
- Kawahara M, Rikihisa Y, Lin Q, et al. Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. *Appl Environ Microbiol.* 2006; 72(2):1102-1109. doi: 10.1128/AEM.72.2.1102-1109.2006.
- Hall T. Bio Edit: a user-friendly biological sequence alignment editor and analysis suite. *Nucleic Acids Symp Ser.* 1999;41:95-98. doi: 10.14601/Phytopathol_Mediterr-14998u1.29.
- Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger

- Datasets. *Mol Biol Evol.* 2016;33(7):1870-1874. doi: 10.1093/molbev/msw054.
17. M'ghirbi Y, Yaïch H, Ghorbel A, Bouattour A. *Anaplasma phagocytophilum* in horses and ticks in Tunisia. *Parasit Vectors.* 2012;5:180. doi: 10.1186/1756-3305-5-180.
 18. Laamari A, Azzag N, Tennah S, et al. Seroprevalence of Antibodies Against *Anaplasma Phagocytophilum* and *Borrelia Burgdorferi* in Horses (*Equus Caballus*) from Northern Algeria. *J Vet Res.* 2020;64(3):413-419. doi: 10.2478/jvetres-2020-0045.
 19. Praskova I, Bezdekova B, Zeman P, Jahn P. Seroprevalence of *Anaplasma phagocytophilum* in horses in the Czech Republic. *Ticks Tick Borne Dis.* 2011;2:111-115. doi.org/10.1016/j.ttbdis.2011.01.002.
 20. Lee SH, Kim KT, Yun SH, et al. Serological and molecular detection of *Anaplasma phagocytophilum* in horse reared in Korea. *Vet Med.* 2015;60:533-538. doi: 10.17221/8491-VETMED.
 21. Laus F, Veronesi F, Passamonti F, et al. Prevalence of tick borne pathogens in horses from Italy. *J Vet Med Sci.* 2013;75:715-720. doi: 10.1292/jvms.12-0449.
 22. Andel AEV, Magnarelli LA, Heimer R, Wilson ML. Development and duration of antibody response against *Ehrlichia equi* in horses. *J Am Vet Med Assoc.* 1998;212:1910-1914. PMID: 9638192
 23. Passamonti F, Veronesi F, Cappelli K, et al. *Anaplasma phagocytophilum* in horses and ticks: a preliminary survey of Central Italy. *Comp Immunol Microbiol Infect Dis.* 2010;33:73-83. doi: 10.1016/j.cimid.2008.08.002.
 24. Altay K, Dumanlı N, Aktas M, Özübek S. Survey of Anaplasma infections in small ruminants from East part of Turkey. *Kafkas Univ Vet Fak Derg.* 2014;20:1-4. doi: 10.9775/kvfd.2013.9189.
 25. Seo MG, Ouh IO, Choi E, Kwon OD, Kwak D. Molecular Detection and Phylogenetic Analysis of *Anaplasma phagocytophilum* in Horses in Korea. *Korean J Parasitol.* 2018;56(6):559-565. doi: 10.3347/kjp.2018.56.6.559.
 26. Haschke-Becher E, Bernauer R, Walleczek AM, et al. First detection of the *Anaplasma phagocytophilum* groEL-A genotype in man. *J Infect.* 2010;60(4):300-305. doi: 10.1016/j.jinf.2009.12.010.
 27. Rymaszevska A. Genotyping of *Anaplasma phagocytophilum* strains from Poland for selected genes. *Folia Biol.* 2014;62:37-48. doi: 10.3409/fb62_1.37.
 28. Domenico MD, Pascucci I, Curini V, et al. Detection of *Anaplasma phagocytophilum* genotypes that are potentially virulent for human in wild ruminants and *Ixodes ricinus* in Central Italy. *Ticks Tick Borne Dis.* 2016;7:782-787. doi: 10.1016/j.ttbdis.2016.03.012.
 29. Kolo AO, Collins NE, Brayton KA, et al. *Anaplasma phagocytophilum* and Other *Anaplasma* spp. in Various Hosts in the Mnisi Community, Mpumalanga Province, South Africa. *Microorganisms.* 2020;8(11):1812. doi.org/10.3390/microorganisms8111812.