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First Worldwide Molecular Detection of *Babesia* Species in Rüppell's Fox (*Vulpes Rueppellii*) From Saudi Arabia: A Novel Species with Zoonotic Potential

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Abstract **Original Research Article**

Wild canids play a crucial role in the natural history of piroplasmoses; however, the contribution of Rüppell's fox (*Vulpes rueppellii*) is unrevealed. Hypothesize that Rüppell's fox residing Saudi Arabia is a competent reservoir for some *Babesia* species. Testing this hypothesis is a multistep project in which the first step is to demonstrate the presence of the parasite in suspected host. Therefore, the aim of this study was to molecularly detect and characterize *Babesia* spp. occurring in Rüppell's fox from KSA. Five archived fox DNA samples were tested using PCR targeting 18S rRNA gene of genus *Babesia*. Amplicons were purified, sequenced and analyzed. Two foxes showed evidence of babesia DNA and the phylogenetic analysis revealed that detected piroplasms belonged to Duncani group. Within this group the parasites clustered with the African lengau species from wild carnivores and Israeli MML species from red fox forming the newly assigned Afro-Asian clade. The pattern of identity, phylogeny and geographic distribution of Afro-Asian clade members exposed relevance to the East Africa West Asian bird flyway. This is the first record of babesia infection in Rüppell's fox of Arabia and worldwide. This molecular study not only demonstrated the presence of a novel potentially zoonotic piroplasm species but also further contributed to the ecology of genus *Babesia*.

Keywords: Afro-Asian clade, *Babesia*, Duncani group, phylogeny, Rüppell's fox (*Vulpes rueppellii*), Saudi Arabia.

INTRODUCTION

Piroplasms are tick-borne hemoprotozoan parasites belonging to genera *Babesia*, *Theileria* and *Cytauxzoon* in the phylum Apicomplexa [1]. These parasites mainly infect animals and occasionally spill over humans [2]. Human piroplasmoses is hitherto caused by species of genus *Babesia* including: *B. microti*, *B. microti*-like parasites, *B. divergens*, *Babesia* spp. KO1, *B. venatorum*, *B. duncani* and *B. duncani*-type organisms [3, 4]. Piroplasmoses are examples for such diseases in which wild animals, including members of family Canidae, play a crucial role in their natural history [5-7].

Red fox (*Vulpes vulpes*), the most abundant wild canid in northern hemisphere, has been recently recognized as a potential reservoir for *Babesia vulpes* in many countries worldwide [8-12]. More recently, a new babesia genotype designated as *Babesia* sp. MML has been reported to infect this wild animal in Israel [13]. This parasite belongs to the human pathogenic duncani clade; nevertheless, its zoonotic potential yet to be determined.

Rüppell's or sand fox (*Vulpes rueppellii*), a closely related wild canid, is distributed across North Africa, the Middle East and southwestern Asia (Fig.1). Within its range, this animal prefers sandy and stony deserts; however, it may introduce into urban and peri-urban areas searching for food [14, 15]. To the best of our knowledge, except for one molecular survey recorded absence of *Babesia* spp. in Rüppell's fox from North Africa [16], there is no information available about piroplasm infections in this animal globally. Hypothesize that Rüppell's fox residing Saudi Arabia is a competent reservoir for some *Babesia* species. Testing this hypothesis is a multistep project in which the first step is to demonstrate the presence of hemoprotozoan in the carnivore host and uncover its molecular identity. Therefore, the aim of this study was to molecularly detect and characterize *Babesia* spp. occurring in Rüppell's fox from Taif district, KSA. Identifying the competent reservoir of any tick-transmitted agent in different geographic areas will facilitate designing and implementation of efficient prevention and control strategies.

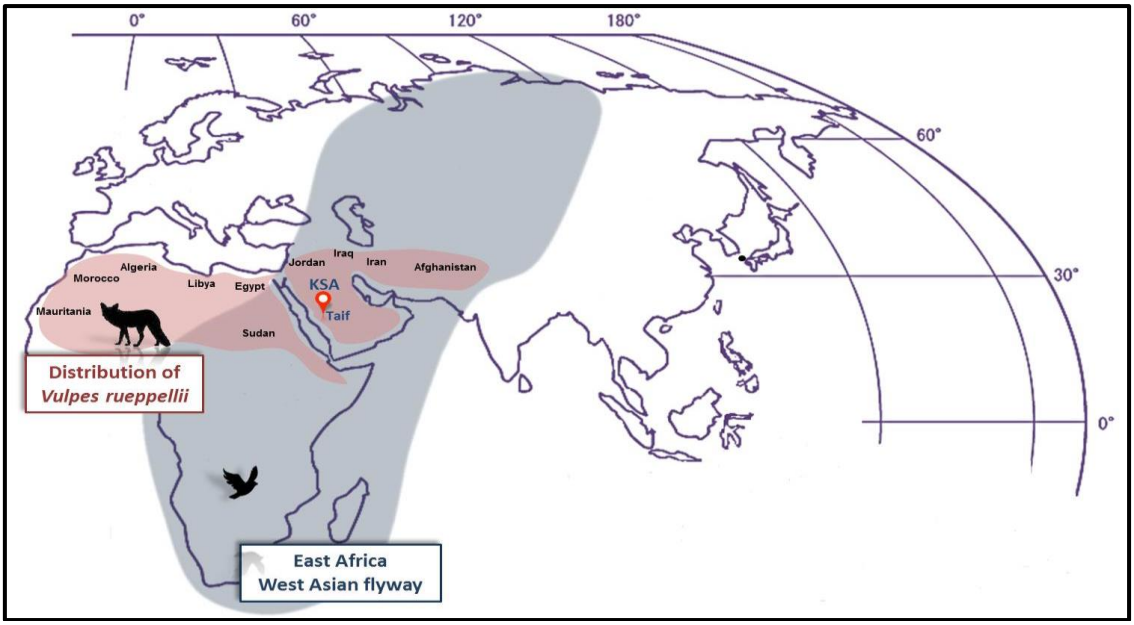

Fig-1: Map showing sampling site, distribution of *Vulpes rueppellii* (pink shaded) and East Africa West Asian bird flyway (grey shaded).

MATERIALS AND METHODS

Samples

Stored 5 leftover Rüppell's fox DNA samples were used in this study. DNA was previously extracted from EDTA-whole blood that was collected during 2013 as a part of a project supervised by the author to investigate the existence of *Anaplasma phagocytophilum* in KSA. These foxes were caught at Taif District (approximately 21° 26' 14" N and 40° 30' 45" E) (Fig. 1) and four of them showed evidence for DNA of unidentified *Anaplasma* sp. [17].

PCR and sequencing

All PCR amplifications were performed in 25-µl reaction mixtures containing 5 µl of each DNA template, 12.5 µl GoTaq Green Master Mix (Promega Corporation, Madison, WI 53711-5399, USA), and 20 pmoles of each *Babesia*-F (GTG-AAA-CTG-CGA-ATG-GCT-CA) and *Babesia*-R (CCA-TGC-TGA-AGT-ATT-CAA-GAC) primer. This oligonucleotide set targets the common sequence of the 18S rRNA gene of the genus *Babesia* [18]. The following thermocycle profile was used: an initial 5-min denaturation at 95°C, 34 cycles (each consisting of a 30-sec denaturation at 95°C, a 30-sec annealing at 55°C, and a 1.5-min extension at 72°C) and a 5-min final extension at 72°C. Amplified PCR products were analyzed on 1.25% agarose gel by electrophoresis and seen under UV with ethidium bromide. Products of ~ 650 bp indicate positive results. Amplicons were purified from agarose gel using Favor Prep Gel Purification Mini Kit (Cat. No. FAGPK001) as directed by the manufacturer. Extracted products were subjected to bidirectional sequencing using Macrogen facilities.

Sequence analysis and phylogeny

The sequenced 18S rRNA gene fragment was analyzed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To construct the most parsimonious phylogenetic trees, two manually aligned files were generated using DNASIS and MacClade v.4 programs [19]. The first file contained our two sequences with selected numbers of designated piroplasm species for the identification of the protozoan to the species level, depending on similarities. To give more detailed information about the genetic and epidemiologic relations of our sequences with other species in the positioned clade, a second file was generated with the highest existing 24 scoring GenBank sequences. The gap-containing and ambiguous sites were deleted from the aligned dataset so that 289 bp were left for phylogeny. The analyses were conducted by maximum-parsimony (MP) and neighbor-joining (NJ) packaged in PAUP* 4.0b10 [20]. The two phylogenetic methods were adjusted with TBR branch swapping algorism and bootstrapping replications of 5,000 for obtaining the appropriate tree topology. The distance option of NJ was Tamura-Nei [21]. The constructed trees were rooted with *Hepatozoon felis* (AY628681).

Accession numbers

The partial 18S rRNA nucleotide sequences obtained in the current study was deposited in GenBank under the following accession numbers: LC368283 for *Babesia* sp. Ghafar VR1 and LC368284 for *Babesia* sp. Ghafar VR2.

RESULTS

Two foxes showed evidence of babesia DNA when analyzed by agarose gel electrophoresis. One of these positive foxes was previously reported to be infected with unidentified *Anaplasma* sp. (a result from

a former study) [17]. Analysis of the partial 18S rRNA gene sequences demonstrated that the detected strains are identical. In addition, BLAST search in existing GenBank database revealed no 100% similar sequences; therefore, these new parasites were designated as *Babesia* sp. Ghafar VR1 and VR2. The query coverage and Identity percentage of unveiled piroplasms with the highest 24 BLAST scoring sequences are displayed in Table 1.

Further phylogenetic analysis with selected designated piroplasm species revealed that our parasites clustered in the known Duncani (Western) clade and distinctly separated from other groups including *Babesia* sensu stricto spp., *Theileria* spp. *Cytauxzoon* spp. and *B. microti* spp. (Fig. 2). Expanded Duncani group demonstrated that Ghafar VR1 and VR2 are closely related to African lengau and Israeli MML species forming a clade which is clearly separated from other American Duncani and Conradae piroplasms (Figs. 3, 4).

Table-1: Query coverage and identity percentage of detected strains with the highest 24 BLAST scoring sequences used in phylogeny. The sequences are shown in order

Accession #	Organism	Isolate/Strain	*Babesia* sp. Ghafar VR1		*Babesia* sp. Ghafar VR2	
			Query cover (%)	Identity (%)	Query cover (%)	Identity (%)
KM025199	Uncultured *Babesia*		100	98	100	98
KX218429	*Babesia* sp. 1 1093 cl9	Lion 1093 cl9	100	97	100	98
KJ956782	*Babesia* sp. MML-2014	913L	100	97	100	98
KX218431	*Babesia* sp. 3 1093 cl8	Lion 1093 cl8	100	97	100	97
KX218430	*Babesia* sp. 2 1092 cl4	Lion 1092 cl4	100	97	100	97
KC790443	*Babesia lengau*	BF226	100	97	100	97
GQ411405	*Babesia lengau*	BF1	100	97	100	97
AF158709	Piroplasmida gen. sp. BH3	BH3	88	99	100	94
AF158708	Piroplasmida gen. sp. BH1	BH1	88	99	100	94
AF158707	Piroplasmida gen. sp. FD1	FD1	88	99	100	94
AF158706	Piroplasmida gen. sp. MD1	MD1	88	99	100	94
AF158705	Piroplasmida gen. sp. CA4	CA4	88	99	100	94
AF158704	Piroplasmida gen. sp. CA3	CA3	88	99	100	94
AF158703	Piroplasmida gen. sp. CA1	CA1	88	99	100	94
HQ289870	*Babesia duncani*	BAB1615	100	95	100	95
AY027816	*Babesia* sp. WA1	CA6	100	95	100	95
AY027815	*Babesia* sp. WA1	CA5	100	95	100	95
AF158701	Piroplasmida gen. sp. WA2	WA2	100	95	100	95
AF158700	Piroplasmida gen. sp. WA1	WA1	100	95	100	95
L13730	Theileria-related sp.		100	95	100	95
AF158702	*Babesia conradae*	118	88	98	100	95
L13729	*Babesia gibsoni*		88	98	100	95
KJ956783	*Babesia* sp. MML-2014	1017L	100	93	100	93
KY684001	*Babesia* sp.		88	96	83	96

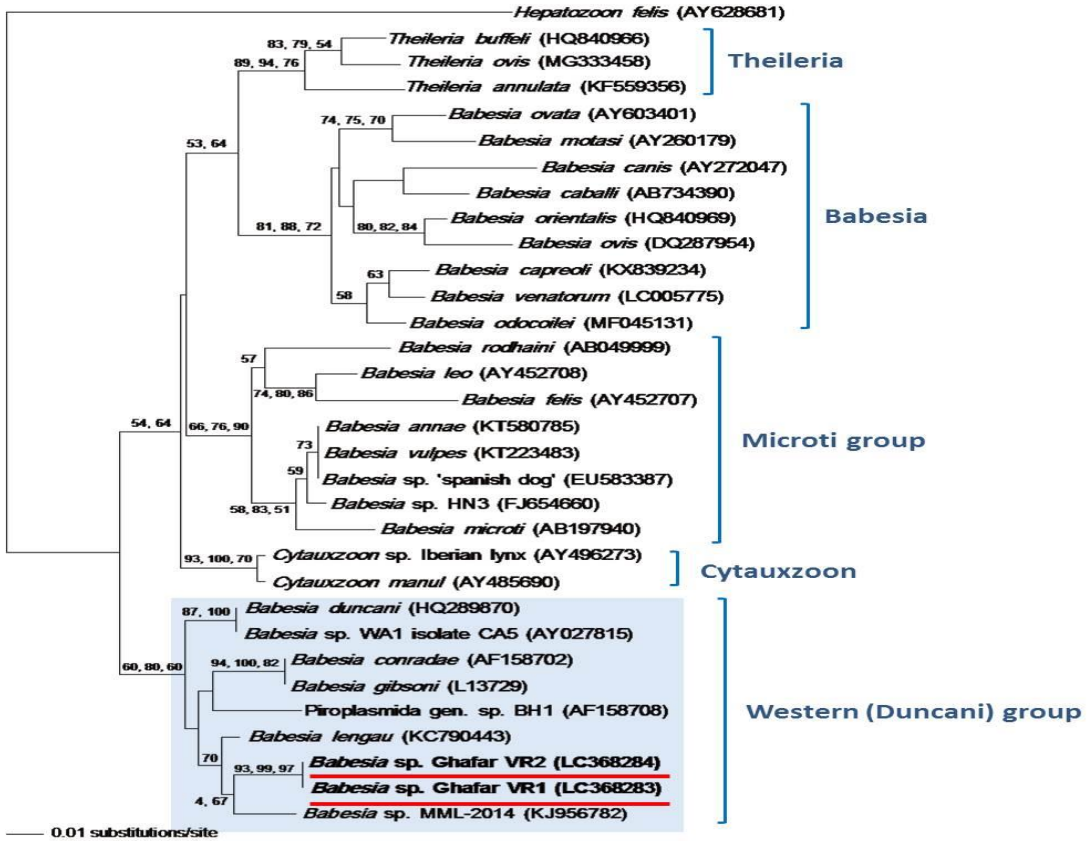

Fig-2: Neighbor-joining phylogenetic analysis of the 18S rRNA gene of detected strains (underlined) with selected designated piroplasm spp. Numbers at the nodes refer to bootstrap probabilities for MP, NJ and ML when they are above 50%. GenBank accession numbers are shown in parentheses and Duncani group is blue shaded

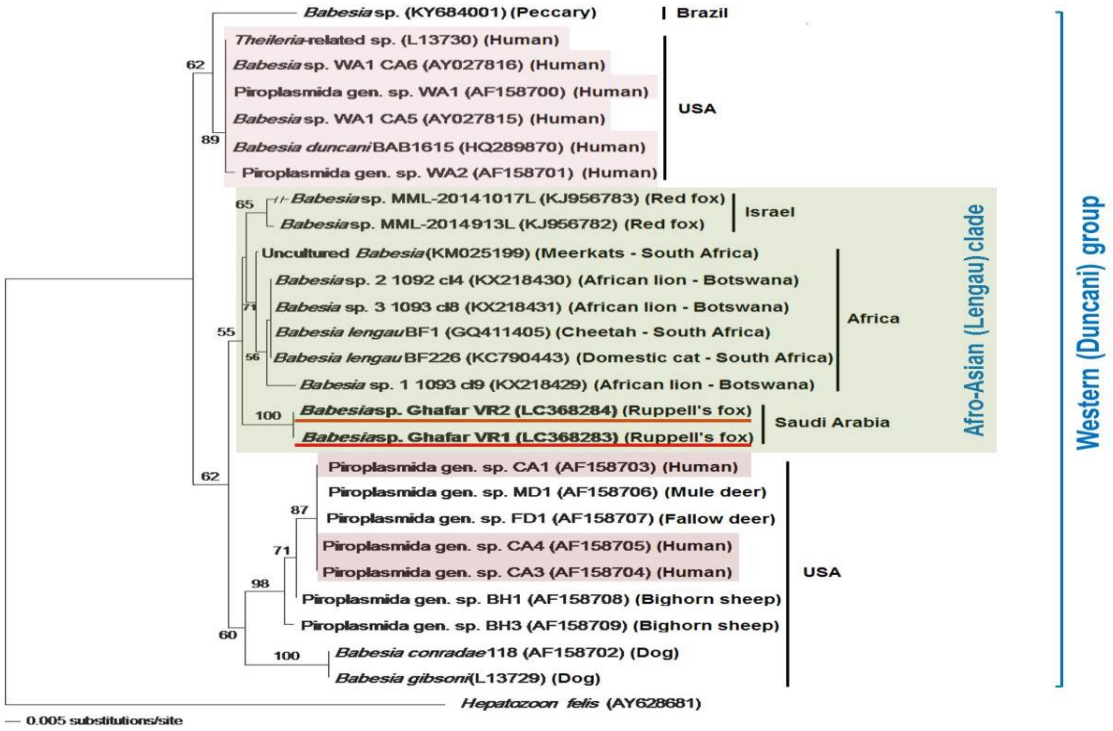

Fig-3: Neighbor-joining phylogenetic analysis of the 18S rRNA gene of detected strains (underlined) with the highest existing 24 BLAST scoring GenBank sequences. Numbers at the nodes refer to bootstrap probabilities when they are above 50%. GenBank accession numbers and animal hosts are shown in parentheses. The Afro-Asian clade is green shaded and the human pathogenic piroplasms are pink shaded

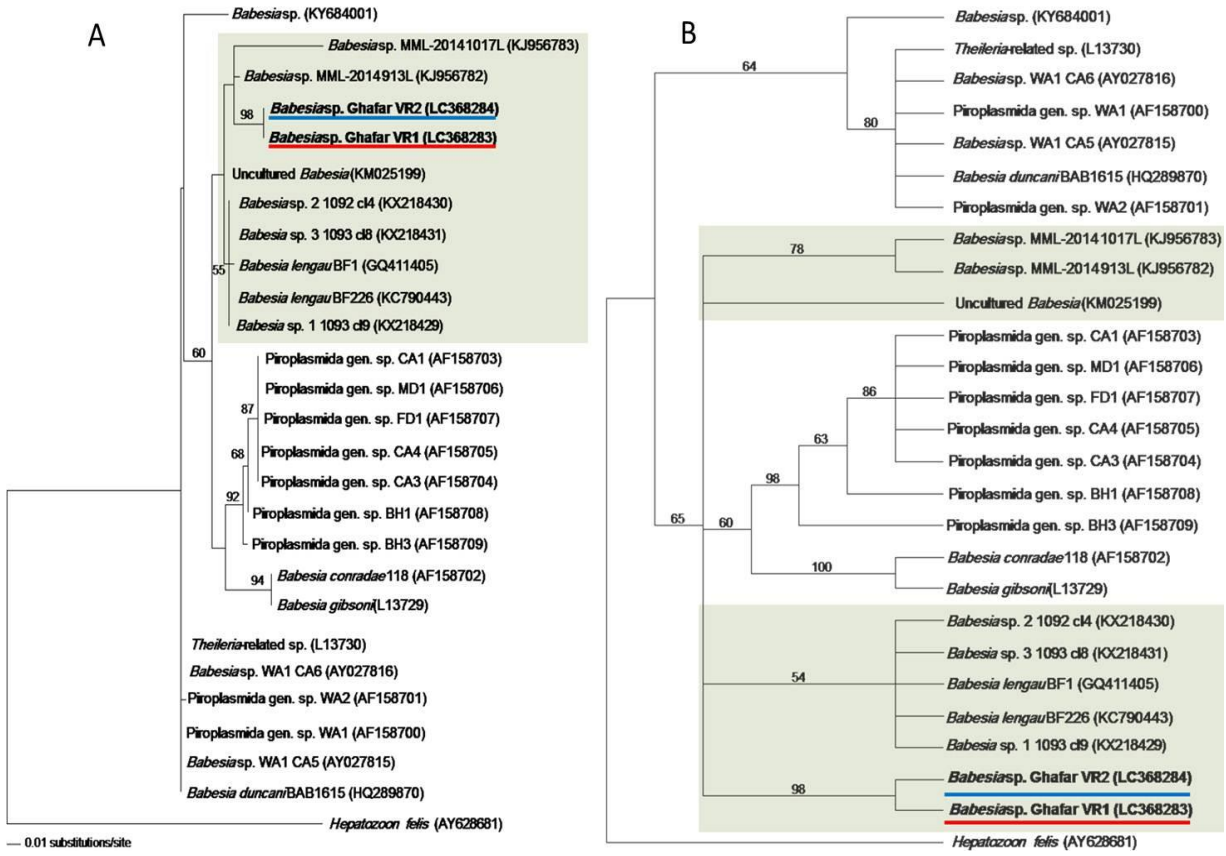

Fig-4: Maximum likelihood (A) and Maximum Parsimony (B) tree analyses of the 18S rRNA gene of detected strains (underlined) with the highest existing 24 BLAST scoring GenBank sequences. The Afro-Asian clade is green shaded and GenBank accession numbers are shown in parentheses. Numbers at the nodes refer to bootstrap probabilities when they are above 50%

DISCUSSION

Indeed, the wild carnivore interface has been shown to play a crucial role in the epidemiology of many piroplasms [22-24]; however, the input of Rüppell's fox is unknown. It is hypothesized that Rüppell's fox is a competent reservoir for some *Babesia* spp. in KSA. Proposing this assumption is based on the following considerations: (1) in a previous study in KSA [17], four of the foxes under investigation showed genomic evidence of anaplasmosis (another tick-borne zoonosis that shares tick vectors with babesiosis [25-27]); (2) anaplasmosis has been reported in Northeast African and East Mediterranean regions [28-30], the eco-niches that share fauna with Arabia and at the same time their geographic coordinates are within the distribution range of Rüppell's fox (Fig. 1); (3) the red fox (*Vulpes vulpes*) has been recognized as a potential reservoir for *B. vulpes* [8-13]. This animal has an overlapping range with sand fox in Arabian Peninsula [31], enhancing the possibility of sharing tick vectors within the same ecological area.

Two (40%) out of the 5 foxes showed evidence of *Babesia* DNA, a figure that does not represent the true overall prevalence rate of infection. This is attributed to the small sized sample and the limited study area as our study was not designed to be in-depth survey. Despite difficulty in obtaining blood samples from wild canid, further investigation using larger number of foxes from different localities is needed to obtain accurate information about pathogen distribution in KSA.

Given that one of the 2 positive animals was concurrently infected with unidentified *Anaplasma* sp., raises the possibility of the presence of a common tick vector simultaneously infected by two or more pathogens. This in turn substantiates the considerations for the poroposed hypothesis. Detection of *Babesia* sp. Ghafar strains in sand fox alone does not mean that this canid is a competent reservoir for this agent; therefore, other complementary reservoir competence studies should be conducted.

Within Duncani clade, phylogenetic analysis placed our strains in a distinct branch with some African and Asian babesias; therefore, the name of "Afro-Asian (Lengau) clade" was assigned (Figs. 3, 4). Noteworthy, it was previously known that all piroplasms from Duncani group, except for *B. lengau*, have been detected in the Western US and the cluster is therefore often referred to as the ‘‘Western group’’ [32, 33]. The results of this study not only expand Duncani group outside the US but also point out that further molecular studies in different geographic areas may add new

species to the list and hence the term "Western" may not be further applicable.

Within the Afro-Asian clade, Ghafar VR1 and VR2 subclustered with *Babesia* sp. MML, which was identified in Red fox from Israel. This may indicate that detected parasites may be genetic variants of MML agents that are evolutionarily accommodated in another related canine host. It is well documented that genetically different strains of the same babesia species may have diverse pathogenicity with varied clinical outcomes [34-36]. Therefore, further molecular analysis of several other genes is needed to obtain more information about relationship with Mediterranean strains and other closely related Duncani pathogens. Unfortunately, the clinical history of the tested foxes was not available to evaluate the pathogenic ability of the detected parasites as it was out of scope of this study. The formerly recorded piroplasms of the Afro-Asian clade have not been documented to infect humans; however, being closely associated with the human pathogenic *B. duncani* organisms (Fig. 3), the possibility of zoonotic infection with this novel species should be considered.

The pattern of identity, phylogeny and geographic distribution of the members of the Afro-Asian clade is relevant with the route of bird migration in East Africa West Asian bird flyway (Table 1; Figs. 3, 5) [37]. This emphasizes the role played by migratory birds in dispersion of tick-borne pathogens [38] and concurrently supports the proposal that detected strains, like other *Babesia* spp., are tick-transmitted agents. Further phylogeographic studies along the bird flyway are needed to confirm the findings and to find out the spatial origin of these piroplasms. The host tropism of Afro-Asian members (Fig. 5) indicates that they are circulating in wild carnivores utilizing tick vectors that allow them to be perpetuated in sylvatic cycles. This could potentially lead to the transmission of infection to domestic carnivores in peri-urban and urban environments posing both veterinary and human public health threats.

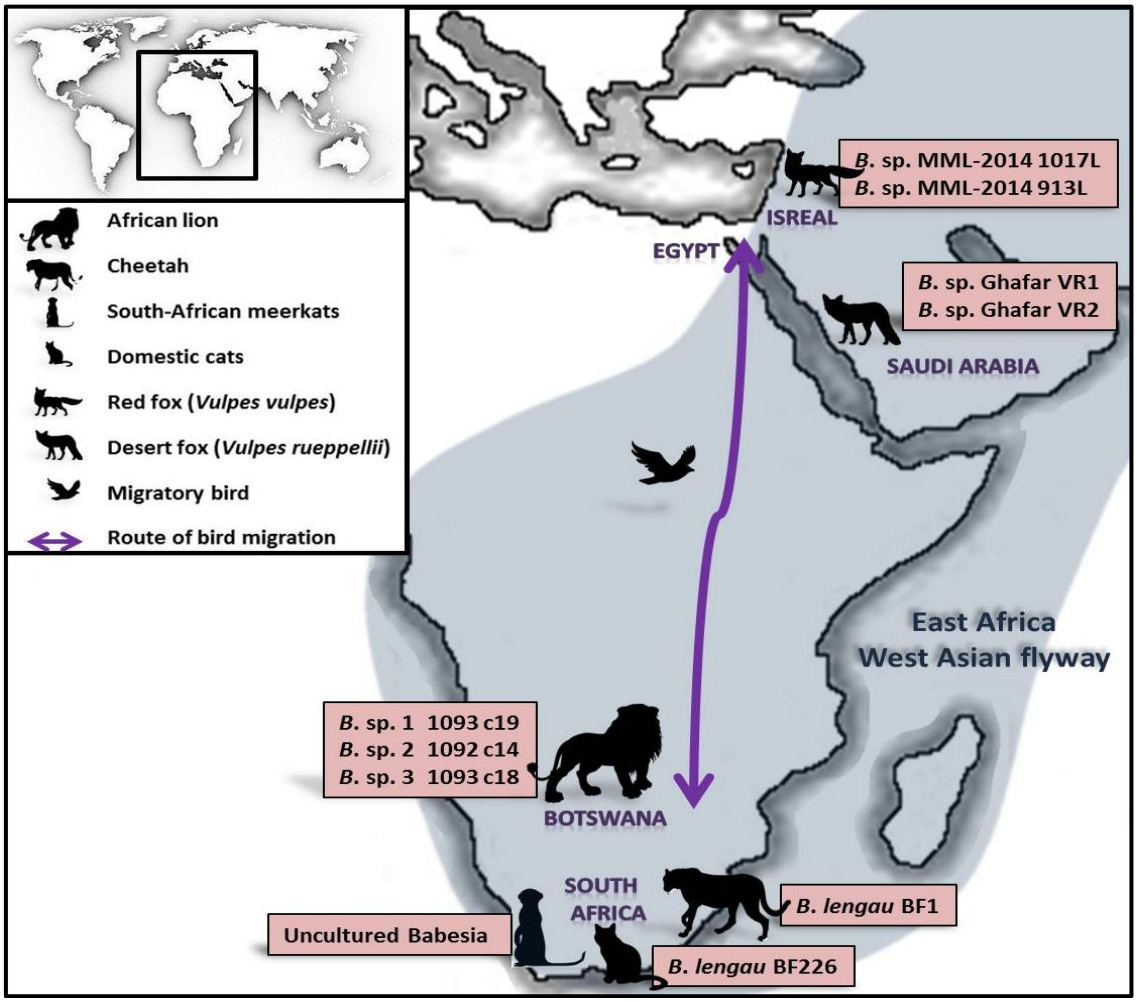

Fig-5: Map showing geographic region of origin and vertebrate host of piroplasm species constituting the Afro-Asian clade in relation to the East Africa West Asian bird flyway (grey)

CONCLUSION

In conclusion, to the best of our knowledge, this is the first record of babesia infection in Rüppell's fox of Arabia and worldwide. This molecular study not only demonstrates the presence of a novel potentially zoonotic piroplasm species in sand fox but also further contributes to the ecology and taxonomy of genus *Babesia*. Many questions regarding genetic variation, reservoir competence, tick vector, geographic range, pathogenicity and clinical relevance yet to be answered.

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