



Prevalence and molecular analysis of *Anaplasma* and piroplasmid species infecting buffaloes in qalyubia governorate, Egypt

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ABSTRACT

This study aimed to investigate the prevalence of *Anaplasma* and piroplasmid species infecting buffaloes with regard to season, age and sex. To achieve this aim, blood samples from a total of 530 apparently healthy and clinically suspected buffaloes in Qalyubia governorate, Egypt were collected throughout the period from June 2013 to May 2014. The blood samples were smeared, stained with Giemsa and examined by conventional microscope. Moreover, PCR assay was applied to amplify DNA of *Babesia* and *Theileria* sp. in 100 microscopically negative buffaloes' blood samples. The microscopic investigation identified the infection in 37.92% of the examined buffaloes. *Anaplasma marginale* was the most prevalent species (30.94%) followed by *Babesia* sp. (26.60%) and *Theileria annulata* (2.08%). The most frequent clinical signs observed during the course of disease, in addition to the effect of seasons, age and sex on the prevalence of infection were presented in the study. PCR assay proved to be the most reliable method for the diagnosis of piroplasmid infections as compared to light microscopy, where it was able to detect *Babesia* and *Theileria* sp. DNA in 25% and 12% with 5% mixed in the microscopically negative blood samples of buffaloes respectively. The high prevalence of infection in buffaloes highlights the need for establishment of a consolidated control program to overcome the economic losses caused by these parasites.

Key words: Buffalo, Piroplasmosis, Prevalence, PCR.

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1. INTRODUCTION

A *Anaplasma*, *Babesia*, *Theileria* and are tick-borne parasites which have a diverse distribution throughout the world, mainly in tropical and subtropical countries and have a great impact on bovine production (Mehlhorn and Schein, 1984). *Anaplasma marginale* is a rickettsial hemolytic disease infecting bovine caused by *Anaplasma marginale* (*Rickettsiales: Anaplasma taceae*) (Kocan, et al., 2000). *A. marginale* can lead to death of adult animals and economic losses including decreased weight gain, abortions, bull infertility as well as costly treatment. The genus *Babesia* includes more than 100 species of pathogenic protozoa that infect RBCs of a

wide range of hosts (Spielman et al., 1985); This includes *B. bigemina* and *B. bovis* which have a significant impact on bovine health and production (Iseki et al 2010), they can result in clinical disease and carrier cases (Goff et al., 2008), However, *B. bovis* is more pathogenic than *B. bigemina* (Chaudhry et al., 2010). *Theileria parva* and *T. annulata* are the most pathogenic species infecting bovine and they are of great economic importance causing mild or severe infection to infected hosts (Altay et al., 2008 and Safieldin et al., 2010). They cause lymphoproliferative disease with high morbidity and mortality rates, generally known as tropical *theileriosis*.

Microscopy is considered the gold standard using Giemsa stained blood films for excellent demonstration of the parasites especially in acutely infected cases (Böse et al., 1995). This aforementioned method is of a low sensitivity which discourages its use in epidemiological surveys (Almeria et al., 2001). So the use of more innovative diagnostic tools are more effective (Weiland and Reiter, 1988). The aim of this current study is to investigate the prevalence of *Anaplasma* and piroplasm infections in buffaloes (*Bubalus bubalis*) using two diagnostic techniques: microscopic examination and PCR molecular tools, together with the evaluation of the effect of seasons, age and sex on the infection rate in Qalyubia governorate, Egypt.

2. MATERIALS AND METHODS

2.1. Animals

This present work was conducted throughout the period from June 2013 to May 2014. A total of 530 Egyptian water buffaloes (*Bubalus bubalis*) (129 clinically suspected and 401 apparently healthy) of different ages and sex (348 Male and 182 Female) were examined for *Anaplasma* and piroplasmid infections. Buffaloes were obtained from three localities in Qalyubia Governorate (Shebin-Elkanater, Toukh and Benha), as well as, from those admitted to the Teaching Hospital in the Faculty of Veterinary Medicine, Benha University.

2.2. Blood Samples

Blood samples were collected directly the jugular vein of each buffalo in anticoagulant test tubes containing Disodium EDTA to be used for preparing of blood smears and for PCR technique. The Blood samples used for PCR assay were stored at -20°C for DNA extraction.

2.3. Microscopic examination

A thin blood films were prepared from each sample and left to dry. The numbered smears were labeled by place of collection, date, age and sex of the examined buffaloes.

In the laboratory, the blood smears were fixed in absolute methanol for 5-7 min, stained with freshly diluted and filtered Giemsa stain for 30-45 minutes, then washed with distilled water and finally, they were air dried (Coles, 1986). The intraerythrocytic stages of *Babesia* and *Theileria* sp. were examined and measured in each slide using eye piece micrometer under 100X objective lens. Calculating the percentage of different forms of *Babesia* sp. was done by counting the different forms of *Babesia* inside 100 RBCs; the process was repeated in 20 slides and the average was taken (Baur et al., 1974). If no parasites were detected in 50 oil-immersion fields in three blood smears, the smears were recorded as negative for piroplasms (Altay et al., 2008).

2.4. Molecular methods

To evaluate the accuracy of molecular diagnosis, one hundred microscopically-negative blood samples obtained from 43 clinically suspected and 57 apparently healthy buffaloes were submitted for examination by PCR methods to amplify *Babesia* and *Theileria* sp. DNA in the tested samples.

2.4.1. DNA extraction

DNA was extracted from whole anticoagulated blood using genomic DNA kits (Peggold, Germany) according to manufacturer's instructions. Multiplex primers (Promega, USA); forward primer BAB GF2 (5-GTCTTGTAATTGGAATGATGG-3) and the reverse primer BAB GR2 (5-CCAAAGACTTTGATTTCTCTC-3) were used for amplification of 350 bp of *Babesia* and 370 bp of *Theileria* (Adaszek and Winiarczyk, 2008). In brief, each reaction mixture (50 µL) contains 1.6 mM of MgCl₂, 100 µM of each dNTP, 0.25 µM of each primer, 5 µL of DNA template and 2.5 ul of Taq polymerase. The amplification was done in a thermal cycler with the following steps: an initial denaturation for 2 minutes at 92°C, 50 cycles of denaturation for 60 seconds at 92°C, annealing for 60 seconds

at 52°C, primary extension for 90 seconds at 72° c, final extension for 5 minutes at 72° c. Negative and Positive controls were included in the assay. PCR results were assessed by ethidium bromide stained gel electrophoresis (2%) and 100-bp DNA ladder (Amersco, Cleveland Ohio, USA).

2.5. Statistical analysis

The data were statistically analyzed using chi-squared X2 test (Steel and Torie, 1981).

3. RESULTS

The microscopic examination of Giemsa stained blood smears of 530 buffaloes revealed that 88/ 129 (68.21%) clinically suspected, 113/ 401 (28.18%) apparently healthy animals were infected with *Anaplasma marginale*, *Babesia* sp., and *Theileria annulata* with an overall prevalence of 37.92%. The frequency distribution of the major clinical symptoms observed in acutely infected and carrier buffaloes were presented in Table (1) and Plate (II). *Anaplasma marginale* was the most prevalent species (30.94%) followed by *Babesia* sp. (26.60%) (*Babesia bigemina* in 12.07%, *Babesia bovis* in 8.49 % and mixed infection of both species was noticed in 6.04% of buffaloes) and *T. annulata* (2.08%) (Table.2). An overall mixed infection between different species was recorded in 27.73% (147/ 530) of buffaloes (Chart.1). The highest prevalence of *A. marginale*, *Babesia* sp. and *T. annulata* (40% and 48.89 and 8.89% respectively, including single and mixed infections) were observed in animals over 5 years old (Table 3), though no significant difference was recorded except at age of 2-5 years ($P < 0.05$). A significant variation ($P < 0.05$) was recorded in different seasons of the year, where *A. marginale* showed the highest prevalence in winter season (39.71%, representing single and mixed infections), whilst *Babesia* sp. and *T. annulata* exhibited the highest rate in summer (36.576 and 2.99% respectively, representing single and mixed infections).

Generally, blood parasites were more prevalent in winter and summer (45.59 and 43.28%, respectively) than autumn and spring (Table 4). Moreover, The highest prevalences of *A. marginale* and *Babesia* sp. were more detectable in females than males, while *T.annulata* showed complete absence of the infection among males (Table.5), nevertheless the significant difference ($P < 0.05$) was observed in *Babesia* sp. and *T. annulata*, but it was not recorded for *A. marginale*.

3.1. Morphology of the detected species

A. marginal is intra erythrocytic rounded bodies 0.29 – 1.0 µm in diameter and mainly situated on or near the margin of the erythrocyte. *B. bovis* was measured 1.6 X 1 µm and *B. bigemina*; 4X 2.1 µm, they were characterized by variable intraerthrocytic forms (doubled pear shape which constituted 32%, single pear shaped form; 23%, rounded form; 13%, amoeboid form; 10%). *Theileria annulata* was mainly diagnosed by their small size within erythrocytes, the piroplasms were mostly round (0.5-2.7µm) or oval (2X 0.5µm) in shape, these forms were more common than rod and comma (1.3X0.4 µm) shape forms which had been rarely recognized. The schizonts of *T. annulata* were circular in shape and observed both as free forms and intracellular forms in the lymphocytes within the blood smears. The detected blood species were demonstrated in Plate I.

3.2. PCR assay

It was confirmed through this study that PCR was more sensitive in detecting low level of infections in carrier buffaloes as compared to light microscopy as it was able to detect piroplasmid infections in 22/43 (51.16%) of clinically suspected buffaloes and in 20/ 57 (35.09%) apparently healthy ones, which were proved to be negatively infected using conventional microscope. *Babesia* and *Theileria* DNA were amplified in 25 and 12% of buffalo blood samples respectively. Mixed infection was noticed in 5% of buffaloes (Table.6 & Fig. 1).

Table.1: Frequency distribution of the major clinical symptoms in buffaloes suffering from *Anaplasma marginale* and Piroplasmid infections

Clinical signs	<i>A. Marginale</i>		<i>Babesia sp.</i>		<i>T. annulata</i>		B + A		B+T
	C=377	I=11	C=111	I=20	C= 2	I=5	C= 63	I=48	I=4
High temperature	-	10	-	12	-	5	-	24	4
Accelerated heart rate	-	3	2	,8	-	4	-	20	3
Increased respiration	-	1		8	-	4	-	20	3
Icterus	-	5		5	-	3	7	13	3
Haemoglobinurea	-	-		10	-	1	-	12	4
Paleness	4	-	2	16	1	5	9	13	4
Alopecia	-	-	-	-	-	1	-	-	1
Cough	-	1	-	6	-	2	1	-	2
Lymphadenopathy	-	1	-	2		5	-	-	4
Inappetance	3	11	2	12	1	5	-	10	4
Recumbency	-	2	-	3	-	2	-	7	1
Corneal opacity	-	-	-	-	-	5	-	-	4
Salivation	-	1	1	-	-	1	-	-	1
Bloody feces	-	8		-	-	2	-	1	-

Abbreviations: C= Carrier I = acutely infected, B: *Babesia*, T: *Theileria*, A: *Anaplasma*.

Table (2) Single and mixed infection of *A. marginale* and piroplasmid species among examined buffaloes

Species	No. infected N= 530	Single infection N=530	Mixed infection N=530
<i>A. Marginale</i>	164 (30.94)	53 (10)	111 (20.94)
<i>Babesia sp.</i>	141 (26.60)	26 (4.91)	115 (21.70)
<i>T. annulata</i>	11 (2.08)	7 (1.32)	4 (0.75)

Values in parentheses represent percentage

Table (3) Prevalence of *A. marginale* and piroplasmid infections among different age groups of buffaloes.

Age(year) Total	<i>A. marginale</i>	<i>Babesia sp.</i>	<i>T. annulata</i>	B+A	B+T
<1 39	4 (10.26)	5 (12.82)	0 (0.00)	5 (12.82)	0(0.00)
1-2 340	30 (8.82)	5 (1.47)	0 (0.00)	76(22.35)	0(0.00)
2-5 106	13 (12.26)	7(6.60)	4 (3.77)	18 (16.98)	3 (2.83)
>5 45	6 (13.33)	9 (20)	3 (6.67)	12 (26.67)	1(2.22)
Total 530	53 (10)	26 (4.91)	7(1.32)	111(20.94)	4 (7.54)

Values in parentheses represent percentage, Abbreviations: B= *Babesia*, T=*Theileria*, A= *Anaplasma*. Statistical analysis using chi-squared X² is considered significant at P< 0.05.

Table (4) Seasonal prevalence of *A. marginale* and piroplasmid infections among buffaloes.

Season	Total	<i>A. marginale</i>	<i>Babesia</i> <i>sp.</i>	<i>T.annulata</i>	B+A	B+T	Total
Winter	136	35 (25.74)	6 (4.41)	0 (0.00)	19(13.97)	2 (1.47)	62 (45.59)
Spring	131	7 (5.34)	7 (5.34)	1 (7.63)	24 (18.32)	2 (1.53)	41 (31.30)
Summer	134	5(3.73)	6 (4.47)	4 (2.99)	43 (32.09)	0 (0.00)	58(43.28)
Autum	129	6 (4.65)	7 (5.43)	2 (1.55)	25 (19.38)	0 (0.00)	40 (31.01)
Total	530	53 (10)	26 (4.91)	7 (1.32)	111 (20.94)	4 (0.75)	201(37.92)

Values in parentheses represent percentage, Abbreviations; B: *Babesia*, T: *Theileria*, A: *Anaplasma*. Statistical analysis using chi-squared X2 is significant at $P < 0.05$.

Table (5) Effect of sex on the prevalence of *A. marginale* and piroplasmid infections among buffaloes.

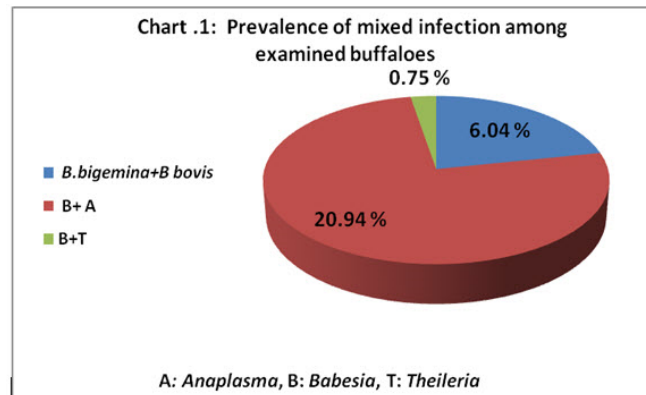
Sex	<i>A. marginale</i>	<i>Babesia</i> <i>sp.</i>	<i>T. annulata</i>	B+A	B+T	Total	
Total							
Female	182	23 (12.64)	21 (11.54)	7(3.85)	37(20.33)	4 (2.20)	92(50.55)
Male	348	30 (8.62)	5 (1.44)	0 (0.00)	74 (21.26)	0 (0.00)	109 (31.32)
Total	530	53 (10)	26 (4.91)	7 (1.32)	111(20.94)	4 (0.75)	201 (37.92)

Values in parentheses represent percentage, Abbreviations; B: *Babesia*, T: *Theileria*, A: *Anaplasma*. Statistical analysis using chi-squared X2 is significant at $P < 0.05$.

Table (6) Piroplamid species detected by PCR analysis of microscopically negative blood samples obtained from buffaloes.

Parasites	Clinically suspected N= 43	Apparently healthy N=57	Total N=100
<i>Babesia</i> <i>sp.</i>	13 (30.23)	12 (21.05)	25 (25)
<i>T. annulata</i>	7 (16.28)	5 (8.77)	12(12)
B+T	2 (4.65)	3 (5.26)	5(5.00)
Total	22 (51.16)	20 (35.09)	42 (42.00)

Values in parentheses represent percentage. Abbreviations; B: *Babesia* *sp.*, T: *Theileria* *sp.*, N: Number examined



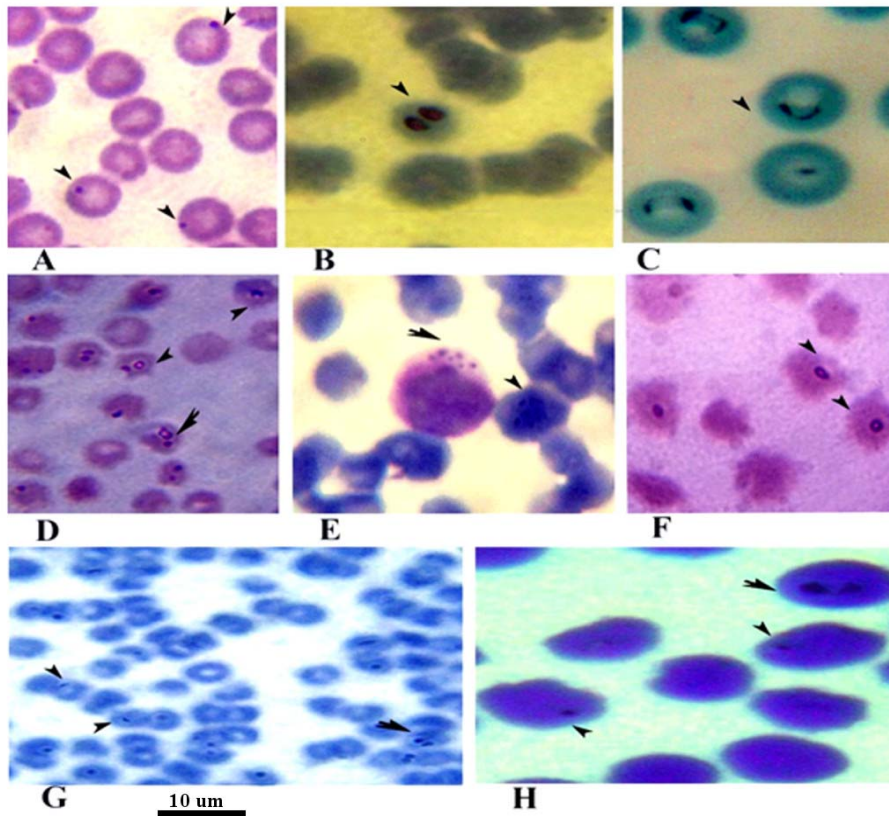


Plate I: A: *A. marginale*, B: *B. bigemina*, C: *B. bovis*, D: different forms of *Babesia* sp., E: Schizont of *T. annulata* in the macrophages (arrow) and multiplying form in RBCs (arrow head), F: different forms of *T. annulata* (round and oval), G: mixed infection *T. annulata* (arrow head) and *B. bovis* (arrow), H: mixed infection *A. marginale* and *B. bovis*.

4. DISCUSSION

In Egypt, tick borne diseases caused by *A. marginale*, *B. bigemina*, *B. bovis* and *T. annulata* are one of the major constraints to livestock and cause serious health problems as reducing of animal productivity and economic losses. The results revealed that *A. marginale* had the highest prevailing rate (30.94%) among the examined buffaloes. The peak of *A. marginale* was in winter season (39.71%). These findings were similar to that observed by Alim et al. (2011) in Pakistan. A lower infection rate was noted by Sajid et al. (2014) (4.17%) and a higher infection rate was encountered by Abou-Elnaga (2005) (59.3%). The prevalence *A. marginale* was significantly higher ($P < 0.05$) at age group of 2-5 years,

which may be attributed to the age resistance which lasts up to 12 months and as the animals get older, become more susceptible to infection (Abou-El-Naga, 2005). In this study, *Babesia* sp. were observed in 26.60% of animals, only two species were identified (*B. Bigemina* in 12.07% and *B. bovis* in 8.49%, mixed infection of both species was noticed in 6.04% of buffaloes). A nearly similar result was obtained by El-Fayomy et al. (2013) (23%) in Port said. A lower incidence of *B. bigemina* was investigated by Rania (2009) (10.44%) in Qalyubia, whereas a higher incidence of *B. bovis* was observed by Mahmmud (2012) (39.8%) in Sharkia governorate. This difference could be explained by the fluctuation of parasitemia in the chronic phase of *Babesia* infection (Gubbels et al., 1999).

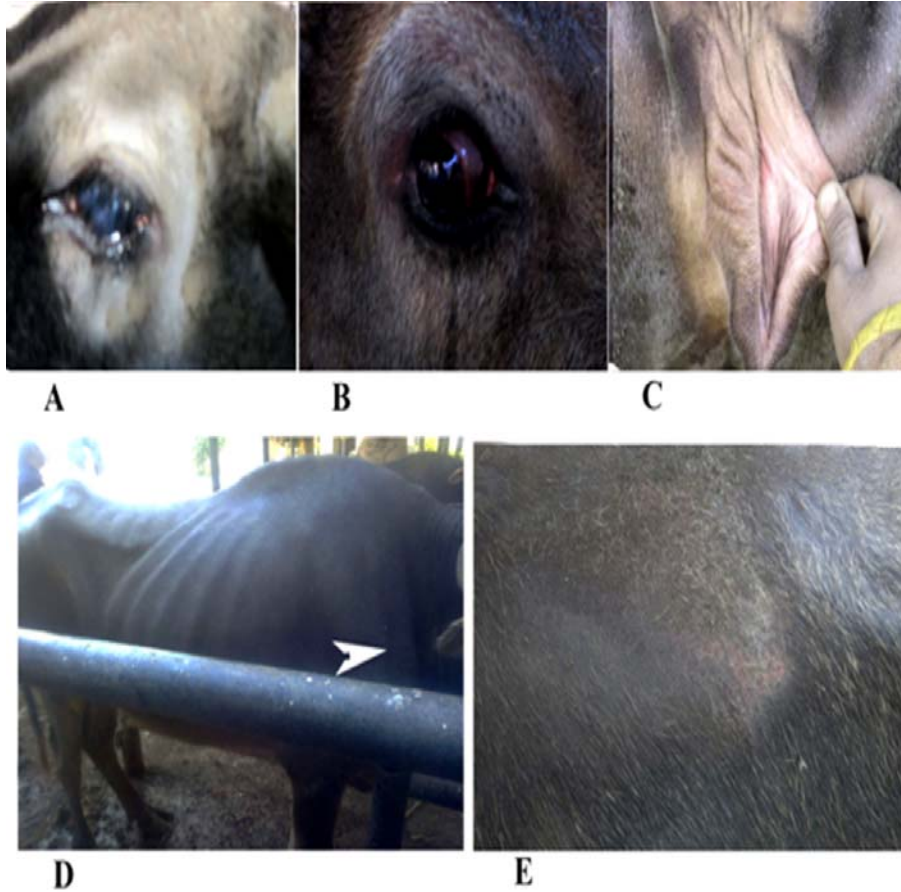
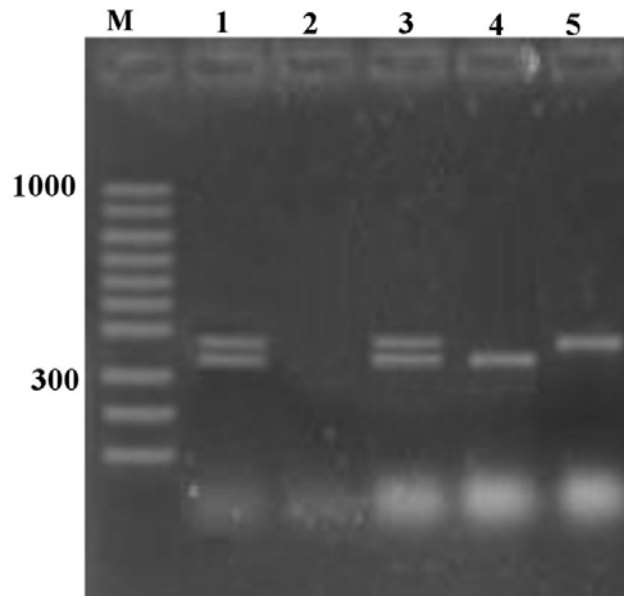


Plate II: The clinical symptoms recorded in clinically infected buffaloes. A: Corneal opacity, B: Lacrimation and redness of eye, C: Paleness of mucous membrane of vagina, D: Emaciation and enlargement of lymph node, E: Skin alopecia.



Giemsa stained thin blood smears revealed different forms of *Babesia*, with the

dominance of the double pear shape form (32%) inside erythrocytes. Those were

morphologically similar to that formerly described by Homer et al., (2000), Bhikane et al., (2001), Ali (2005) and Rania (2009).
 Fig. 1: PCR assay for *Babesia* and *Theileria* sp. M: DNA ladder (100 bp), lane 1: Positive control for *Babesia* and *Theileria* sp., lane 2: Negative control, lane 3: Positive mixed infection, lane 4: Positive *Babesia* sp., Lane 5: Positive *Theileria* sp.

A significantly higher infection ($P < 0.05$) of *Babesia* sp. in summer season (36.57%) could be explained by the highest abundance of the ticks in the month of July (Sajid et al., 2008). This observation agreed with that previously reported by Alim et al., (2011). Conversely, El Moghazy et al. (2014) established a highest infection rate for *Babesia* in autumn in the same area of study. This variation may be caused by the frequent use of acaricides and antipiroplasmid drugs by the owners at intervals that has an effect on the parasite prevalence in different seasons (Safiedin et al., 2010).

An observed increase of *Babesia* infection with increasing animal age, where a significant difference was recorded at age of 2-5 years ($P < 0.05$). This was mainly due to the postponed infection caused by restriction of calf movement by keeping them indoors, which is practiced by 86.5% of the farmers (Rubaire-Akiiki, 2004). Females were significantly ($P < 0.05$) more susceptible (34.06 %) to *Babesia* sp. than males (22.70%), this came in agreement with the results of Alim et al. (2011) and Atif et al. (2012).

The low prevalence of *T. annulata* recorded in this study (2.08%) were consistent with other studies in Egypt (El-Moghazy, 2011 and Elsify et al., 2015), while a higher incidence was reported by Ghanem et al. (2013) (23.82%). The prevalence of *Theileria annulata* was significantly higher ($P < 0.05$) during summer (2.99%), this was previously observed by El Mentenawy, (2000) and Alim et al., (2011) in cattle, while El-Moghazy (2011) reported a higher rate among buffaloes in spring. It was also noted in the study that adult buffaloes were more prone to *theileriosis* than their calves,

which as well be explained by the age dependent immunity to *Theileria* and *Babesia* sp. infection (Norval et al., 1992 and Mahoney and Ross, 1972). The absence of *Theileria* infection in male buffaloes investigated in this study as compared to female could be ascribed to the immunosuppression status of female during pregnancy and/ or lactation (Kocan et al., 2010). *T. annulata* piroplasms were identified by their round and oval shape forms which were more common than rod and comma shapes, which are the common forms of *T. parva* infection (Koch, 1898).

The molecular prevalence based on PCR amplification of *Babesia* and *Theileria* DNA in the blood of 100 buffaloes proved to be microscopically negative, showed a positive infection in 25% and 12% of buffaloes respectively and mixed infection was noticed in 5% of buffaloes. The high infection rate of *Babesia* sp. as compared to *Theileria* sp. could be attributed to carrier animals, which are known to exhibit fluctuating low parasitemia which sometimes even escapes detection (Ros-García et al; 2013). Generally, PCR was more sensitive for the detection *Babesia* and *Theileria* infections in subclinical, acutely infected and carrier animals, which were difficult to be detected by conventional microscope.

5. CONCLUSIONS

The high prevalence of *Anaplasma marginale* and piroplasmid infections, besides, the presence of the infection among apparently healthy animals indicates that these diseases are prevalent among buffaloes in Qalyubia governorate, Egypt, with the occurrence of carrier cases which act as a main source of infection for ticks. PCR assay provided more accurate data on the prevalence of *Babesia* and *Theileria* infections, allowed direct identification of carrier cases and diagnosis of piroplasmosis in animals in the early phase, which is very helpful for epidemiological investigations and surveillance programs.

6. ACKNOWLEDGMENT

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