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Original Article

Serological and molecular surveys of *Anaplasma* spp. in Egyptian cattle reveal high *A. marginale* infection prevalence

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Abstract

Background: Bovine anaplasmosis is an infectious disease with worldwide distribution. It spreads by various routes mainly through tick bites. **Aims:** This study aimed to investigate bovine related *Anaplasma* spp. in cattle from three northern governorates of Egypt by serological and molecular assays, to evaluate the associated risk factors and to analyze the phylogeny of revealed *A. marginale* isolates. **Methods:** During 2020, a total of 650 blood samples were collected from asymptomatic cattle in the governorates of Kafr El-Sheikh (n=240), Menofia (n=230), and Al-Gharbia (n=180). Sera samples were examined using the *Anaplasma* antibody test kit, cELISA v2. Blood genomic DNA of seropositive cattle was then examined by PCRs specific to *A. marginale*, *A. centrale*, and *A. bovis*. Selected positive samples were subjected to nucleotide sequencing. Risk factors (i.e. geographical area, breed, type of production, sex, age, herd size, season, husbandry system, tick infestation, and application of acaricides) were evaluated by logistic regression approach. **Results:** In total, 130 cattle (20%, 95% CI: 17.1–23.3) were recorded seropositive for *Anaplasma* species. Major risk factors associated with seropositivity were being crossbred, dairy cattle, aged more than 5 years, summer season, herd size of below 300, pasture grazing, tick infestation, and not being subjected to regular treatment with acaricides. By using species-specific PCR, only *A. marginale* was detected. Nucleotide sequencing showed the occurrence of two different *msp4* genotypes. **Conclusion:** This study shows the high prevalence of *A. marginale* in cattle of Kafr El-Sheikh, Al-Gharbia, and Menofia. However, the connection between *Anaplasma* species and their tick vectors remains unknown in Egypt and merits further investigations. Since these infections primarily spread through ixodid tick bites, effective ectoparasite control strategies, regular examination of cattle and successful chemoprophylaxis are recommended.

Key words: *Anaplasma*, Cattle, Molecular detection, Phylogeny, Seroprevalence

Introduction

Tick-borne diseases (TBDs) are of economic significance to the livestock industry (Jongejan and Uilenberg, 2004). Globally, 80% of the world's cattle population are at risk of TBDs with economic losses estimated at US$30 billion per year (Lew-Tabor and Valle, 2016). One of the most economically important hemoparasitic TBDs of bovine in the world is undoubtedly anaplasmosis (Uilenberg, 1995) transmitted by several species of hard ticks (Ixodidae) belonging to the genera *Ixodes*, *Haemaphysalis*, *Amblyomma*, *Rhipicephalus*, *Hyalomma*, and *Dermacentor* (Dantas-Torres and Otranto, 2017).

Anaplasmosis is a tick-borne disease caused by bacteria of the genus *Anaplasma*, which infects a wide range of wild and domestic animals (Ben Said *et al*., 2018b, 2019). Although some of the nine recognized and possible *Anaplasma* species i.e. *A. phagocytophilum*, *A. marginale*, *A. bovis*, *A. centrale*, *A. ovis*, *A. mesaeterum*, *A. platys*, *A. caudatum*, *A. odocoilei*, *A. capra*, and "*Candidatus* Anaplasma camelii" exhibit a certain degree of host specificity, some of them (e.g., *A. phagocytophilum*, and *A. platys*) may infect more than one animal species, including humans (Dantas-Torres and Otranto, 2017; Sharifiyazdi *et al*., 2017; Sazmand *et al*., 2019; Selmi *et al*., 2019; Alanazi *et al*., 2020; Selmi *et al*., 2020). Bovine anaplasmosis is mainly caused by

erythrocytes-infecting *A. marginale*, monocytes-infecting *A. bovis* (Belkahia *et al*., 2015; Ben Said *et al*., 2018a), and strains genetically related to granulocytes-infecting *A. phagocytophilum* (*A. phagocytophilum*-like 1 and 2) (Ben Said *et al*., 2015; Ben Said *et al*., 2017b), and related to platelets-infecting *A. platys* (*A. platys*-like) (Zobba *et al*., 2014; Battilani *et al*., 2017; Ben Said *et al*., 2017a; Selmi *et al*., 2019). However, *A. marginale* is responsible for almost all outbreaks of clinical bovine anaplasmosis (OIE, 2018). In cattle, the infection is spread mainly by ixodid ticks, but also by other arthropod vectors such as biting flies, and blood-contaminated objects e.g. needles, ear tags, dehorning and castration equipment. Transplacental transmission may also contribute to the epidemiology of the disease in some regions (Aubry and Geale, 2011). The disease is widespread in tropical and subtropical regions and is characterized by fever, anemia, weakness, enlarged lymph nodes, abortion, decreased milk production, jaundice, and sometimes death (Kocan *et al*., 2010). Cattle that recover from acute infection remain persistently infected carriers for whole life and may act as a source of infection in naïve cattle populations, causing endemic disease stability (Kocan *et al*., 2015).

In Egypt, previous studies reported *A. marginale* infection in cattle, buffaloes, and camels (El-Naga and Barghash, 2016; Elhariri *et al*., 2017; AL-Hosary *et al*., 2020; El-Dakhly *et al*., 2020; Nasreldin *et al*., 2020; Parvizi *et al*., 2020). In addition, DNA of *A. marginale* has been detected in *Hyalomma anatolicum*, and *Rhipicephalus annulatus* collected from cattle (Loftis *et al*., 2006). However, information on bovine anaplasmosis is still incomplete in some parts of the country. Therefore, the objectives of the present study were as follow:

I) Investigation of the seroprevalence of *Anaplasma* spp. in cattle from three northern governorates,
II) Evaluation of the associated risk factors,
III) Molecularly detection of *A. marginale*, *A. centrale*, and *A. bovis* by PCR,
IV) Genetically characterizing the positive samples.

Materials and Methods

Study area

Egypt is a transcontinental country that stretches across northeastern Africa and southwestern Asia. It is divided into 27 governorates. The large regions of the Sahara desert, which make up most of Egypt's territory, are sparsely inhabited. This research was carried out in three northern governorates namely Kafr El-Sheikh (31.1107°N, 30.9388°E), Al-Gharbia (30.8754°N, 31.0335°E), and Menofia (30.5972°N, 30.9876°E) (Fig. 1). These governorates are geographically located between two branches of the Nile River in northern Egypt, and are characterized by hot desert climate with common temperature range of 15 to 35°C. However, high temperatures varying between 35 and 45°C is usually observed between July and August. These governorates have variable average rainfall in the range of 100–200 mm that mainly occurs during the winter months. The study areas are agriculture area and have many exchange pools suitable for the multiplication of arthropod vectors.

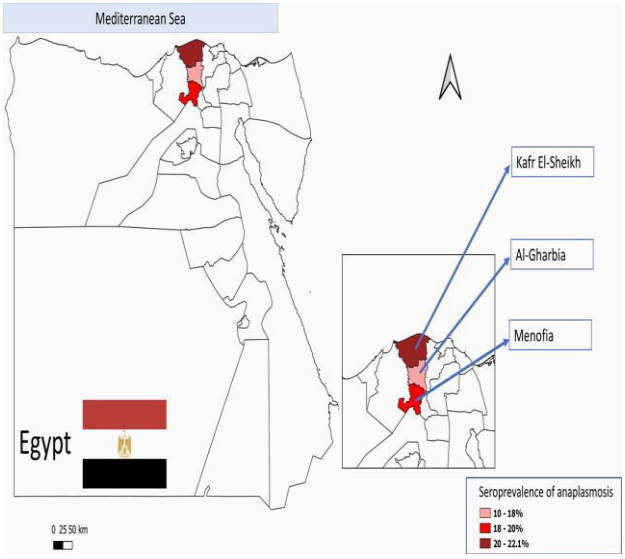

Fig. 1: Map of Egypt indicating governorates where the studied animals are located

Samples collection

The sample size (n=650) was calculated based on the described formula (Thrusfield and Christley, 2018) with an expected prevalence of 18.5% (Parvizi *et al*., 2020), a confidence interval of 95%, and an accuracy of 5%. During four seasons in 2020, jugular vein blood samples were collected from individual cattle using clean sterilized vacuum tubes with and without EDTA for molecular and serological assays. No individual was sampled more than once. The sera were separated by centrifugation at 3500 ×g for 10 min. A clinical examination including measurement of rectal temperature, pulse and respiratory rates was performed on all animals before sampling. The examined cattle were grouped according to breed (Holstein, crossbreed, native), type of production (dairy, beef), gender (male, female), age (<2, 2–5, >5 years old), and herd size (<100, 100–300, >300). Also, for each cattle, the season, the husbandry system (stall feeding, pasture grazing, pasture grazing plus stall feeding), the tick infestation, and the application of acaricides were recorded.

This study was approved by the Ethical Research Committee, Faculty of Veterinary Medicine, Benha University, Egypt.

Serological analysis

All blood sera samples were examined using *Anaplasma* antibody test kit, cELISA v2 (VMRD, Pullman, Washington, USA) according to the manufacturer's instructions. This competitive ELISA (cELISA) based on the recombinant major surface protein 5 (rMSP5) is licensed for the detection of antibodies directed against the MSP5 protein of *A. marginale*, *A. centrale*, and *A. ovis* (Dreher *et al*., 2005).

It has a diagnostic sensitivity of 100% and a specificity of 99.7% (Chung *et al*., 2014). The results were expressed as percent inhibition (%I) and calculated as follow:

1 - (OD620 of sample /OD620 of negative control) × 100

The sample was considered positive if %I ≥30.

Genomic DNA extraction and PCR assay

Genomic DNA were extracted from 200 µL aliquots of EDTA-treated blood samples of serologically positive cattle using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA samples were then examined by conventional PCRs using primers AmargMSP4Fw: 5´-CTG AAG GGG GAG TAA TGG G-3´ and AmargMSP4Rev: 5´-GGT AAT AGC TGC CAG AGA TTC C-3´ for *A. marginale* (Torina *et al*., 2012), AC1f: 5´-CTG CTT TTA ATA CTG CAG GAC TA-3´ and AC1r: 5´-ATG CAG CAC CTG TGT GAG GT-3´ for *A. centrale* (Kawahara *et al*., 2006), and AB1f: 5´-CTC GTA GCT TGC TAT GAG AAC-3´ and AB1r: 5´-TCT CCC GGA CTC CAG TCT G-3´ for *A. bovis* (Kawahara *et al*., 2006). All the PCR reactions were performed using the Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X) (Thermo Scientific, Waltham, US) in a T100™ thermal cycler (BioRad, California, USA). For all reactions, DNA from blood samples positive for the pathogen served as a positive control. The amplified PCR products were separated on a 1.5% agarose gel (UltraPure™ Agarose, Thermo Scientific, Waltham, USA) stained with ethidium bromide and visualized by a UV transilluminator (Gel Doc™ XR+, BioRad, California, USA).

DNA sequencing

None of the examined cattle were infected with *A. centrale* or *A. bovis*. Two positive amplicons of *A. marginale* sized ca. 344 bp from 3 years old female cattle from Al-Gharbia governorate and 3.5 years old female cattle from Kafr El-Sheikh governorate were randomly selected, extracted from the gel, and purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The amplicons were then sequenced in both directions using BigDye™ Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) in an ABI-PRISM 3500 automated sequencer (Thermo Scientific, Waltham, US). Sequence reads were analyzed with BioEdit® Sequence Alignment Editor (Hall, 1999), assembled into consensus sequences and compared to those available in the GenBank® database using the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

Partial sequences of the *msp4* gene isolated from *Anaplasma marginale* with the size of 301 bp were aligned with the corresponding sequences available from the GenBank® database, using Clustal W (http://www.clustalw.genome.jp). Similarity searches were performed using BLAST (http://blast.ncbi.nlm.nih.gov) (Altschul *et al*., 1997). DNAMAN program (ver. 5.2.2; Lynnon Biosoft, Que., Canada) was also used to calculate genetic distances computed by the maximum composite likelihood method (Tamura and Nei, 1993). Neighbor-Joining trees were built using the same software (Saitou and Nei, 1987). Statistical support for internal branches was established by bootstrap analysis with 1000 replications (Felsenstein, 1985).

Statistical analysis

The results were transferred to SPSS software (ver. 24.0, IBM, USA) for further analysis. The Chi-square test was used to compare seropositivity to *Anaplasma* species and the results were considered significant if P≤0.05. Univariable logistic regression analysis was used to evaluate the association between anaplasmosis seroprevalence and variables of location (Kafr El-Sheikh, Al-Gharbia, Menofia), breed (Holstein, crossbreed, native), type of production (dairy, beef), gender (male, female), age (<2, 2–5, >5 years old), herd size (<100, 100–300, >300), season, husbandry system (stall feeding, grazing, grazing plus stall feeding), tick infestation, and application of acaricides. Variables with a P≤0.05 in the univariable analyses were evaluated with multivariable models to determine the risk factors, odds ratio (OR), and confidence interval (CI) of each significant variable in univariable analyses.

Results

Of 650 cattle tested by cELISA, 130 (20%, 95% CI: 17.1–23.3) were found to be seropositive for targetted *Anaplasma* spp.. None of the analyzed animals showed typical clinical signs of anaplasmosis e.g. anemia, fever, pale mucous membranes, weakness. Univariate statistical modeling revealed that the risk of seropositivity was significantly associated with breed and age of cattle, production type, husbandry system, herd size, the season of sampling, tick infestation, and not the application of acaricides. However, the geographic region and the gender of cattle did not have a significant role in seropositivity to *Anaplasma* spp. in this study (Table 1). Multivariate logistic regression analysis of eight risk factors with P≤0.05 in univariable analysis revealed a higher probability of seropositivity in crossbreed cattle, dairy cattle, and animals over the age of 5 years. Furthermore, seropositivity was highest during the summer, in grazing cattle, in animals infested with ticks, and in those not receiving acaricides regularly (Table 2).

PCR examination of blood samples from 130 seropositive cattle using specific primers revealed that all tested animals (100%) were infected with *A. marginale*. None of the examined cattle were infected with *A. centrale* or *A. bovis*.

Anaplasma marginale infections were validated by sequencing of 301 bp *msp4* gene from two randomly selected positive cattle samples. Sequences alignment revealed two distinct genotypes differed by one nucleotide (data not shown). The average nucleotide

identity was 99.6% among genotypes. Genotypes were 99.0 to 100% homologous in comparisons with previous *A. marginale* genotypes existing in the GenBank. The new nucleotide sequences generated from AS01 and AS02 *A. marginale* isolates were deposited in the GenBank® (http://www.ncbi.nlm.nih.gov/) under the accession numbers MZ695054 and MZ695055, respectively. When compared to the *A. centrale* reference sequence (GenBank accession number AF428090), nucleotide identities were estimated to be 83.4 and 83.1%, respectively.

Phylogenetic analysis, based on the alignment of the two genotypes of this study with partial *msp4* sequences from the GenBank, originated three main clusters with the robustness of nodes' rates estimated at 78 and 76% (Fig. 2). The first cluster included two isolates from Hungary. The second cluster contained strains from Latin America (e.g. Mexico, Brazil, and Argentina), Asia (e.g. Taiwan and China), and Southern Europe (e.g. Spain and Italy). The third cluster included isolates mainly from Africa (Nigeria, Zimbabwe, and South Africa), North America (represented exclusively by the USA), and Southern Europe (Italy, Spain). The last included two isolates from Mexico (Fig. 2). Egyptian strains were assigned to the second cluster. In particular, isolate AS01 clustered in a second sub-cluster with two strains from Mexico, and isolate AS02 clustered in the first sub-cluster with 5 strains from Latin America, one strain from Spain, and two others from Asia (Fig. 2).

Discussion

Presented data indicate that cattle populations (i.e. 20%) in three northern governorates of Kafr El-Sheikh,

Table 1: Risk factors associated with seroprevalence rates of anaplasmosis in 650 cattle in Egypt according to different variables

Variables	No.	No of positive animals (%)	95% CI[a]	Statistics
Governorate				
Kafr El-Sheikh	240	53 (22.1)	17.3–27.7	χ^2=1.234
Menofia	230	45 (19.6)	15–25.2	df[b]=2
Al-Gharbia	180	32 (17.7)	12.8–24	P=0.5
Breed				
Holstein	200	49 (24.5)	19.1–30.9	χ^2=11.692
Crossbreed	350	73 (20.9)	16.9–25.4	df=2
Native	100	8 (8.0)	4.1–15	P=0.003
Age				
<2 years	150	12 (8.0)	4.6–13.5	χ^2=28.569
2–5 years	380	77 (20.3)	16.5–24.5	df=2
>5 years	120	41 (34.2)	26.3–43	P=0.0001
Sex				
Male	61	8 (13.1)	6.8–23.8	χ^2=1.955
Female	589	122 (20.7)	17.6–24.2	df=1
				P=0.16
Season				
Winter	120	11 (9.2)	5.2–15.7	χ^2=56.783
Spring	180	19 (10.6)	6.9–15.9	df=3
Summer	280	94 (33.6)	28.3–39.3	P=0.0001
Autumn	70	6 (8.6)	3.9–17.5	
Production type				
Dairy	500	115 (23.0)	19.5–28.9	χ^2=12.188
Beef	150	15 (10)	6.2–15.8	df=1
				P=0.0001
Husbandry system				
Stall feeding	150	15 (10)	6.2–15.8	χ^2=13.820
Pasture grazing	155	41 (26.4)	20.1–33.9	df=2
Pasture grazing plus stall feeding	345	74 (21.4)	17.5–26.1	P=0.001
Herd size				
<100	340	81 (23.8)	19.6–28.6	χ^2=6.883
100–300	170	29 (17.1)	12.2–23.4	*df*=2
>300	140	20 (14.3)	9.5–21	*P*=0.03
Tick infestation				
Yes	380	95 (25.0)	20.9–29.6	χ^2=14.294
No	270	35 (12.9)	9.5–17.5	df=1
				P=0.0001
Application of acaricides				
Every three months	201	21 (10.5)	6.9–15.5	χ^2=19.531
Irregular	405	94 (23.2)	19.4–27.6	df=2
Not applied	44	15 (34.1)	21.8–48.8	P=0.0001
Total	650	130 (20)	17.1–23.3	

[a] CI: Confidence interval, and [b] df: Degree of freedom. Significant variables (P≤0.05)

Table 2: Multivariant logistic regression analysis of risk factors associated with seroprevalence rate of anaplasmosis in 650 cattle in Egypt according to different variables

Variable	ß[a]	SE[b]	OR[3]	95% CI[4]	P-value
Breed					
Holstein	1.317	0.404	3.7	1.9–8.2	0.001
Crossbreed	2.419	0.384	11.2	5.3–23.8	0.0001
Native (constant)	–	–	–	–	–
Type of production					
Dairy	0.989	0.292	2.7	1.5–4.6	0.001
Beef (constant)	–	–	–	–	–
Age					
2–5	1.072	0.327	2.9	1.5–5.5	0.001
>5	1.786	0.357	5.9	2.9–12	0.0001
<2 years (constant)	–	–	–	–	–
Herd size					
<100	0.334	0.240	1.4	0.8–2.2	0.0001
100–300	0.334	0.240	0.7	0.4–1.1	0.0001
>300 (constant)	–	–	–	–	–
Season					
Winter	0.074	0.531	1.1	0.4–3.1	0.001
Spring	0.230	0.491	1.3	0.5–3.3	0.0001
Summer	3.181	0.446	24.1	10–57.6	0.0001
Autumn	–	–	–	–	–
Grazing system					
Pasture grazing	1.175	0.327	3.2	1.7–6.2	0.0001
Pasture grazing plus stall feeding	0.899	0.302	2.5	1.4–4.4	0.003
Stall feeding (constant)	–	–	–	–	–
Presence of ticks					
Yes	0.806	0.216	2.2	1.5–3.4	0.0001
No (constant)	–	–	–	–	–
Application of acaricides					
Irregular	0.952	0.259	2.6	1.6–4.3	0.0001
Not applied	1.489	0.393	4.4	2.1–9.6	0.0001
Every three months (constant)	–	–	–	–	–

[a] ß: Wald statistic, [b] SE: Standard error, [c] CI: Confidence interval, and [d] OR: Odds ratio. Significant variables (P≤0.05)

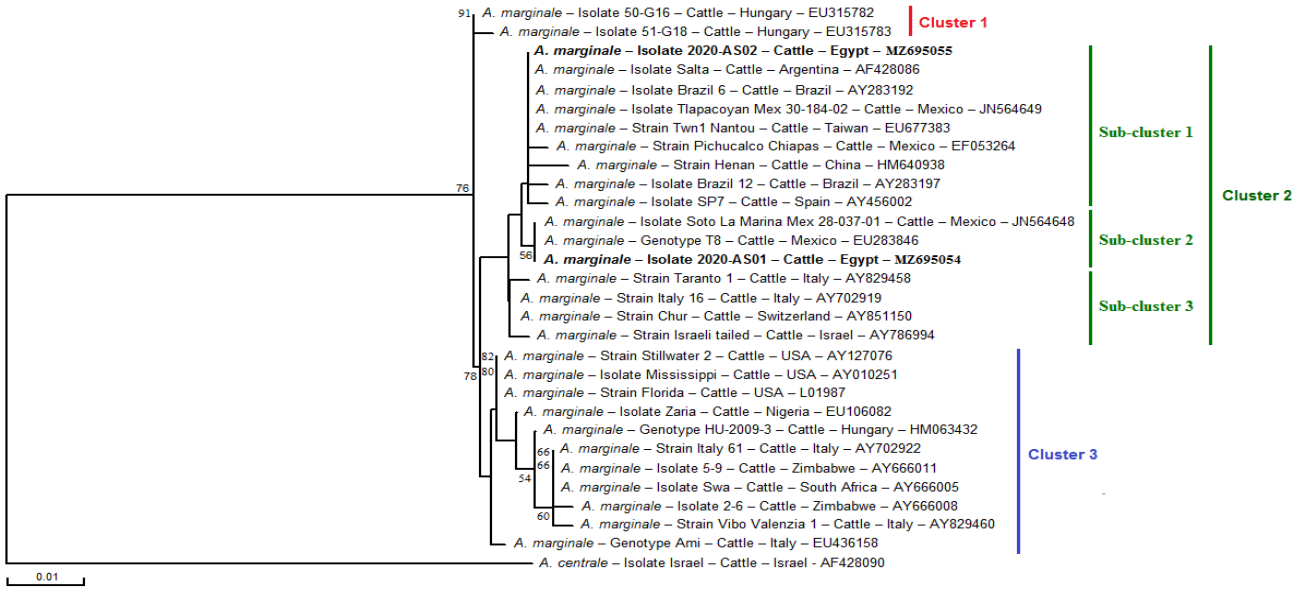

Fig. 2: Phylogenetic relationships of *Anaplasma marginale* isolates of the present study and other *A. marginale* isolates and strains available in the GenBank based on *msp4* partial sequence of 301 bp. The analyses were performed using the Neighbor-Joining method based on the maximum composite likelihood method (Tamura and Nei, 1993) in the DNAMAN program (ver. 5.2.2, Lynnon Biosoft, Que., Canada). Sequences are presented by isolate, genotype or strain name, host species, country of origin, and GenBank® accession number. One *A. centrale msp4* partial sequence was added as an out-group. Numbers associated with the nodes represent the percentage of 1000 bootstrap iterations supporting the nodes (only percentages greater than 50% were represented). The sequences of *A. marginale* newly obtained in the present study are in bold

Table 3: Reports of *Anaplasma marginale* in cattle (*Bos taurus*) and their ticks in Egypt

Host/vector	Governorate/city	No. of examined population	% Prevalence	Method	Year of study	Reference
Cattle	Dakahlia and Damietta	3310	3.5 3.7	Blood smear IFAT	2005–2006	Younis *et al*. (2009)
Cattle	Dakahlia Damietta	650 4640	6.3 9.05	Blood smear	2005-2007	Salm *et al*. (2011)
Cattle	Qalyoubia	100	60	Blood smear	2011	Radwan *et al*. (2013)
Cattle	Dakahlia	164	20.1	cPCR	2012–2013	El-Ashker *et al*. (2015)
Cattle	Qena	90	28	ELISA	2014–2015	Fereig *et al*. (2017)
Cattle	24 governorates	758	18.5	ELISA	2015–2016	Parvizi *et al*. (2020)
Cattle	Beni-Suef El-Fayoum El-Wadi El-Gadid	50 50 50	4 16 12	cPCR[a]	2015–2018	El-Dakhly *et al*. (2020)[b]
Cattle	Menofia	92	15.2	cPCR	2017	Tumwebaze *et al*. (2020)
Cattle	New Valley	31	61.3	Blood smear	2017–2018	Nasreldin *et al*. (2020)
Cattle	EL-Minia, Assiut, EL-Fayoum, New Valley	309	16.2 54.8[c] 68.3 50.2	Blood smear ELISA qPCR[d] RLB[e]	2018	AL-Hosary *et al*. (2020)
Cattle	Kharga, EL-Fayoum, Assuit	41	92.7	RLB and cPCR	2018	AL-Hosary *et al*. (2021)
Cattle	Kafr El-Sheikh Al-Gharbia Menofia	240 180 230	22.1 17.7 19.6	ELISA and cPCR	2020	This study
Hyalomma excavatum	Siwa	NS[e]	NS	cPCR	2002–2003	Loftis *et al*. (2006)
Rhipicephalus annulatus	Wadi el Natroun	NS	NS	cPCR	2002–2003	Loftis *et al*. (2006)
Hyalomma excavatum	Assuit, El-Fayoum	NS	NS	RLB and cPCR	2018	AL-Hosary *et al*. (2021)
Rhipicephalus annulatus	Assuit, El-Fayoum	NS	NS	RLB and cPCR	2018	AL-Hosary *et al*. (2021)

[a] cPCR: Conventional PCR, [b] Overall prevalence was 10.6%, [c] 188 cattle were included in the serology, [d] qPCR: Real-time PCR, [e] RLB: Reverse line blot, and [e] NS: Not stated

Menofia, and Al-Gharbia are exposed to *A. marginale*, and *A. centrale*. As summarized in Table 3, previous studies have reported antibodies against *A. marginale* in 18.5% to 54.8% of cattle in different regions of Egypt (Fereig *et al*., 2017; AL-Hosary *et al*., 2020; Parvizi *et al*., 2020). Interestingly, the seroprevalence of anaplasmosis was 22.1% and 19.6% in Kafr El-Sheikh, and Menofia, however, in a previous study in 2015–2016, no seropositive cattle were recorded in these governorates (Parvizi *et al*., 2020). Since none of the animals showed clinical signs of anaplasmosis, the examined cattle should have been infected subclinically or persistently (Aubry and Geale, 2011), so unrestricted transport of these animals could contribute to both the spread of anaplasmosis and the increase in strain diversity (Kocan *et al*., 2015).

Compared to other North African countries, the seroprevalence in our study remains higher than that observed in Algeria at 7.4% (Ziam and Benaouf, 2004). Moreover, our finding was similar to that observed in Morocco with a prevalence rate of 16.5% (Ait Hamou *et al*., 2012). In Sudan, anaplasmosis prevalence occurs at much higher levels which are estimated at 37.8 and 38.9% (Salih *et al*., 2008; Salih *et al*., 2009). However, prevalence rates reported for countries must be taken with caution, since one standardized assay such as sampling procedure was not used in each study, and infection rates may vary even between neighboring farms (Ait Hamou *et al*., 2012).

In this study, seropositivity to *Anaplasma* spp. was more frequent in older cattle and dairy animals that are usually kept longer for production compared to beef cattle that are slaughtered at young ages. Similarly, dairy cattle under 1 year of age have been shown to have the lowest risk of anaplasmosis compared to other age groups i.e. cattle from 1–3, 3–5, and >5 years old (Noaman and Moradi, 2019). These observations may be explained by the fact that older animals are more exposed to arthropod infestations as they live longer and went through more vector seasons (Ben Said *et al*., 2018b).

Furthermore, cattle of native breeds had a significantly lower risk of anaplasmosis. In contrast, in Tunisia, Holstein cattle were less infected with *A. marginale* than other breeds (Schwyz and crossbreeds) (Belkahia *et al*., 2015; M'ghirbi *et al*., 2016). The lower

prevalence of anaplasmosis in native breeds compared to pure breeds, that are mainly imported from Europe, might be due to greater resistance to anaplasmosis or the circulation of host-adapted strains in the north Africa (M'ghirbi *et al*., 2016; Ben Said *et al*., 2018b).

Larger herds had lower rates of anaplasmosis possibly due to stricter hygienic measures and more effective management and control strategies. Consistent with our findings, the incidence of anaplasmosis within herds was negatively related to herd size in the United States (Alderink and Dietrich, 1983). Previous studies have shown that owners of larger flocks and herds have a significantly better understanding of parasites and diseases (Sazmand *et al*., 2020). This factor is crucial in the monitoring and treatment of sick animals.

We found a statistically higher incidence of anaplasmosis in cattle with risk factors associated with ticks and tick bites. In particular, 73.08% of seropositive animals were infested with ticks, 83.8% did not receive regular acaricides treatment, and 88.5% went out to graze. Furthermore, a significantly higher number of seropositive cattle was recorded in the summer season. These factors are recognized risks in the epidemiology of bovine anaplasmosis (Aubry and Geale, 2011; Ben Said *et al*., 2018b). *Anaplasma marginale* is transmitted essentially by ticks, however, mechanical transmission through bites of flies and by instruments frequently used in veterinary practice may also occur (Kocan *et al*., 2015). It has also been suggested that transplacental transmission may contribute to the epidemiology of bovine anaplasmosis in some regions (Aubry and Geale, 2011). However, the burden of alternative routes of *Anaplasma* transmission in Egypt requires further investigations.

Species-specific PCRs for the detection of active infection with *A. marginale* revealed that the pathogen was actively circulating in 20% of examined population. In molecular epidemiology studies in Egypt, 10.6–92.7% of tested cattle were PCR positive (Table 3). In other north African countries, infection rates in cattle based on molecular studies were 4.7–25.4% in Tunisia, 21.9% in Morocco, 6.1% in Sudan, and 11.1% in Algeria (Ben Said *et al*., 2018b). However, the different prevalence rates reported for these countries could be explained in part by the difference in sampling seasons, i.e. late spring and early summer, which is favorable to the spread of ticks compared to the winter season with minimal tick activity (Aubry and Geale, 2011).

Understanding the phylogenetic relationships between *A. marginale* isolates is important for performing an informative intraspecific diversity analysis contributing to better prevention and control of this bacterium. Thus, the sequencing of the partial *msp4* gene was used for an analysis of the diversity of our Egyptian *A. marginale* isolates. The detection of two different *A. marginale* isolates in this study, in the second cluster, suggests that phylo-geographical resolution may be obtained at the regional level (de la Fuente *et al*., 2003; de la Fuente *et al*., 2004), but not when the analysis is conducted worldwide. This heterogeneity could be explained, in part, by the importation of live cattle and/or the dissemination of *Anaplasma* spp. infected ticks with migratory birds which are proven by several studies from different countries (Alekseev *et al*., 2001; Ogden *et al*., 2008; Hildebrandt *et al*., 2010; Kang *et al*., 2013).

When examining 130 cattle for *A. centrale* and *A. bovis*, no cases of infection were detected. In accordance with our result, these *Anaplasma* species were not diagnosed in previous studies in Egypt (El-Ashker *et al*., 2015; AL-Hosary *et al*., 2020; Nasreldin *et al*., 2020). *Anaplasma centrale*, which is less pathogenic than *A. marginale*, causes mild signs in cattle and is considered a naturally attenuated subspecies. It has therefore been widely used as a live vaccine against *A. marginale* (Kocan *et al*., 2010). In North Africa, the molecular prevalence of up to 15.1% and 39.4% have been recorded in Tunisia and Algeria (Belkahia *et al*., 2015; Rjeibi *et al*., 2017). *Anaplasma bovis* that infects circulating monocytes and tissue macrophages of domestic and wild ruminants, is also usually asymptomatic however, can cause a variety of clinical signs, including a reduction in body weight, fever, anemia, depression, lymphadenopathy, rarely abortion, and death in some cases (Noaman and Shayan, 2010). Although it has not been detected in Egypt, the DNA of *A. bovis* was reported in all domestic ruminant species i.e. cattle, sheep, and goats in Tunisia and Algeria (Ben Said *et al*., 2018b). Given the low prevalence of *A. bovis* in cattle populations in North Africa and West Asia (Noaman *et al*., 2016), a higher number of tested samples from more regions are required in Egypt.

Although cattle have been traditionally studied for *A. marginale*, buffaloes and camels can also be infected with hemoparasites from cattle and transmit them to cattle in mixed grazing areas (Sazmand *et al*., 2016; Elhariri *et al*., 2017; Sazmand *et al*., 2019). Moreover, wild species such as hedgehogs and their ticks and fleas could act as reservoirs for anaplasmosis (Khodadadi *et al*., 2021; Bezerra-Santos *et al*., 2021). Hence, for a sustainable control of anaplasmosis in Egypt, different domestic and wild mammals should be involved.

As a limitation of this study, only seropositive cattle were selected for PCR. However, it was shown that PCR is capable to detect more infected cattle (AL-Hosary *et al*., 2020). Hence, the burden of bovine anaplasmosis in the investigated areas is possibly higher. Furthermore, infection of Egyptian cattle with *A. platys*, and *A. platys*-like bacteria (AL-Hosary *et al*., 2020; Tumwebaze *et al*., 2020) and exposure of dogs to *A. phagocytophilum* and *A. platys* (Selim *et al*., 2021) suggest that various *Anaplasma* species are present in the country; so investigation of both classified and unclassified *Anaplasma* spp. in future studies in Egypt would shed light on the true incidence of bovine anaplasmosis.

This serological and molecular surveillance study showed the presence of *A. marginale* in cattle of Kafr El-Sheikh, Al-Gharbia, and Menofia. However, the connection between *Anaplasma* species and their tick vectors remains largely unknown and merits further investigation. Considering that the infection spreads

mainly through bites of ixodid ticks, effective ectoparasite control strategies, regular examination of cattle, and successful chemoprophylaxis are advocated.

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Conflicts of interest

The authors declare no conflict of interest.

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