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First Serological Detection of *Theileria Annulata*in Buffaloes in Iraq

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Abstract

In Iraq, no data are available about the prevalence of *Theileria annulata* in buffaloes. For this reason, 184 blood samples were collected from buffaloes of different areas in Maysan province (Iraq) during June and September (2020), to be subjected for microscopic and serological testing using ELISA. The findings revealed that the total positive animals were 6.52% by microscopy and 16.85% by ELISA. Among seropositives, mild infection was increased significantly when compared to moderate and severe infections. Concerning sex factor, no significant differences were detected between females and males by microscopy, however, ELISA were revealed a significant increase in positive females compared to males. In seropositive females, there were significant increases in values of ELISA in comparison to those reported by light microscopy; while in males, no significant variation was showed between values of microscopy and those of ELISA. Regarding age factor, significant higher seropositives were reported in buffaloes aged ≥ 2- < 6 years by microscopy and ELISA when compared to buffaloes aged < 2 years and ≥ 6 years. Significantly, higher rates of prevalence were reported among all age's groups by ELISA when compared to microscopic results. In conclusion, this appears to be the first serological study concerned serological investigation of *T. annulata* in buffaloes in Iraq. Therefore, additional studies using advanced diagnostic techniques are of great importance to detect the actual prevalence of infection among buffaloes of other Iraqi provinces.

Keywords: *Theileria annulata*, Bovine, Enzyme-linked immunosorbent assay, Risk factor

Introduction

In ruminants, the genus of *Theileria* consists of tick-borne haeoprotozoan parasites, among them;*T. annulata* infects and circulates in cattle and buffalo causing a disease called as bovine theileriosis that more prevalencethroughout a wide geographical regionsworldwide (Bilgic et al., 2010; Ullah et al., 2021). Theielriosis can transmit by many types of hard tick (Ixodidae), andcharacterized clinically by fever, enlarged lymph nodes, jaundice and sometimes death (Ali et

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al., 2013; Abdel Rahman and Ismaiel, 2018).In endemic countries, substantial economic lossfor dairies and livestock industries can occur due to decreasing milk production, losing weight and increasing sensitivity to secondary bacterial and fungal infections (Gharbi et al., 2006; Inci et al., 2007).

Worldwide, bovine theileriosis is one of the most common tick-borne diseases with several outbreaks noticed each year. Hence, accurate and early detection of infection is cruciallyto controllingthe disease. In clinically acutely infections, microscopic examination is applied usually to detect eitherthe piroplasms in Giemsa-stained blood smear or macroschizont in lymph node biopsies (Shayan and Rahbari, 2005; Dehkordi et al., 2012). This diagnostic technique is not enough in sensitivity to allowingareliabledetectionthe parasite in chronic carriers (Sharifi et al., 2016). Alternatively, several serological tools were developed to behigh in sensitivity and specificity than microscopic identification of *T. annulata* infections such as such as indirect fluorescent antibody (IFA) technique (Salih et al., 2007), and different types of enzyme-linked immunosorbent assay (ELISA) (Mans et al., 2015; Sumbria et al., 2016; Mohmad et al., 2018).

In middle and southern parts of Iraq, riverine-type of buffalo (*Bubalus bubalis*) represents a fundamental and irreplaceable resourcefor milk production and secondarily for meat. Some researchers suggested that buffaloesmight be more resistance to tick-borne infections due to their low-exposure chance to tick infestation and long-existence time in water (Benitez et al., 2012; Obregón et al., 2020).Other studies were demonstrated exposure of buffaloes to tick- invasion and the possibility of transmission of different pathogens through these external parasites (Anderson et al., 2013; Alkefari et al., 2017). In Iraq, there were no previously or recently online available data about the prevalence of *T. annulata* in buffaloes.Hence, this study appear to be the first one aimed for microscopic and serological diagnosis of theileriosis caused by *T. annulata*, and evaluation association of positive rates of infection to animal risk factors included age and sex.

Materials and methods

Study samples

A total 184 buffaloes of different sexes and ages were selected randomly from different areas in Maysan province (Iraq) during June and September (2020). Under aseptic conditions, each study animal was subjected for draining 5 ml ofjugular venous blood into a vacutainer-glass tube that coated with EDTA as an anticoagulant,and transported to the laboratory under cooling

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condition using an icebox. At laboratory, the tubes of blood were centrifuged (5000 rpm, 15 minutes) and the obtained sera were kept frozen into labeled eppendorf tubes until be used for serology by ELISA. The slides of blood smear were directly prepared, air-dried, fixed with the absolute methyl alcohol (BDH, England), air-dried, and transported to the laboratory using slide box to be stained with Giemsa.

Microscopic examination

Following the method described by Al-Abedi and Al-Amery (2020), the slides of blood smear were stained by diluted Giemsa satin (BDH, England), air-dried, and examined under light microscopic using the oil immersion lens at 100×.

Serological testing

According to the manufacturers' instructions of Bovine Theileria ELISA Kit (SunLong Biotech, China), the serum samples, reagents and microelisa stripplates were prepared and processed. Absorbancewas measured at an optical density (OD) of 450 nm using the Microplate Photometer Reader (BioTek, USA).The OD of blank well was set at zero, and then, the test effectiveness was determined. The critical value (CUT OFF) was calculated at 256 nm. To detect the severity of infection, the positive ODs were divided into three categories as weak (256-349 nm), moderate (350-499 nm) and strong (≥500 nm).

Statistical analysis

Using the GraphPad Prism version 6.0.1 (GraphPadSoftware Inc, USA), results of microscopy and serology were compared statistically by the Chi-Square (x^2) test; whereas, values for levels of positive ODs were compared using One-Way ANOVA. Correlation between positivity and the animal risk factors, sex and age, were analyzed using the Odds Ration. Differences between values were considered significant at a value of $P<0.05$ (Petrie and Watson, 2013; Al-Gharban, 2016;Kim, 2017).

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Results

Out of 184 blood smears examined by light microscopy, 12 (6.52%) were positive for *Theilria* infection, whereas 31 of 184 (16.85%) buffaloes were seropositiveby ELISA (Table 1, Figure 1).

Table (1): Total results of diagnostic assays applied for detection of *T. annulata* in buffaloes

Total No.	Diagnostic assay	Positive		p-value
		No.	%	
184	Microscopy	12	6.52	0.038
	ELISA	31	16.85 *	

Significance * (P<0.05)

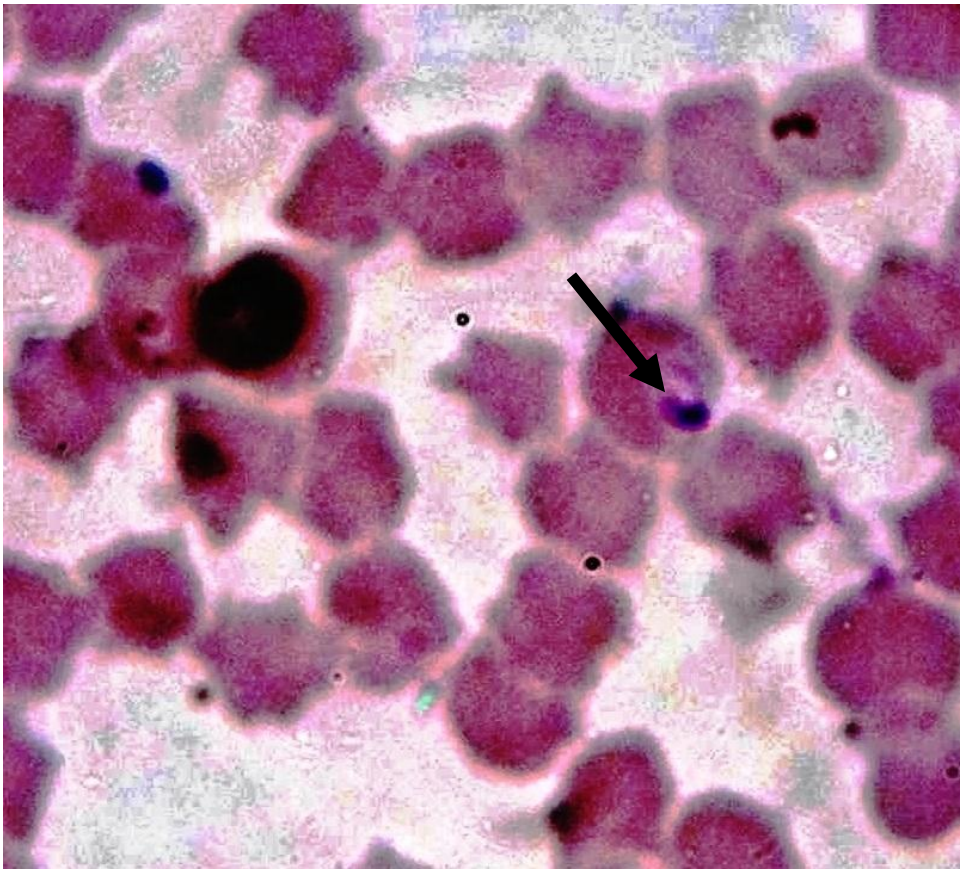

Figure (1): Positive blood smears for *Theileria* infection (Giemsa stain, 100×)

Among positive samples by ELISA, significant increases (P≤0.21) in mild infection (58.07%) were seen in comparing with the moderate (29.03%) and severe (12.9%) infections (Table 2).

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Table (2): Severity of infection based on OD values of positive buffaloes

Category	Positive	p-value
Mild	18 (58.07%) *	
Moderate	9 (29.03%)	0.21
Severe	4 (12.9)	
Total No.	31 (16.85%)	-

Significance * (P<0.05)

In this study, distribution of positive findings among groups of sex and age of study animals were showed a significant variation (P<0.05). Concerning sex factor, no significant differences (P≤0.083) were detected between females and males by microscopy,6.38% (9/141) and 6.98% (3/43), respectively (Figure 2).However, the findings of ELISA were revealed a significant elevation (P≤0.041) in values of positive females, 16.77% (27/141), compared to males,9.3% (4/43) (Figure 3).

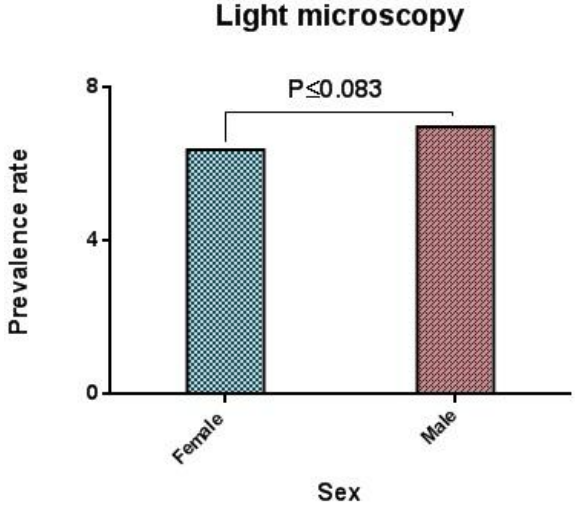

Figure (2): Distribution of positive results by light microscopy among females and malesof study buffaloes

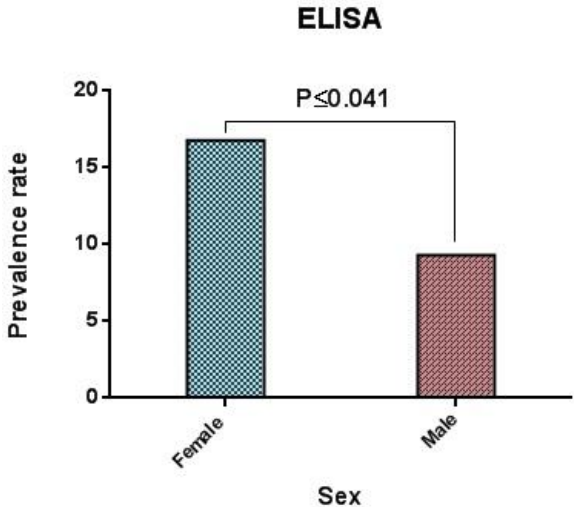

Figure (3): Distribution of positive results by ELISA among females and malesof study buffaloes

Among female buffaloes, there were significant increases (P≤0.032) in positive values of ELISA, 16.77% (27/141), in comparison with those reported by light microscopy, 6.38% (9/141), (Figure 4). In males, no significant variation (P≤0.059)was showed in values of light microscopy, 6.98% (3/43), and those of ELISA, 9.3% (4/43), (Figure 5).

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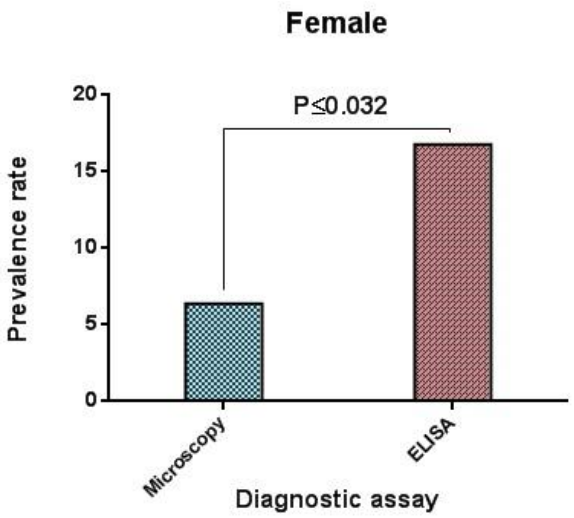

Figure (4): Distribution of positive results by light microscopy and ELISA among femalesof study buffaloes

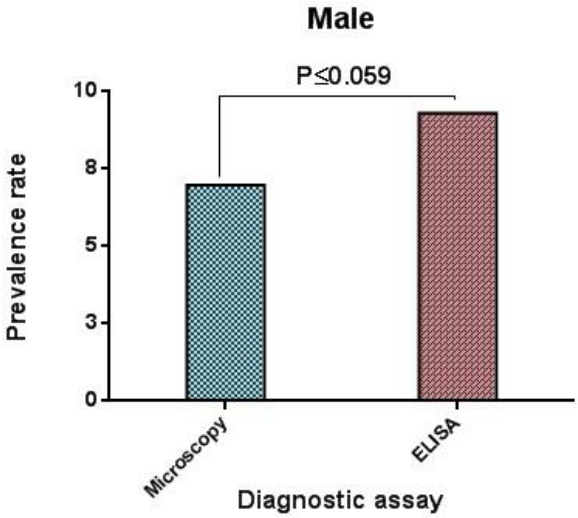

Figure (5): Distribution of positive results by light microscopy and ELISA among malesof study buffaloes

Regarding age factor, significant differences (P<0.05) were observed in rate of prevalence of positivity among the three age groups: < 2 years (total No. 51), ≥ 2- < 6 years (total No. 35) and ≥ 6 years (total No. 98). Significantly, higher positive findings were reported in buffaloes aged ≥ 2- < 6 years by light microscopy (14.29%) and ELISA (34.29%) when compared to other age groups; < 2 years (0% and 5.88%, respectively) and ≥ 6 years (7.14% and 16.33%, respectively), (Figures 6, 7).

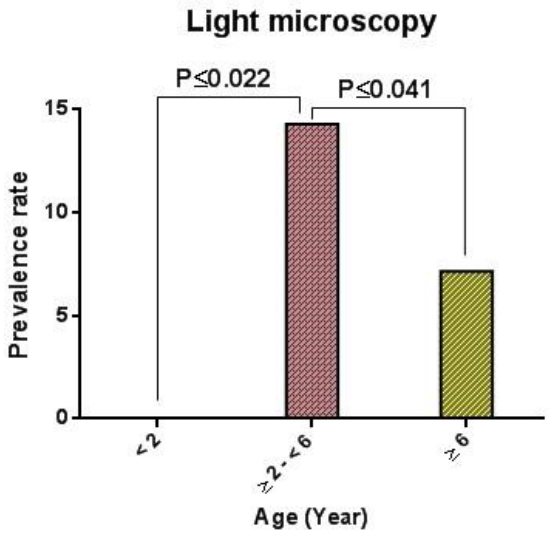

Figure (6): Distribution of positives by light microscopy among different age groupsof study buffaloes

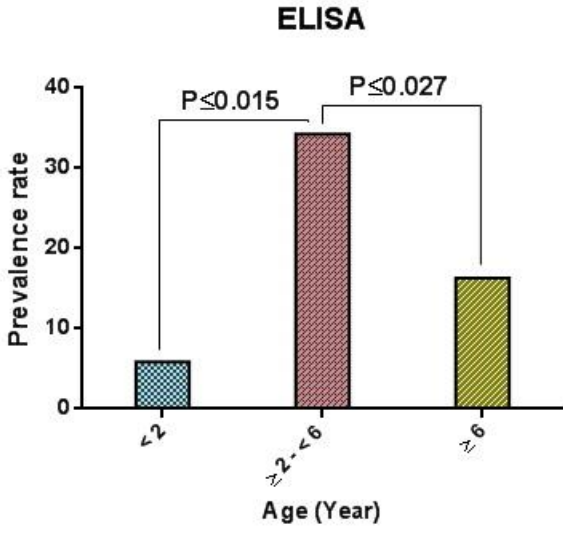

Figure (7): Distribution of positives by ELISA among different age groupsof study buffaloes

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In comparison between the applied diagnostic assays, significant increases (P<0.05) were reported in ELISA results among buffaloes aged < 2 (5.88%), ≥ 2- < 6 (34.29%) and ≥ 6 (16.33%) years when compared to light microscopic results (0%, 14.29% and 7.14%, respectively), (Figures 8-10).

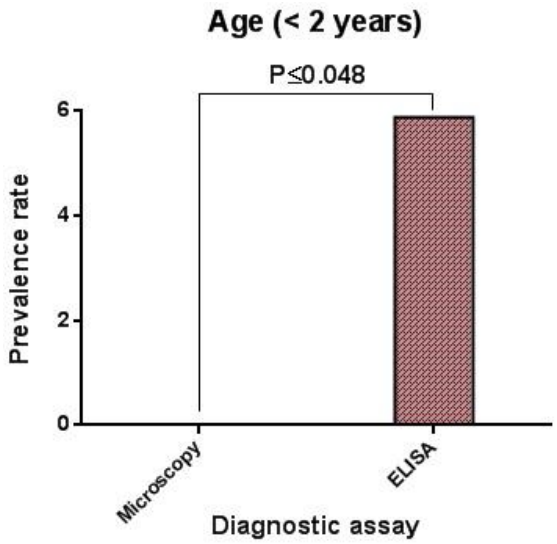

Figure (8): Distribution of positive results by light microscopy and ELISA among study buffaloes aged < 2 years

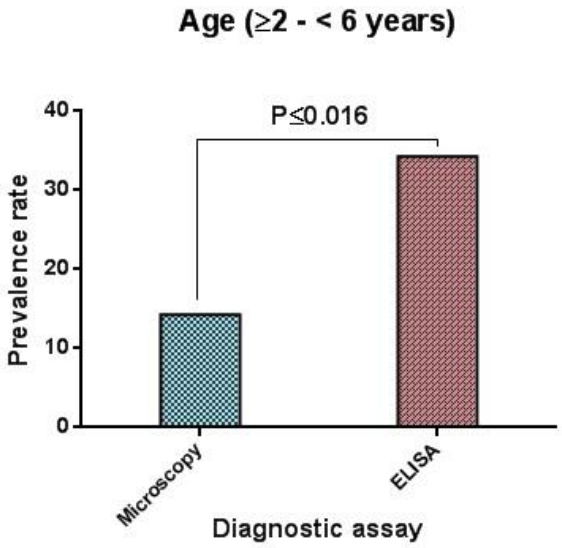

Figure (9): Distribution of positive results by light microscopy and ELISA among study buffaloes aged ≥ 2-<6 years

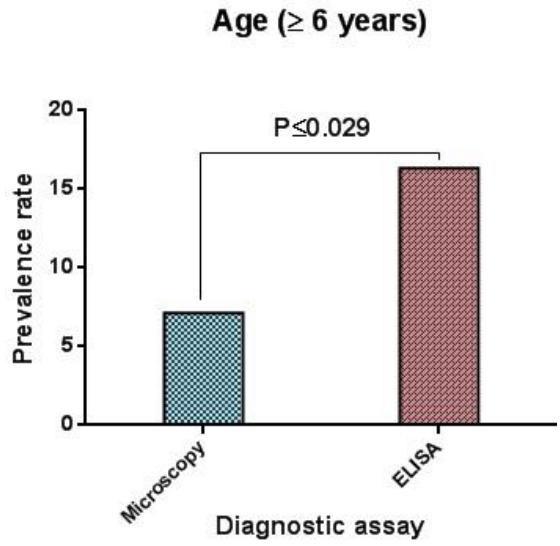

Figure (10): Distribution of positive results by light microscopy and ELISA among study buffaloes aged ≥ 6 years

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Discussion

Buffalo farming is a key activity and an important part of livestock sector, which plays a crucial role in the agricultural economy of Iraq. Tick-borne pathogens including *T. annulata* are endemic in tropical and subtropical regions of the world such as Iraq and constitute a potential threat to livestock farming. This study has identified and reported *T. annulata* infection in buffalo's population from the study area based on blood smear microscopy and ELISA. Giemsa-stained blood smears microscopic technique revealed on overall prevalence rate of 6.25% for theileriosis. In comparison to findings of other studies, microscopic prevalence of theileriosis in buffaloes was 12.8% in Pakistan (Ullah et al., 2021), 9.31% in Egypt (El-Dakhly et al., 2018), 3.04% in Iran (Soosaraei et al., 2018) and 1.6% in India (Bhosale et al., 2020).

In this study, the findings of light microscopy were revealed a significant lowering in comparison to serological testing by ELISA. These results might be explained by the low levels of parasitemia or due to the role of buffaloes as carrier for theileriosis. Worldwide, acute phase of the infection, when the level of parasitemia is high, is easier to diagnose the infection by microscopy for thin blood smear stained by Giemsa that consider as the most common traditional method for diagnosis of blood parasites (Çöl and Uslu, 2007; Eamens et al., 2013). Many studies demonstrated that the smear method is associated with technical problems and even wrong diagnosing and has low sensitivity in diagnosing carrier animals (Nayel et al.,2012; Nourollahi-Fard et al., 2015). However, serological assays are more suitable for the diagnosis of the disease during the chronic phase of the infection, where the animals serve as a carrier that having a high titers of specific antibody against *T. annulata* while the level of parasitemia is low and microscopically hardly undetectable (Salihet al., 2007; Al-Hosary et al., 2015; Gargano et al., 2021).

Significant prevalence of mild infection among seropositive animals observed in this study might be attributed to low exposure of buffaloes to tick infestation, thick skin and difficulties in incidence of infection, genetic-resistance of buffaloes to haemoparasitic infections. Wang et al. (1999) summarized that clearance of trypanosomes from the blood of infected buffaloes with the development of responses; complement-independent activity that was not restricted by clone or species, and complement-dependant and clone-specific lytic activity.

An association of animal risk factors to results of light microscopy and ELISA was showed a significant difference in their values. In concerning with sex factor, the light microscopic results

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showed that the rate of prevalence of infection among females and males was differed insignificantly. This may be caused by the low numbers of examined male buffaloes, or to that both sexes are exposed to same level of infection. However, the findings of ELISA found that females were exposed greatly to *T. annulata* infection than males, which in agreement with that detected by other researchers in cattle (Tuli et al., 2015; Goyal, 2018; Ziam et al., 2020). This may be explained by the fact that the milch animals have higher hormonal stress, carry more ticks, stress due to milk production and calving, and are at higher risk of exposure to the infection during milking time.

In regarding to age factor, our study microscopic and serological findings reported that the higher rates of infection were seen in adult buffaloes aged ≥ 2-< 6 years than younger (<2 years) and elderly (< 6 years) buffaloes. Similar results were reported by other studies, in which, these findings attributed to a natural resistance in younger buffaloes in particular those aged 6-9 months, the absence of exposure to exophilic ticks associated with low infestation rates, and due to colostral immunity (Figueroa et al., 2010;Ziam et al., 2020). On the other hand, a lower prevalence rate of theileriosis in older animals may be due to their multiple recurrent infections and the development of concomitant immunity during their lifetime (Ilhan et al., 1998; Gharbi and Darghouth, 2014).

Conclusion

For our knowledge, thisrepresents the first serological study concerned with the serological investigation of *T. annulata* in buffaloes in Iraq. Itappears that microscopic description of *Theileria* spp. solely on the basis of morphology is challenging task and should be supported by a more sensitive and reliable diagnostic alternative assay. ELISA showed a high efficacy in detection of carrier animals as well as in identification the prevalence rate of theileriosis among apparently healthy buffaloes. Therefore, additional studies using advanced diagnostic techniques are of great importance to detect the actual prevalence of infection among buffaloes of other Iraqi regions.

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