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Molecular survey of zoonotic *Anaplasma phagocytophilum* and genetic evidence of a putative novel *Anaplasma* species in goats from Taif, Saudi Arabia

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Abstract

Aim: Genus *Anaplasma* is of veterinary and public health importance, and its members utilize ruminants as key hosts in their epidemiology. To date, information about the occurrence and molecular identity of *Anaplasma phagocytophilum* and other *Anaplasma* species in Saudi Arabian goats is scarce. This study aimed to molecularly detect and characterize zoonotic *A. phagocytophilum* and other *Anaplasma* spp. in goats from Taif District, KSA.

Materials and Methods: Blood samples collected from 67 goats were polymerase chain reaction tested using common and *A. phagocytophilum*-specific primers targeting 16S rRNA and *msp4* genes, respectively. Amplicons of common reactions were purified, sequenced, and analyzed.

Results: Six goats yielded positive results with common primers, whereas all animals proved negative for *A. phagocytophilum*. Analysis of the two successfully sequenced amplicons revealed the presence of a variant strain of *Anaplasma ovis* (99.52% ID) and a new *Anaplasma* organism, which was clustered with *Anaplasma bovis* (95.9% ID) and *Aegyptianella pullorum* (94.99% ID) and distinctly separated from all other recognized species of the genus *Anaplasma*.

Conclusion: The tested goats proved negative for *A. phagocytophilum*; however, we could not confirm that the area is pathogen free. A variant strain of *A. ovis* and a putative novel *Anaplasma* spp. were reported raising the concern of veterinary and zoonotic potential. Other genes should be sequenced and analyzed for complete identification of the detected organisms.

Keywords: *Anaplasma ovis*, *Anaplasma phagocytophilum*, goats, phylogeny, Saudi Arabia.

Introduction

Anaplasmataceae (Rickettsiales) encompasses five recognized genera: *Anaplasma*, *Ehrlichia*, *Aegyptianella*, *Neorickettsia,* and *Wolbachia*. Organisms of this family are obligatory intracellular Gram-negative bacteria of veterinary and public health importance [1,2]. *Anaplasma* includes seven species: *Anaplasma marginale*, *Anaplasma centrale*, *Anaplasma ovis*, *Anaplasma platys*, *Anaplasma bovis*, *Anaplasma phagocytophilum*, and *Anaplasma capra* [3,4]. The cellular tropism, host range, vectors, and pathogenicity of these species are variables [1].

A. marginale, *A. centrale,* and *A. ovis* are closely related intraerythrocytic pathogens of ruminants [5-7]. *A. marginale* is known to be highly pathogenic in cattle and can result in considerable economic losses [8,9]. *A. centrale* is less pathogenic, and, therefore, it has been used as a live vaccine against *A. marginale* in cattle [10,11]. *A. ovis* has moderate pathogenicity for sheep and goats; however, it can cause severe disease in animals exposed to stress factors [12-15]. Interestingly, variant strains of *A. ovis* were implicated in human disease in Cyprus and Iran [16,17]. *A. platys* is known to infect platelets and causes canine cyclic thrombocytopenia in dogs [18,19]; moreover, new closely related strains have been detected in camels, cattle, sheep, and goats, postulating that ruminants are a likely alternative host for *A. platys* [4,20-24]. Genomic evidence of *A. platys* was also identified in human patients from Venezuela, suggesting a potential public health risk [25]. *A. bovis*, a monocytotropic species, has been commonly recorded in cattle and buffalo from different countries [26-28]; noteworthy, 16S rRNA gene sequences of *A. bovis* have been identified in Chinese goats [24]. *A. phagocytophilum* is a zoonotic pathogen which replicates in granulocytes of many host species, including domestic ruminants, deer, horse, dog, rodents, and humans. The pathogen causes human, canine, and equine granulocytic anaplasmosis and tick-borne fever in ruminants [29-36]. *A. capra*, a newly recorded novel species, was identified in goats, sheep, ticks, and humans in China;

however, its vectors and infected cell types are unclear [3,4,37]. Other several candidates and unclassified *Anaplasmataceae* species were recently molecularly described [38-44].

According to what was stated above, it is obvious that ruminants (including goats) represent key hosts in the epidemiology of *Anaplasma* including zoonotic species. To date, information about the occurrence and molecular identity of *Anaplasma* species in Saudi Arabian domestic ruminants is scarce [45]. To the best of our knowledge, there is only one molecular survey of *A. ovis* and *A. phagocytophilum* in goats from Al Madinah region [46].

This study aimed to molecularly detect and characterize zoonotic *A. phagocytophilum* and other *Anaplasma* spp. in goats from Taif District, KSA.

Materials and Methods

Ethical approval

Blood samples were collected while slaughtering the goats at Taif abattoir; therefore, no ethical permission was needed.

Blood samples and DNA extraction

Blood samples were collected from 67 goats while slaughtering at Taif abattoir. These animals were residing at Taif district (approximately 21° 26' 14'' N and 40° 30' 45'' E), KSA. The samples were sent under refrigeration to Biotechnology Laboratory at Taif University and stored at −20°C until DNA extraction. According to the manufacturer's protocol, purification of DNA was executed using AxyPrep Blood Genomic DNA Miniprep Kit (Cat. No. AP-MN-BL-GDNA-250).

Polymerase chain reaction (PCR) and sequencing

All samples were examined using PCR technique with common primer pair: ECC (5'-AGA ACG AAC GCT GGC GGC AAG CC-3') and ECB (5'-CGT ATT ACC GCG GCT GCT GGC A-3'). These oligonucleotides were used to amplify the target sequence of 16S rRNA gene of *Anaplasma* spp. [47,48]. Animals were also tested using MAP4AP5 (5'-ATG AAT TAC AGA GAA TTG CTT GTA GG-3') and MSP4AP3 (5'- TTA ATT GAA AGC AAA TCT TGC TCC TAT G-3') primers which target *msp4* gene of *A. phagocytophilum* [49]. PCR reactions were implemented in 25-µl mixtures containing 12.5 µl GoTaq Green Master Mix (Promega Corporation, Madison, WI 53711-5399, USA), and 20 pmoles each primer. The thermocycle profile used in common reactions included 2-min denaturation at 94°C, 40 cycles (1-min denaturation at 94°C, 2-min annealing at 55°C, and 30-s extension at 72°C), and additional step of 5-min final extension at 72°C. The cycling program for the specific PCR using MAP4AP5 and MSP4AP3 primers implemented the following profile: initial 30-s denaturation at 94°C, 35 cycles (each consisting of 30-s denaturation at 94°C and combined 1-min annealing and extension at 55°C), and 5-min final extension at 72°C. Positive control samples obtained from a previous study using ECC and ECB primers [45] and negative "NO DNA" controls were included in each run. Amplicons were analyzed by agarose gel electrophoresis. Amplicons of ~500 bp and 849 bp indicate positive results for common and specific reactions, respectively.

Sequencing and analysis

According to the manufacturer's instructions, target amplicons of positive common primer samples were extracted from agarose gel using FavorPrep Gel Purification Mini Kit (Cat. No. FAGPK001). Purified products were subjected to bidirectional sequencing using Macrogen facilities.

Sequence analysis

BLAST search was performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to investigate homologies with sequences available in database. The obtained DNA sequences were aligned using MAFFT [50]. The unalignable and gap-containing sites were deleted so that 300 bp were left for the analysis. The neighbor-joining phylogenetic tree was constructed with bootstrap analysis of 1000 replicates to represent the evolutionary history of the taxa analyzed [51]. The accession numbers used for comparison with our detected strains are shown in the phylogenetic tree.

Nucleotide sequence accession numbers

The partial 16S rRNA nucleotide sequences obtained in the current study were registered at GenBank under the following accession numbers: LC467272 *(Anaplasma* spp. MWG-2019, Ghafar-G24 strain) and LC467273 *(Anaplasma* spp. MWG-2019, Ghafar-G25 strain).

Results

Of 67 goats, 6 (9%) yielded positive results when PCR tested using common primers, whereas all of animals proved negative for *A. phagocytophilum*. Two positive common reaction amplicons were successfully sequenced, and BLAST search of their partial 16S rRNA gene sequences showed that there were no 100% identical sequences; therefore, the new names "*Anaplasma* spp. MWG-2019, Ghafar-G24" and *Anaplasma* spp. MWG-2019, Ghafar-G25" were assigned. Ghafar-G24 possessed highest similarity (100% QC, 0.0 E-value, 99.52% ID) with *A. ovis* strain (JQ917900) detected in ticks from China. However, Ghafar-G25 showed highest identity (100% QC, 0.0 E-value, 96.13% ID) with *A. bovis* strain (KP314239) detected in Chinese ticks and with uncultured *Anaplasma* spp. (LC066137) detected in ticks from Bangladesh. Similarity features of our detected strains with species used in the phylogenetic tree are presented in Table-1. Phylogenetic analysis with recognized species representing *Anaplasmataceae* (Figure-1) revealed that Ghafar-G24 strain is closely related to and clustered

Table-1: Similarity features of the detected *Anaplasma* organisms to recognized species used in the phylogenetic tree. (Accessed March 16, 2019).

Accession	Organism	Isolate/strain	*Anaplasma* spp. Ghafar-G24			*Anaplasma* spp. Ghafar-G25		
			QC (%)	E	ID (%)	QC (%)	E	ID (%)
JQ917900	*Anaplasma ovis*	WYG59	100	0.0	99.52	100	0.0	94.53
AY262124	*Anaplasma ovis*	-	100	0.0	99.28	100	0.0	94.53
JF514507	*Anaplasma ovis*	54y-SV161	73	3e-164	99.67	72	7e-156	96.86
KU686784	*Anaplasma centrale*	Uganda KT5	100	0.0	98.81	99	0.0	94.76
AF414869	*Anaplasma centrale*	South Africa	100	0.0	98.81	100	0.0	94.76
AB916498	*Anaplasma marginale*	Ghafar-1Catl-KSA	100	0.0	98.33	99	0.0	94.95
AB916499	*Anaplasma marginale*	Ghafar-6Catl-KSA	100	0.0	97.85	100	0.0	95.01
KM206273	*Anaplasma capra*	HLJ-14	100	0.0	95.71	100	0.0	93.64
MH762077	*Anaplasma capra*	AK-Rm-429	100	0.0	95.71	100	0.0	93.64
JN558824	*Anaplasma bovis*	G49	100	0.0	94.03	100	0.0	95.90
KP314251	*Anaplasma bovis*	tick 18/China/2013	100	0.0	94.27	100	0.0	95.90
U02521	*Anaplasma phagocytophilum*	Webster	100	0.0	93.82	100	0.0	95.01
KC800985	*Anaplasma phagocytophilum*	9B13	100	0.0	94.30	99	0.0	95.45
AY125087	*Aegyptianella pullorum*	-	100	0.0	93.79	100	0.0	94.99
AY077619	*Anaplasma platys*	Okinawa	100	0.0	93.81	99	0.0	95.44
MF289478	*Anaplasma platys*	YY36	100	0.0	93.57	100	0.0	95.67
KF843825	*Candidatus* Anaplasma camelii	Camel_7	100	0.0	93.57	100	0.0	95.69
KF843823	*Candidatus* Anaplasma camelii	Camel_2	100	0.0	93.57	100	0.0	95.69
AB196302	*Ehrlichia muris*	FN2619	100	8e-162	90.61	100	2e-173	91.05
U96436	*Ehrlichia ewingii*	95E9-TS	87	8e-162	94.02	100	2e-173	91.05
EU826516	*Ehrlichia chaffeensis*	clone 16S_Echaf_Ap	87	2e-163	94.29	100	6e-173	91.01
U03777	*Ehrlichia ruminantium*	Ball3	100	1e-155	89.83	100	4e-175	91.42
M73221	*Ehrlichia canis*	-	87	4e-160	93.75	100	5e-179	91.89
EU810404	*Candidatus* Neoehrlichia mikurensis	-	100	6e-163	90.93	100	1e-179	92.05
AF179630	*Wolbachia pipientis*	-	99	4e-135	87.08	100	7e-158	89.12
U12457	*Neorickettsia helminthoeca*	-	100	3e-121	84.96	100	3e-136	86.10
L36105	*Rickettsia conorii*	Moroccan	100	9e-117	84.49	100	9e-122	84.28

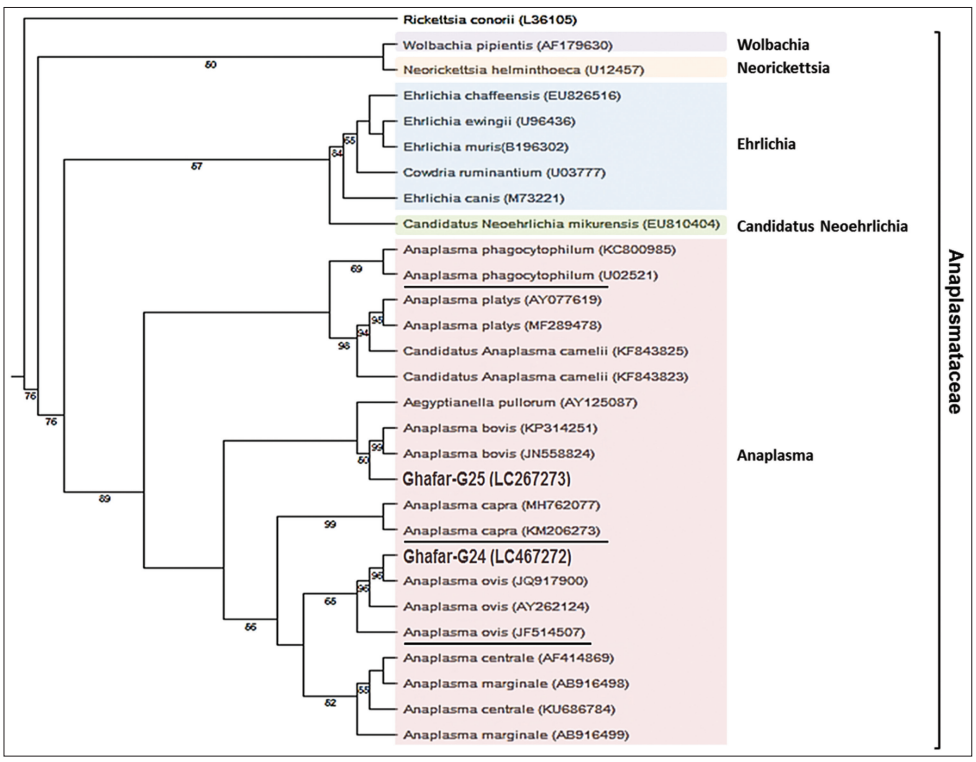

Figure-1: Neighbor-joining phylogenetic analysis of the 16S rRNA gene of detected *Anaplasma* spp. strains (bold) with selected designated *Anaplasmataceae* spp. Numbers at the nodes refer to bootstrap probabilities when they are above 50%. GenBank accession numbers are shown in parentheses and human pathogens are underlined.

with *A. ovis* of both animal and human origin. The phylogeny also placed Ghafar-G25 strain on a distinct, separate branch within a clade containing *A. bovis* and *Aegyptianella pullorum*.

Discussion

To date, very little is known about the magnitude of *Anaplasma* pathogens in Saudi Arabia. Few studies concerned microscopic examination of blood smears detected these bacteria in camel (40.5%), cattle (0.98%, 1%, and 3.4%), and sheep (2%) [52-54]. Other two serological surveys demonstrated the occurrence of *A. marginale* in camel (8.57% and 14%) [55], as well as *A. ovis* and *A. phagocytophilum* in sheep and goats [46]. In addition, only three molecular studies were performed to elucidate the molecular identity of *Anaplasma* spp. in the Kingdom [20,45,46]. Noteworthy, to the best of our knowledge, there is no study of any kind was performed on anaplasmosis in the human population. Therefore, the role of *Anaplasma* spp. in both animal and human medicine in KSA is not clear. In the present study, we tried to molecularly identify zoonotic *A. phagocytophilum* and other occurring *Anaplasma* spp. in goats residing in Taif district, KSA.

16S rRNA gene-based PCR and sequencing were employed in our experiment. This molecular technique proved invaluable in the detection and taxonomic classification of newly discovered bacteria and organisms that are difficult to grow in the laboratory. This is attributed to the fact that 16S rRNA gene is less variable and therefore is sensitive to phylogenetically discriminate between different species [1,56].

The negative detection of *A. phagocytophilum* in this study is consistent with the previous study conducted in the area to detect this pathogen in camel, cattle, and sheep [45]. Several plausible explanations could account for this negative result. The first, most likely, explanation is that Taif district is free of the disease due to the absence of competent vector in the area. The second, least likely, explanation is that the pathogen is present in low prevalence rate, but using of relatively small-sized sample (67 goats) led to the production of a biased result. The third, unlikely, explanation is that blood samples were collected after a short duration of bacteremia, and therefore, detection of the organism was impossible.

Six goats yielded positive results in PCR using common primers. BLAST search and phylogeny of the two successful sequences (Ghafar-G24 and Ghafar-G25) showed that the detected organisms belong to *Anaplasma* but distinct from all established species.

Ghafar-G24 clustered with *A. ovis* strains of tick, sheep, and human origin with identity ranged from 99.28% to 99.67% (Figure-1 and Table-1), suggesting that this organism is a variant strain of *A. ovis*. The variation in the short sequenced fragment (300 bp) may have a great impact on ecology and pathogenicity of the present strain, especially when associated with other genetic differences in protein-coding genes. Unfortunately, the clinical history of the tested goats was unavailable. Given the previous information, we cannot confirm that Ghafar-G24 strain can cause animal or human disease; however, the veterinary and human public health impact should be considered.

Ghafar-G25 strain showed genetic distance from other known *Anaplasma* species with highest relatedness (96.13% identity) to *A. bovis* and uncultured *Anaplasma* spp. Phylogeny clustered Ghafar-G25 strain with strains of *A. bovis* and *A. pullorum* (Figure-1). Noteworthy, *A. pullorum* is still needed to be clarified whether it belongs to *Anaplasma* or remains in a distinct genus under *Anaplasmatacea* [2]. According to its level of 16S rRNA gene divergence and the cutoff value (99.0%) for species delineation [57], this strain can be potentially classified as novel species as sequence identities varied from 93.64% to 95.9% (Table-1) when blasted with all officially recognized *Anaplasma* species. Interestingly, the divergence seen in 16S rRNA gene between this Saudi Arabian strain and all known *Anaplasma* species is greater than the divergence seen between the established genera of *Anaplasmataceae*, providing strong evidence for the recognition of a putative new taxon at the genus level [41]. Given all the previous information, we cannot confirm that Ghafar-G25 constitutes a new genus or even a novel species as the formal description requires analysis of multiple other genes. Unfortunately, the amount of DNA available was limited and did not allow additional sequencing.

Detection of the novel *Anaplasma* agent in goats does not confirm that this animal species is a competent reservoir for this pathogen; however, this study is a crucial initial step in reservoir competence studies. Molecular detection and phylogeny of new *Anaplasma* species from different hosts and geographic areas are still needed for elucidating the taxonomic and phylogenetic relationships among *Anaplasmataceae* species. We cannot confirm that Ghafar-G25 bacteria can cause disease in animals or human; however, the veterinary health and zoonotic potential of this strain should be considered.

Conclusion

This study reports for the first time the presence of a potentially zoonotic variant strain of *A. ovis* and a putative novel *Anaplasma* spp. in goats from Saudi Arabia. Other multiple genes should be sequenced and analyzed to reach the formal description of the detected organisms. Other investigations are also required to elucidate the epidemiology of the newly discovered agent including competent vector and reservoir, as well as geographic distribution. Pathogenicity to animals and zoonotic importance of the organism should also be determined.

Authors' Contributions

MWG designed the study, collected the samples and materials, and performed the experiments. SAMA and MWG conducted molecular and phylogenetic analyses. Both authors wrote the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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