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Insights into some tick-borne pathogens in cows

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ABSTRACT

Vector-borne infections are gaining attention in public health and veterinary fields due to their increasing effects on humans and animals. In livestock, multiple hemoparasite infections can cause severe anaemia, affecting health and productivity. Cows are especially susceptible to theileriosis, leading to symptoms like anaemia, jaundice, fever, and reduced milk production, particularly when combined with *Hemoplasma* species. This study investigated the molecular detection of Piroplasma (*Babesia* species, and *Theileria* species), haemotropic Mycoplasma, and *Bartonella* species in the blood of apparent healthy cows, including identification, sequencing, and phylogenetic analysis of positive samples. The total prevalence of *Theileria annulata* and *Hemoplasma* spp. was 27.6%, 15.4%, and 37.4%, 43.1% by microscopic examination and PCR, respectively, mixed infection with *Theileria annulata* and *Hemoplasma* spp were detected in 9.8% of examined samples by the two techniques. While *Babesia* spp. and *Bartonella* spp. never be detected either by ME or by PCR. The sequencing of six isolated *Theileria* spp. was identified as *Theileria annulata*. Meanwhile, eleven positive haemotropic mycoplasma samples revealed five *Mycoplasma wenyonii* and six '*Candidatus Mycoplasma haemobos*'. The sequence analysis of *Theileria annulata*, illustrated a high similarity of examined isolates to strains previously deposited in the GenBank. Moreover, *Mycoplasma wenyonii* and '*Candidatus Mycoplasma haemobos*' sequence analysis showed Homology with strains from cattle, ticks, cats, and dogs. It was concluded that *T. annulata* and bovine haemotropic mycoplasma infections are widespread in cattle. Our findings may be significant for the development of control programs.

Introduction

Livestock plays a crucial role in the economy and sustenance, with cattle being significant worldwide. These animals are not only a primary source of nutritious milk and rich meats but also contribute to the cultural practices and agricultural traditions of the communities (El-Dakhly *et al*., 2018). Tick-borne pathogens (TBPs) are all potential threats to global cow productivity. Every year, millions of cattle die from TBP infections, which leads to productivity losses and costly veterinary care and tick control expenses that finally overwhelm cattle ranchers worldwide. Tick and TBP-related losses in Asian and African nations have been estimated to be between 3.1 and 57.2 million US dollars per year (Garcia *et al*., 2022).

Piroplasms are apicomplexan parasites transmitted by ticks and are distributed globally. These parasites cause piroplasmosis, including theileriosis and babesiosis, in vertebrates (Almazán*et al*., 2022). Species within the genus *Theileria* are responsible for bovine theileriosis and are obligate intracellular parasites. Their sporozoites target red and white blood cells, inducing a reversible transformation to an uncontrolled proliferative state during the schizont stage. This process leads to anaemia, fever, leukopenia, and lymphoproliferative disorders. The pathogen *T. annulata* infects host monocytes, macrophages, and B lymphocytes, causing tropical theileriosis, also known as Mediterranean theileriosis (Woods *et al*., 2021).

Babesia infection has significant economic impacts on cattle. It can lead to high mortality rates, reduced productivity, decreased feed intake, and lower feed conversion efficiency. Additionally, it may cause miscarriages, increased expenses related to tick management, and costs associated with disease prevention measures. The most common clinical signs of *Babesia* species infections in cattle include anaemia, hemoglobinemia, fever (pyrexia), and hemoglobinuria (Agina *et al*., 2020).

Haemotropic mycoplasmas, commonly referred to as *Hemoplasma*s, are bacteria that infect humans and other animals. While they can lead to serious illnesses, they typically result in subclinical infections. The two main species identified in cattle are *Mycoplasma wenyonii*, discovered in 1934, and *Candidatus Mycoplasma haemobos*, which was identified more recently using molecular techniques (Kim *et al*., 2024). Infected cattle may show several signs, including anaemia, fever, hemoglobinuria, lymphadenopathy, edema, and reduced milk production. Although *Hemoplasma*s are linked to regenerative anaemia, the exact mechanisms by which they adversely affect cattle remain unclear. Transmission can occur through direct blood contact and may even involve perinatal transmission during pregnancy. Additionally, arthropods like ticks and mosquitoes can facilitate the spread of these bacteria (Arendt *et al*. 2024). In association with *Hemoplasma* species, which produce comparable symptoms, results in a more severe condition. Bartonellosis is a rising vector-borne illness that impacts both humans and animals across the globe. This disease is instigated by intracellular, rod-shaped, Gram-negative bacteria known as *Bartonella* species (Obiegala *et al*., 2021).

The primary vectors responsible for spreading the disease are arthropods, particularly those associated with rodents and other small mammals, such as shrews and bats (Mhamphi *et al*., 2024). It can be transmitted to humans and other mammals through blood-feeding arthropods, including fleas, lice, ticks, and mosquitoes. Transmission primarily occurs via the feces of fleas and lice, mosquito bites, and potentially through tick bites (Ashtiani *et al*., 2024). The bacterium infects red blood cells, causing symptoms that range from mild to severe. These symptoms include endocarditis, myocarditis, neuroretinitis in immunocompromised individuals, anaemia, splenomegaly, lymphadenopathy, and fever, which neurological issues may sometimes accompany (Osman *et al*., 2024).

The goal of this study was to detect Piroplasm (*Babesia* spp. and *Theileria* spp.), *Hemoplasma* spp., and *Bartonella* spp. in cows' blood using molecular methods. This was in addition to precise identification, sequencing, and phylogenetic analysis of the positive samples.

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Materials and methods

Ethical Approval

This study was carried out in strict accordance with the Guidelines of Institutional Animal Care and Use Committee Vet. Cu (IACUC) under no. ARC AHRI 163 24.

Sample collection

Blood samples were collected from 123 backyard cows in the Fayoum and Giza governorates from April to September 2023. Data about previous tick infestation and acaricide treatment, mucous membrane color, viability, and any disease conditions of the cows were recorded. Samples were drawn from the jugular vein into 10 mL EDTA tubes and then divided into two portions. One portion was stored at -20°C until DNA extraction, while the other was used to prepare thin blood smears.

Blood smear

The smears were fixed with 100% methyl alcohol and stained with a 10% Giemsa solution. The stained slides were examined under an oil immersion lens at 1000X magnification, following the method described by Sengupta *et al*. (2010).

DNA extraction

Following the manufacturer's instructions, 200 µL of EDTA-blood samples were used to extract total genomic DNA using the Trans Pure DNA Kit. After elution in 20 µL of elution buffer, the DNA was stored at -20°C until use. To ensure reliability, each set of samples was extracted concurrently with negative control using phosphate-buffered saline (PBS).

Molecular amplification

PCR reactions were performed in a total volume of 50 µL using 25 µL of Cosmo PCR red master mix (Willowfort.CO.UK.) with 20 picomoles of each primer, 5µL of extracted DNA and complete with sterile nuclease-free water. Amplification was performed using a programmable conventional thermocycler (GTC96S cleaver scientific) with the following conditions: 10 minutes at 95ºC, followed by 35 cycles, 30 seconds at 95ºC, 30 seconds of specific primer annealing (Table 1), and 30 seconds at 95ºC, then 5 minutes at 72ºC.

The amplified products were electrophoresed on 1.5 % agarose gels in 1X Tris Borate EDTA (TBE). The agarose gel was stained with ethidium bromide and visualized by UV transillumination. The amplified fragments' size was compared to a 100 bp DNA molecular weight marker (Genedirex 100 bp DNA ladder H3 RTU). Nuclease-free water was used as a negative control.

Sequencing and phylogenetic analysis

Specific sizes of six and eleven bands of *Theileria* spp. and *Haemoplasma* spp. were excised from the gel, and the Wizard DNA Purification Kit was utilized to purify these specific DNA bands. The resulting PCR product was sequenced using a two-way Sanger sequencer provided by Macrogen®. The sequencing data were analyzed with the NCBI Blast tool and subsequently compiled and edited using BioEdit version 7.1.5 (Hall, 1999). Following this, MEGA X software was used to conduct maximum likelihood phylogenetic analyses as part of the Molecular Evolutionary Genetics Analysis (Tamura *et al*., 2021).

Results

Out of 123 examined cows, 45% (55/123) showed pale mucous membranes, a history of tick infestation, adenitis, and abortion cases. Microscopic examination of thin blood smears stained with Giemsa stain described different stages of TBPs in the examined blood samples. *Theileria* spp. were identified as piroplasms within red blood cells (RBCs) and macro and micro schizonts in lymphocytes. The parasites resembled tiny organisms with a single signet ring and measured between 1.5 and 2.8 µm. while, no additional piroplasms, such as *Babesia* spp., were seen; instead, *Hemoplasma* spp. were described as tiny, spherical, basophilic entities that were present on erythrocytes and occasionally visible free in the background.

Molecular amplification results

All microscopically positive *Theileria* spp. and *Hemoplasma* spp. showed specific bands of 560 bp and 595 bp, respectively. However, all examined samples tested negative for *Bartonella* spp.

The overall TBP prevalence in the present study was 52.8% and 90% using microscopic examination (ME) and PCR respectively. Additionally, in Al Fayoum and Giza governorates, data were displayed in Table 2. *Theileria annulata* and *Hemoplasma* spp. were detected in 27.6%, 15.4%, and 37.4%, 43.1% using ME and PCR respectively, mixed infection with *Theileria annulata* and *Hemoplasma* spp. were detected in 9.8% of examined samples by the two techniques. While *Babesia* spp. and *Bartonella* spp. never be detected either by ME or by PCR.

Sequencing and phylogenetic analysis

Sequencing analysis of the seven *Theileria* spp. bands were submitted to GenBank under accession numbers PQ344761.1 to PQ344766.1, while the sequencing analysis of the eleven bands of *Haemoplasma* spp. was submitted to GenBank under accession numbers PQ304803.1 to PQ304813.1.

The detected T. annulate strains showed 99%- 100% homology with each other and others in Egypt and other countries from cattle and ticks from cattle (Fig. 1).

The detected Ca. *M. hemobovis* and *M. wenyonii* strains showed 99%-100% homology with each other and others from cattle, goats, dogs, and cats in Egypt and other countries (Fig. 2).

Discussion

Vector-borne infections are receiving increasing attention from pub-

Table 1. Primer sequences we reused in the current study.

Target gene	Primer Sequence 5'-3'	Annealing temperature	Product size (bp)	Reference
Ss-*rRNA* (*Theileria* and *Babesia* spp.)	F: GTCTTGTAATTGGAATGATGG R: CCAAAGACTTTGATTTCTCTC	49 °C	560	(Beck *et al*., 2009)
16S-*rRNA* (*Heamoplasma* spp.)	F: ATACGGCCCATATTCCTACG R: TGCTCCACCACTTGTTCA	60 °C	595	(Mesquita *et al*. 2021)
*rpo*B *Bartonella* spp.	F: CGCATTGGCTTACTTCGTATG R: GTAGACTGATTAGAACGCTG	53°C	825	(Halajian *et al*., 2016)

Table 2. Prevalence of detected TBPs. in cattle from Al Fayoum and Giza governorates.

Study area	TBPs.	*Theileria annulata*		*Hemoplasma* spp.		Mixed infection	
		ME	PCR	ME	PCR	ME	PCR
Al Fayoum		(28/76) 30.3%	(30/76) 39.50%	(11/76) 14.5%	(35/76) 46.1%	(8/76) 10.5%	(8/76) 10.5%
Giza		(11/47) 23.4%	(16/47) 34%	(8/47) 17%	(18/47) 38.3%	(4/47) 8.5%	(4/47) 8.5%
Total		(34/123) 27.6%	(46/123) 37.4%	(19/123) 15.4%	(53/123) 43.1%	(12/123) 9.8%	(12/123) 9.8%

Fig. 1. Phylogenetic tree based on the partial ssu-rRNA nucleotide sequence of six T. annulate strains detected in this study. The green circles represent strains from this study. Bootstrap values of ≥ 70% are reported.

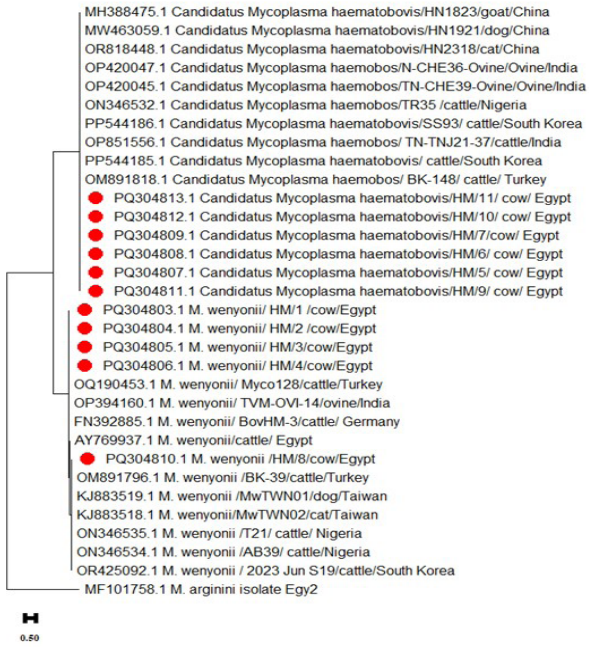

Fig. 2. Phylogenetic tree based on the partial 16S rRNA nucleotide sequence of eleven haemotropic mycoplasma (Ca. *M. hemobovis* and *M. wenyonii*) strains detected in this study. The red circles represent strains from this study. Bootstrap values of ≥ 70% are reported.

lic health and veterinary professionals. The range of these infections has expanded, affecting both humans and animals. Accurate diagnosis and identification are crucial to create an effective epidemiological map of these diseases. Various tick species flourish in Egypt due to the changing climate and inadequate preventive and control measures. Consequently, the impact of tick-related illnesses in the country is not well understood (Hashad *et al*., 2024).

Unfortunately, this study could not detect ticks because the examined cattle had recently undergone acaricide treatment due to a prior tick infestation.

Currently, there is no comprehensive report on the genomic properties of haemotropic mycoplasma, despite advancements in genotyping and molecular proteomics related to various parasitic diseases. The increasing prevalence of haemotropic mycoplasmosis presents an economic concern for cattle producers. Our understanding of how bovine haemotropic mycoplasmas are transmitted is still developing. Potential transmission routes include direct contact with contaminated blood and vectors such as fleas, hard ticks, and mosquitoes (Tatsukawa *et al*., 2021). Infections caused by haemotropic mycoplasmas are often associated with nonspecific clinical symptoms, which can lead to frequent misdiagnoses.

The current study aimed to detect tick-borne pathogens (TBPs) in blood samples collected from cows in the Giza and Al-Fayoum governorates. The findings revealed prevalence rates of 23.4% for *T. annulata* and 30.3% for Haemotropic mycoplasma, as observed through microscopic examination. In contrast, studies by El-Dakhly *et al*. (2020) and Al-Hosary *et al*. (2018) reported higher prevalence rates of 54.0% and 63.6%, respectively for *T. annulata* in different regions of Egypt. Furthermore, mixed infections involving both pathogens were identified in 9.8% of the samples, aligning with other studies that confirmed the presence of co-infections in cattle blood linked to ticks (Al-Hosary *et al*., 2018; 2021). These co-infections suggest that cattle infested with ticks are susceptible to multiple pathogens.

Babesia species were not detected in the examined animals, which contrasts with previous findings in Egypt. El Moghazy *et al*. (2014) and Abdel-Rahman and Ismaiel (2018) reported higher prevalence rates of 22.47%, 35%, and 20% in cattle from the Qaluobia, Ismailia, and Sharkia governorates, respectively. The fluctuation in prevalence rates may be attributed to variations in environmental conditions and different study areas that affect both the parasites and their vectors (Nayel *et al*., 2012).

The molecular results indicated significantly higher diagnostic sensitivity in the detection of tick-borne pathogens using PCR compared to conventional microscopic examination of blood smears, with sensitivity of 90% and 52.8%, respectively. This finding aligns with the study by Al-Hosary *et al*. (2018), which demonstrated the advantages of using PCR for detecting a wide range of pathogens, particularly in sub-clinically infected cattle and their associated ticks. None of the examined blood samples tested positive for *Babesia* spp. by PCR, despite the current study targeting the small subunit rRNA genes used for the identification of both *Babesia* and *Theileria* spp. in the same reaction. A probable explanation for this finding is that *Babesia* and *Theileria* spp. do not share the same vector. Theileriosis is transmitted by ixodid ticks of the genus *Hyalomma*, while Babesiosis is primarily transmitted by *Rhipicephalus* (*R. microplus* and *R. decoloratus*) (Soulsby, 1982; Al-Hosary *et al*., 2021). Also, *Bartonella* bacterium wasn't detected in the examined cows either by ME or PCR. This finding nearly agreed with El-Alfy *et al*. (2022) who considered *Bartonella* spp. as miscellaneous TBPs recorded with a low incidence of 2.6% in examined native cattle breeds. This result is not surprising as there is little evidence that *Bartonella* spp. can replicate within ticks and no definitive evidence of transmission by a tick to a vertebrate host (Angelakis *et al*., 2010; Bai *et al*., 2024).

Sequencing and Phylogenetic analysis of PCR products revealed two types of haemotropic mycoplasmas (*M. wenyonii* and *Candidatus Myco-*

plasma haematobovis) in 43.1% of examined cattle. In a similar investigation conducted by Khudhair and his colleagues in 2022, PCR testing confirmed the presence of haemotropic mycoplasmas in 9% of blood samples collected from calves with pale mucous membranes in areas where ticks are highly endemic. From these, PCR-positive samples were identified *M. wenyonii* and *Ca. M. haemobos*.

A study by Díaz-Sánchez *et al*. in Cuba in 2019 found that 66.2% of the cattle and 95% of the buffalo analyzed positive for the bovine haemotropic mycoplasma (*M. wenyonii* and *Ca. M. haemobos*). Additionally, research conducted in Japan by Tatsukawa *et al*. (2021) found that 91.5% of the inspected cows tested positive for bovine *Haemoplasma*. Furthermore, Niethammer al. (2018) reported that 60.24% of cows tested positive for hemotrophic mycoplasma using PCR, specifically identifying strains such as *M. wenyonii* and *Ca. M. haemobos*.

It is still challenging to determine the risk of pathogenic bovine *Haemoplasma*s. The *Haemoplasma*-positive cows in our study may be considered chronic carriers, as they exhibited no outward symptoms. During the acute phase of *Hemoplasma* infection, there have been reports of anaemia, abortion, and decreased milk production in affected cows. Additionally, cows with a persistent *Haemoplasma* infection have produced less milk and had lower birth weights for their calves (de Souza *et al*., 2024). Since decreased reproductive efficiency is the main economic loss for breeding cows, further studies are required to elucidate the effect of subclinical *Hemoplasma* infection on reproductive performance in native cattle.

It was observed that a connection existed between the infestation of tick-borne pathogens and haemotropic mycoplasmosis. Animals infected with tick-borne pathogens, such as *Theileria*, are more susceptible to haemotropic mycoplasmosis because the presence of multiple co-infecting pathogens causes a complex, divergent, or similar response, allowing for synergy and more successful colonization in the host (Paul *et al*., 2020).

Significant uncertainties exist regarding the potential modes of transmission and the epidemiology of chemotropic mycoplasmas in cattle. *Haemoplasma*s can be transmitted through contact with contaminated blood, and various arthropod vectors are suspected of being possible carriers. Many researchers have hypothesized that ticks may serve as transmission vectors for *Hemoplasma*s (De Souza and Ruegg, 2023).

Mycoplasma molecular typing has proven to be highly effective in detecting hard-to-cultivate mollicutes. The 16S rRNA group-specific PCR assay amplifies all mollicute species within the genera Mycoplasma, Acholeplasma, Ureaplasma, and Spiroplasma (Ahmed *et al*. 2023).

From the sequence of small subunit-rRNA genes common and specific for Piroplasm (*Theileria annulata*) detected the strains PQ344761.1, PQ344762.1, and PQ344764.1showed high nucleotide identity 100% with each other and OP542455.1, OP542445.1, OP542456.1, OP542458.1, MN732742.1, MN223728.1, from Egypt, MW165671.1 from Pakistan, and MK089831.1 from China. While PQ344766.1 and PQ344765.1 strains showed 100% homology with each other and 99% with PQ344761.1, PQ344762.1, PQ344763.1 and PQ344764.1 (study strains), OP542455.1, OP542445.1, OP542456.1, OP542458.1, MN732742.1, MN223728.1, from Egypt, MW165671.1 from Pakistan, and MK089831.1 from China.

The 16s rRNA gene sequences shared among bovine haemotropic mycoplasmas (*M. wenyonii* and *Ca. M. haemobos*) showed that the detected strains *M. wenyonii*PQ304805.1 and PQ304806.1 had 100% identity with strain OP394160.1 from ovine in India, FN392885.1 from cattle in Germany, and AY769937.1 from cattle in Egypt. Moreover, the detected *M. wenyonii* strain PQ304810.1 displayed 100% homology with OM891796.1 from cattle in Turkey, KJ883519.1 from a dog in Taiwan, KJ883518.1 from a cat in Taiwan, and multiple instances from cattle in Nigeria, specifically ON346535.1 and ON346534.1, as well as OR425092.1 from cattle in South Korea.

Furthermore, the strains identified *Ca. M. haemobos* PQ304807.1, PQ304808.1, PQ304809.1, PQ304811.1, PQ304812.1, and PQ304813.1 exhibited 100% homology with each other and with the strains in OM891818.1 from cattle in Turkey, additionally, *Ca. M. haemobos* strains PP544185.1 from cattle in South Korea, OP851556.1 from cattle in India, PP544186.1 from cattle in South Korea, ON346532.1 from cattle in Nigeria, OP420045.1 from sheep in India, OP420047.1 from sheep in India, and OR818448.1 from a cat in China were also noted.

Conclusion

Comprehensive and systematic monitoring of tick-borne pathogens (TBPs) infections in cattle is essential across different regions of Egypt to accurately assess the prevalence and distribution of these infections. Additionally, the implementation of targeted and efficient tick control programs is crucial. These programs should include integrated pest management approaches, regular veterinary assessments, and effective treatment regimens aimed at reducing both the incidence of TBP infections and the resulting economic losses suffered by the livestock industry. By addressing these factors, it is possible to improve animal health, enhance productivity, and support the overall economic stability of affected communities.

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Conflict of interest

The authors have no conflict of interest to declare.

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