Molecular detection and characterization of *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis, in some animals suspected to be competent reservoirs in Taif district, Kingdom of Saudi Arabia

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Abstract: *Anaplasma phagocytophilum* is the causative agent of human granulocytic anaplasmosis (HGA), an emerging tick-borne zoonosis. The pathogen utilizes cattle, sheep, goats, and wide variety of animal species as reservoirs and members of genus *Ixodes* tick as vectors. Although conditions for transmission of the agent in Kingdom of Saudi Arabia do exist, there is no work of any kind was done to address the existence and epidemiology of the disease in the country. The objectives of this study are to molecularly detect and characterize *A. phagocytophilum* in some animals suspected to be competent reservoirs in Taif district, KSA. DNA was extracted from EDTA whole-blood samples from 44 dromedary camels, 20 cattle, 50 sheep, 5 desert foxes (*Vulpes rueppellii*), and 10 Dabb lizards (*Uromastyx ornata*). For screening purposes, PCR targeting 16S rRNA gene using the common primer pair ECC and ECB was conducted to all samples. All purified DNA preparations were also tested using heminested (targeting 16S rRNA gene) and standard (targeting *msp4* gene) species-specific PCR. All animals were negative to *A. phagocytophilum* while the prevalence of unidentified species belonging to family *Anaplasmataceae*, when common primers were used, was 95.5%, 95%, 100%, 80%, and 100% for camels, cattle, sheep, foxes, and Dabb lizards respectively. To confirm our results that tested animals do not harbor *A. phagocytophilum* while other related organisms do exist, we had selected some cattle samples that yielded positive in screening PCR for downstream sequencing experiment. Purified double stranded DNA from the agarose gel of selected samples was subjected to bidirectional sequencing. Partial 16S rRNA sequence and phylogenetic analysis revealed that cattle residing Taif district are infected with 2 strains of *Anaplasma* spp. closely related to *A. marginale* (99% identity). In conclusion, this study is considered not only the first molecular survey of *A. phagocytophilum* in the kingdom, but also the first report that molecularly addressed existence of *A. marginale* in cattle from KSA. Extensive molecular surveys are needed to address the prevalence and geographical distribution of *A. marginale* in cattle from all-over the country. Moreover, additional sequencing and species-specific PCR experiments are needed to elucidate the molecular identity of the organisms detected when common primers were used.

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1.Introduction

Human granulocytic anaplasmosis (HGA) is an emerging tick-borne zoonosis. It is a febrile systemic illness and its severity ranges from asymptomatic or non-specific flu-like symptoms to death. Headache, malaise, myalgia, lethargy, arthralgia, leucopenia, thrombocytopenia, and elevated levels of hepatic enzymes are the most encountered clinical and laboratory findings (1). The first report of HGA came from United States in 1994 (2), and since that initial record, an increasing number of cases has been described in the US, Europe, and Asia (3-5). The causative agent of HGA is *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), a Gram-negative obligatory intracellular bacterium, that replicates within neutrophilic granulocytes (6). *A. phagocytophilum* has been designated after reorganization of order Rickettsiales, joining together the three previously characterized species, the agent of human granulocytic ehrlichiosis (HGE), *Ehrlichia phagocytophila* (the causative agent of tick-borne fever in cattle and sheep), and *E. equi* (the causative agent of equine and canine granulocytic ehrlichiosis). This new designation was based on the similarities in 16S rRNA and *groESL* gene sequences as well as antigenic and biological characteristics (7). However, genetic diversity among *A. phagocytophilum* strains from different geographical areas has been described (8). It is noteworthy to mention that, agents of HGA with different 16S rRNA sequence are associated

with variable biological and ecological characteristics including pathogenicity and vector specificity (8, 9). Several members of genus *Ixodes* have been implicated in the natural transmission cycle of *A. phagocytophilum*; including *I. scapularis* and *I. pacificus* in the US (10, 11), *I. ricinus* in Europe (12), and *I. persulcatus* in Asia (13).

In Saudi Arabia, in a single study, a closely related tick-borne zoonotic pathogen, *Ehrlichia canis*, has been serologically and molecularly detected in dogs. At the same time the competent vector of the disease, *Rhipicephalus sanguineus* tick, was found to parasitize these infected canine hosts (14). It is noteworthy to mention that *R. sanguineus* was reported to be a potential competent vector for *A. phagocytophilum* in Egypt (15, 16), the country that shares fauna and ecology with the Kingdom. Given the previous information and the fact that animal reservoir hosts of HGA agent are abundant and sometime become in close proximity with people, we can conclude that the conditions for transmission of *A. phagocytophilum* do exist in the KSA.

For efficient prevention and control of any tick-borne zoonosis we have first to elucidate its epidemiology and identify its competent vectors and reservoirs. To date, and to the best of our knowledge, no work of any kind has been executed to address existence and epidemiology of the human granulocytic anaplasmosis in the Kingdom. Achieving this goal is a multistep project, where its first initial experiments are to detect and identify the organism of concern in its natural animal reservoir hosts. Therefore, the objectives of this study are: 1) to molecularly detect *A. phagocytophilum* in some suspected animal reservoir hosts such as camels, cattle, sheep, goats, or wild animals living in Taif district, Saudi Arabia. 2) to molecularly characterize the detected organism and compare its sequence with other known worldwide human pathogens.

2. Material and Methods

Blood samples

EDTA-whole blood samples were collected from 44 dromedary camels, 20 cattle, 50 sheep, 5 foxes (*Vulpes rueppellii*), and 10 Spiny-tailed Dabb lizards (*Uromastyx ornata*). Camel and cattle specimens were collected at Taif Slaughter House, while ovine samples were obtained from sheep raised in a private farm. Wild animals were purchased from Haraj Animal market at Taif, transported to the lab and sacrificed for sampling. All animals subjected to blood sampling were residing at Taif district, KSA. Once arrived to the lab, samples were stored at -20 ^{o}C until DNA extraction. Aseptic procedures during sampling and handling of specimens were implemented to avoid contamination of specimens.

DNA extraction

Purification of genomic DNA from whole blood was performed using AxyPrep Blood Genomic DNA Miniprep Kit; Axygen Biosciences, CA, USA (Cat. No. AP-MN-BL-GDNA-250) according to the manufacturer protocol. In case of Dabb lizard's, 10 µl of blood combined with 200 µl of PBS were used as working sample. Eluted genomic DNA was stored at –20 ^{o}C till used in PCR.

Primers selection

The primers used in PCR to detect and molecularly identify *A. phagocytophilum* are presented in Table 1. For screening purposes, the common primer pair, ECC and ECB, was used. These primers amplify a target sequence of 16S rRNA gene of almost all members belonging to family *Anaplasmataceae* including *E. phagocytophilum*, *E. equi*, and human granulocytic ehrlichiosis agent (the elderly combined 3 species of *A. phagocytophilum*) (17, 18). A specific heminested PCR amplification was performed with primers designed to amplify the 16S rRNA gene of *A. phagocytophilum*. Primers GE9f and GE10r were applied for the initial amplification while the primer pair GE9f and GE2 was used in the heminested round (2, 13). Another specific standard PCR amplification using the primer pair MAP4AP5 and MSP4AP3 that target *msp4* gene of *A. phagocytophilum* was also used for further molecular characterization of the detected organism (19).

Table 1. Sequences, target genes and size of expected amplicons for primers selected for molecular detection and identification of *A. phagocytophilum*.

Target gene	Primer name	Sequence (5'→3')	Expected product (bp)
16S rRNA	ECC	AGA ACG AAC GCT GGC GGC AAG CC	~ 450-500
	ECB	CGT ATT ACC GCG GCT GCT GGC A	
16S rRNA (1st round heminested PCR)	GE9f	AAC GGA TTA TTC TTT ATA GCT TGC T	919
	GEl0r	TTC CGT TAA GAA GGA TCT AAT CTC C	
16S rRNA (2nd round heminested PCR)	GE9f	AAC GGA TTA TTC TTT ATA GCT TGC T	546
	GE2	GGC AGT ATT AAA AGC AGC TCC AGG	
***msp4* gene**	MAP4AP5	ATG AAT TAC AGA GAA TTG CTT GTA GG	849
	MSP4AP3	TTA ATT GAA AGC AAA TCT TGC TCC TAT G	

PCR amplification and agarose gel electrophoresis

All amplifications were performed in 25-µl reaction mixture containing 12.5 µl GoTaq Green Master Mix (Promega Corporation, Madison, WI 53711-5399, USA), and 20 pmoles each primer. The following thermocycle profile was used in reactions using ECC and ECB primer pairs: an initial 2-min denaturation at 94°C, 40 cycles (each consisting of a 1-min denaturation at 94°C, a 2-min annealing at 55°C, and a 30-s extension at 72°C) and a 5-min final extension at 72°C. The cycling program for both 1st and 2nd round of heminested PCR using the primer pairs (GE9f and GE10r) and (GE9f and GE2) respectively was the same and included the following profile: an initial 2-min denaturation at 95°C, 35 cycles (each consisting of a 1-min denaturation at 94°C, a 75-s annealing at 55°C, and a 1-min extension at 72°C) and a 7-min final extension at 72°C. The cycling program for the specific standard PCR using the primer pair MAP4AP5 and MSP4AP3 implemented the following profile: an initial 30-s denaturation at 94°C, 35 cycles (each consisting of a 30-s denaturation at 94°C, a combined 1-min annealing and extension at 55°C) and a 5-min final extension at 72°C. Owing to unavailability of positive control we had relied on preliminary screening to obtain positive samples using common primers. The first obtained positive samples were used as internal positive controls in subsequent reactions. In addition, one "NO DNA" negative control (water was added instead of any DNA) was included in each run. Amplified PCR products were analyzed on 1.25% agarose gel by electrophoresis and seen under UV with ethidium bromide.

Purification of PCR products from agarose gel

Amplicons of ~ 500-bp generated using common primers of some positive cattle samples were selected for downstream sequencing application. Target bands were purified from agarose gel using FavorPrep Gel Purification Mini Kit; Favorgen Biotech Corp., Ping-Tung, Taiwan (Cat. No. FAGPK001) according to the manufacturer protocol.

Sequencing of PCR products

Purified double-stranded PCR products were subjected to bidirectional sequencing using Macrogen facilities. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using our common primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequence analysis

A BLAST search was performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the consensus sequence of this study. The obtained DNA sequences were aligned separately and manually using MacClade v.4. The unalignable and gap-containing sites were deleted and the aligned data were then concatenated so that 393 bp were left for the analyses. The tree analysis was done using Neighbor-Joining (NJ) methods with PAUP* 4.0b10 (20) by heuristic searches with the TBR branch swapping and 10 random taxon additions, respectively. The bootstrapping replicates were set to be 1000 with simple additions for the two methods. The 16S rRNA gene *Rickettsia rickettsii* (accession: U11021) was used as out-group.

Nucleotide sequence accession numbers

The accession numbers of nucleotide sequences used for comparison with detected cattle strains are indicated beside the organism's name in the phylogenetic tree. The partial 16S rRNA nucleotide sequences obtained in the current study were registered at GenBank under the following accession numbers: AB916498 for *Anaplasma marginale* Ghafar-1Catl-KSA and AB916499 for *Anaplasma marginale* Ghafar-6Catl-KSA.

3. Results

PCR

Results of different PCR experiments for different animal species are presented in Table 2 and Figure 1.

Table 2. Results of different PCR experiments for different animal species tested.

Animal species	*# tested*	*PCR results targeting*		
		16S rRNA gene		*msp4* gene
		Standard PCR using common primers	Heminested PCR	
		No. positive (%)	No. positive (%)	No. positive (%)
Camel	44	42 (95.5)	0 (0)	0 (0)
Cattle	20	19 (95)	0 (0)	0 (0)
Sheep	50	50 (100)	0 (0)	0 (0)
Fox	5	4 (80)	0 (0)	0 (0)
Lizard	10	10 (100)	0 (0)	0 (0)

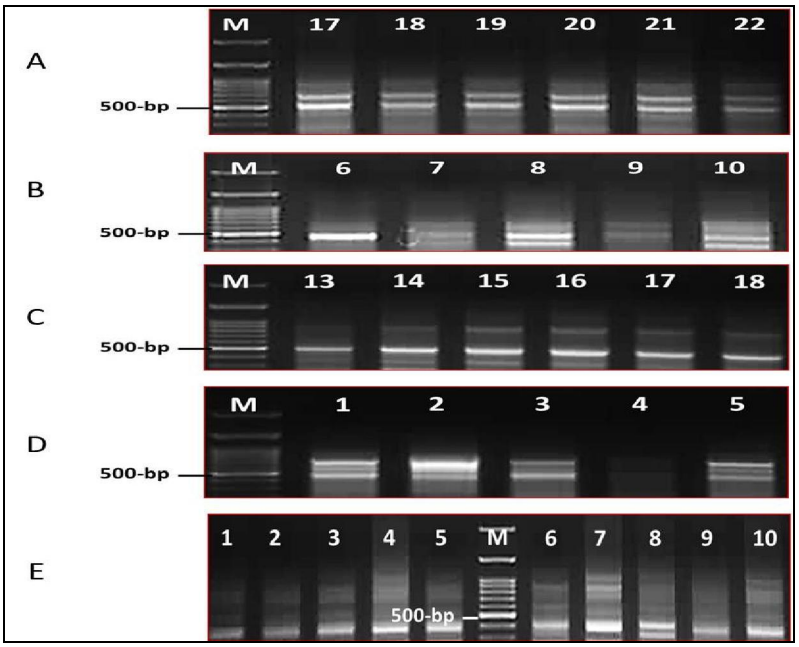

Figure 1. Agarose gel electrophoresis of PCR products obtained by amplification of DNA of camel (A), cattle (B), sheep (C), foxes (D), and Dabb lizard (E) samples using the common primers. Lane M, molecular size standard marker, 100bp DNA Ladder RTU. Numbers of corresponding tested animals are shown.

Sequencing, homology and phylogeny

Alignment of gene sequences and phylogenetic analysis with selected sequences from the GenBank revealed that the anaplasmal 16S rRNA gene from cattle belongs to the *A. marginale* (Figure 2). Identities of our strains (Ghafar-1Catl-KSA and Ghafar-6Catl-KSA) to the organisms used in phylogenetic tree are presented in Tables 3 and 4. Nucleotide and some epidemiological aspect differences between present strains and other selected ones used in the phylogenetic tree are summarized in Table 5.

Table 3. Identity, query coverage, and total and maximum score of *A. marginale* Ghafar-1Catl-KSA strain to organisms used in phylogenetic tree.

Organism	Max score	Total score	Query Cover	Identity	Accession #
Rickettsia rickettsii	455	455	98%	85%	U11021
Neorickettsia helminthoeca	484	484	98%	86%	U12457
Neorickettsia sennetsu	457	457	98%	85%	M73225
Neorickettsia risticii	462	462	98%	86%	M21290
Candidatus Neoehrlichia mikurensis	628	628	100%	92%	EU810404
Wolbachia pipientis	540	540	99%	88%	AF179630
Ehrlichia ruminantium	614	614	100%	91%	U03777
Ehrlichia ewingii	619	619	100%	91%	U96436
Ehrlichia muris	625	625	100%	91%	AB196302
Ehrlichia chaffeensis	623	623	100%	91%	EU826516
Ehrlichia canis	621	621	100%	91%	M73221
Anaplasma marginale strain Florida	828	828	100%	99%	NR_074556
Anaplasma marginale isolate ZJ02/2009	828	828	100%	99%	HM439433
Anaplasma marginale isolate C6A	828	828	100%	99%	JQ839012
Anaplasma central from *R. simus*	806	806	100%	99%	AF414869
Anaplasma central strain 14	800	800	98%	99%	EF520689
Anaplasma ovis	795	795	100%	98%	AY262124
Anaplasma ovis isolate Zhangjiachuan	784	784	100%	98%	AJ633051
Anaplasma bovis isolate G49 clone 49	699	699	100%	94%	JN558824
Anaplasma platys isolate Okinawa	701	701	100%	95%	AY077619
Anaplasma phagocytophilum isolate 9B13	719	719	100%	95%	KC800985

Table 4. Identity, query coverage, and total and maximum score of *A. marginale* Ghafar-6Catl-KSA strain to organisms used in phylogenetic tree.

Organism	Max score	Total score	Query Cover	Identity	Accession #
Rickettsia rickettsii	460	460	96%	85%	U11021
Neorickettsia helminthoeca	494	494	99%	86%	U12457
Neorickettsia sennetsu	466	466	99%	85%	M73225
Neorickettsia risticii	472	472	99%	85%	M21290
Candidatus Neoehrlichia mikurensis	654	654	99%	92%	EU810404
Wolbachia pipientis	556	556	100%	89%	AF179630
Ehrlichia ruminantium	636	636	100%	92%	U03777
Ehrlichia ewingii	641	641	100%	92%	U96436
Ehrlichia muris	647	647	100%	92%	AB196302
Ehrlichia chaffeensis	651	651	100%	92%	EU826516
Ehrlichia canis	632	632	100%	91%	M73221
Anaplasma marginale strain Florida	833	833	100%	99%	NR_074556
Anaplasma marginale isolate ZJ02/2009	833	833	100%	99%	HM439433
Anaplasma marginale isolate C6A	833	833	100%	99%	JQ839012
Anaplasma central from *R. simus*	811	811	100%	98%	AF414869
Anaplasma central strain 14	797	797	97%	99%	EF520689
Anaplasma ovis	800	800	100%	98%	AY262124
Anaplasma ovis isolate Zhangjiachuan	789	789	100%	98%	AJ633051
Anaplasma bovis isolate G49 clone 49	715	715	100%	95%	JN558824
Anaplasma platys isolate Okinawa	728	728	100%	95%	AY077619
Anaplasma phagocytophilum isolate 9B13	741	741	99%	96%	KC800985

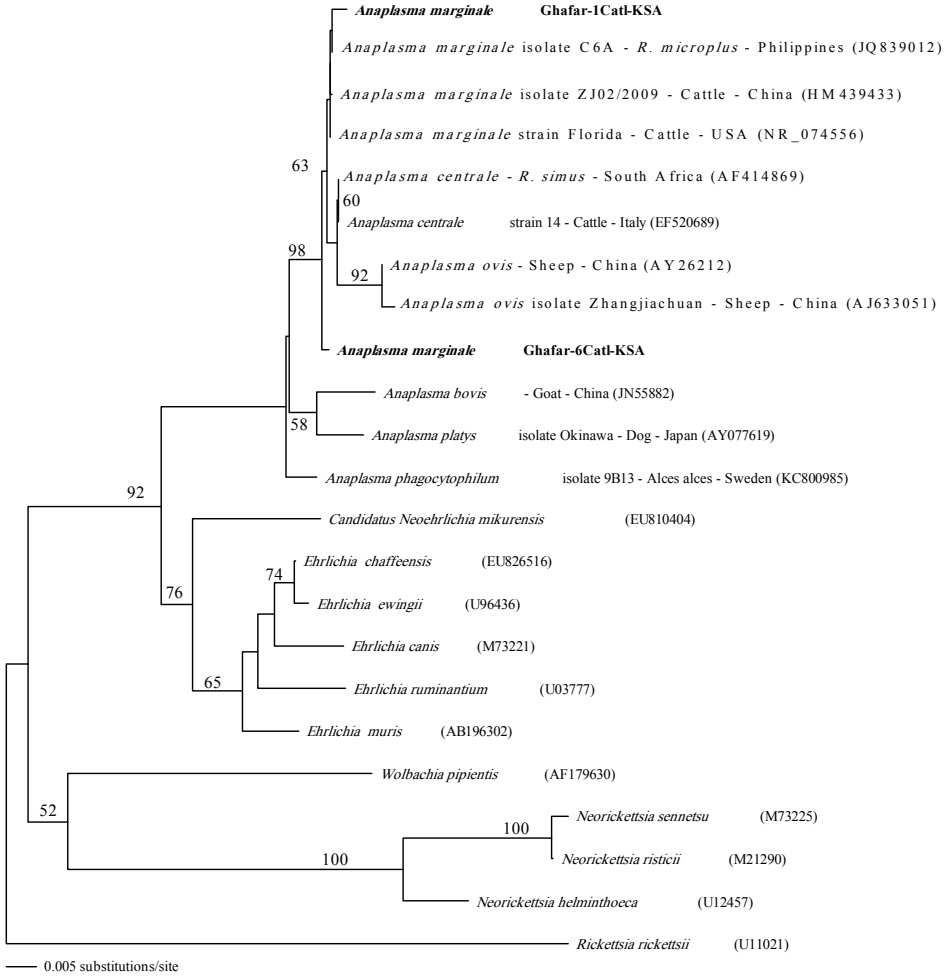

Figure 2. Neighbor-joining tree based on partial (393-bp) 16S rRNA sequences obtained with bootstrap analysis of 1000 replicates. Numbers on branches indicate bootstrap probabilities that reproduced the topology for each clade when it was over 50%. Parentheses enclose GenBank accession numbers of the sequences used in the analysis.

Table 5. Nucleotide and some epidemiological aspect differences between *A. marginale* detected in cattle from Taif, KSA and other selected ones used in the phylogenetic tree.

Organism	*Host*	*Country*	*Nucleotide difference at position*							*Accession #*
			34	46	109	142	154	163	386	
***A. marginale* Ghafar-1Catl-KSA**	**Cattle**	**KSA**	**A**	**G**	**T**	**G**	**T**	**G**	**G**	**AB916498**
***A. marginale* Ghafar-6Catl-KSA**	**Cattle**	**KSA**	**A**	**G**	**T**	**A**	**T**	**G**	**C**	**AB916499**
***A. marginale* isolate C6A**	***R. microplus***	**Philippines**	**A**	**G**	**T**	**G**	**T**	**G**	**C**	**JQ839012**
***A. marginale* isolate ZJ02/2009**	**Cattle**	**China**	**A**	**G**	**T**	**G**	**T**	**G**	**C**	**HM439433**
A. centrale	***R. simus***	**South Africa**	**A**	**A**	**T**	**G**	**T**	**G**	**C**	**AF414869**
***A. centrale* strain 14**	**Cattle**	**Italy**	**A**	**A**	**T**	**G**	**T**	**G**	**C**	**EF520689**
A. ovis	**Sheep**	**China**	**G**	**A**	**T**	**G**	**C**	**T**	**C**	**AY262124**
***A. ovis* isolate Zhangjiachuan**	**Sheep**	**China**	**G**	**A**	**C**	**G**	**C**	**T**	**C**	**AJ633051**

4. Discussion

The objectives of this study were to molecularly detect and characterize *A. phagocytophilum* in some animals suspected to be competent reservoirs in Taif district, KSA. Three domestic (camels, cattle, sheep) and 2 wild (foxes and Dabb lizard) animal species were tested using PCR technology targeting 16S rRNA and *msp4* genes (Table 2). Our finding that all tested samples yielded negative results for *A. phagocytophilum*, when using specific primers, was somewhat surprisingly. However, this result could be explained by the assumption that the organism does not exist in the region and these animals do not harbor the pathogen but do serve as reservoirs for other related organisms. This view is supported by the results obtained from both PCR using common primers (Table 2 and Figure 1) and sequencing experiments (Figure 2). Nevertheless, these findings do not exclude the presence of the HGA agent in the country as testing larger sized samples from the same tested species or other animals may confirm its presence.

The high positivity rate together the appearance of more than one band in screening PCR (Figure 1) indicates high infection rate with more than one evolutionary related organism. This could be attributed to the fact that ECC and ECB primer pair amplifies sequences from almost entire members of family *Anaplasmataceae* (17, 18). Inclusion of positive and negative controls and confirmation with sequencing experiment has eliminated the risk of occurrence of false positive and false negative results.

Previously, it has been demonstrated that 16S rRNA gene sequence analysis is useful in phylogenetic determination and identifying newly discovered bacteria as well as redefining existing taxonomy (21). Our 393-bp sequence analysis has revealed presence of 2 strains that are closely related to *A. marginale* (99% identity). Although bovine anaplasmosis due to *A. marginale* has been diagnosed microscopically in cattle from regions of KSA (22), this report is considered the first molecular identification of the pathogen in the country.

It is well known that cattle anaplasmosis due to *A. marginale* is a tick-borne disease that occurs in temperate areas of all six continents and is manifested clinically by severe hemolytic anemia resulting in marked economic losses (23). Unfortunately, we were not able to assess the health status of tested cattle as the samples were collected during slaughtering and the health data of the herd was not available. Since geographic strains of *A. marginale* have been shown to differ in their genome and pathogenicity (24), the relevance of these findings to veterinary medicine deserves further investigation.

Recording *A. marginale* infection in cattle from different areas in KSA raises the assumption that the disease is widespread in the country. Therefore, further extensive molecular surveys on large number of samples covering the whole country are needed in order to correctly address the prevalence and geographical distribution of the disease. This in turn will help in designing and implementation of effective preventive and control measures.

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