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Original Article

Molecular and microscopical identification of bovine *Theileria* species isolates in Sharkia Governorate, Egypt

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Abstract:

Theileria species are tick-borne protozoal parasites that infect many domestic and wild animals worldwide. *Theileria annulata* (*T. annulata*) is the most economically important species affecting cattle and buffaloes. This work was aimed to detect the prevalence of *Theileria species* in cattle and buffaloes during the period extended from January 2018 till December 2018 at different localities of Sharkia Governorate, Egypt. Blood samples were collected randomly from 174 (86 cattle and 88 buffaloes) apparently healthy and clinically infected animals. Samples were examined microscopically using Giemsa stained blood films. The prevalence of *T. annulata* was 34.88% and 32.95% in cattle and buffaloes respectively. *T. annulata* infection was higher in male (44.68% &48.08%) than female (23.08% &11.11%) in cattle and buffaloes respectively. Furthermore, *T. annulata* prevalence infection was higher in old animal (>6 years) (61.29% &48.48%) than 1-6 years (25% &37.5%), 1- 2 months (18.18% & 20%) and 3-12 months (16.67% &21.62%) in cattle and buffaloes respectively. The tick infestation status showed a potential risk factor in developing infection. The PCR product length was 721 bp from *T. annulata* positive samples using the specific primer. Sequencing of the PCR products and subsequent blast analysis to detect their identities were clarified with the previous studies.

Key words Cattle and buffaloes, *Theileria annulata*, prevalence, PCR, Phylogenetic analysis

INTRODUCTION

Theileriosis caused by *Theileria* species is a protozoal disease transmitted by the tick, which infect domestic ruminants and wild bovine. *Theileria annulata* is one of the most economically significant species that transmitted by *Hyalomma* tick species, which highly spread from northern Sudan and the Mediterranean countries to the Middle East, India, southern Asia and China **(Delves, 1998, Zachary, 2017 and Mullen and Durden, 2019).**

Recording to *Theileria* species life cycle; the sporozoites which formed during the cyclical development in ticks was beening injected with saliva of tick into the mammalian host. They develop in white blood cells into schizonts and then piroplasms (merozoites) in red blood cells **(Constable et al., 2017).**

Clinically, the diseased animals show the signs of depression, lacrimation, diarrhea, anorexia and weight loss. The most common observed signs are abortions, corneal opacity, severe pulmonary edema with dyspnea and a frothy nasal discharge. The superficial lymph nodes enlargement was associated with these signs. Due to the destroying of erythrocytes by the parasites, theileriosis is characterized by icterus, anemia and occasionally hemoglobinuria **(Maxie, 2015)**.

The diagnosis of *T. annulata* infection depends on clinical findings and microscopic examination of Giemsa-stained blood smears. Recently, Polymerase Chain Reaction (PCR) has been reported as the best diagnostic tool for the detection of *T. annulata* due to its higher sensitivity and specificity than any other techniques **(Shahnawaz et al., 2011)**.

The aim of our study was detecting the prevalence of *Theileria* species and risk factors (age, sex and tick infestation) associated with the infection in cattle and buffaloes at different localities of Sharkia Governorate, Egypt. In addition, molecular and phylogenetic analyses of isolated species were performed. The genetic character of the isolates and their phylogenetic relationships with the available sequences on the Gen Bank was also carried out.

MATERIAL AND METHODS

Study area and blood samples collection:

A study was conducted on different localities of Sharkia Governorate (Zagazig, Deyrb Negm and Al-Ibrahimia), Egypt **(Figure 1)** from a period extended from January 2018 till December 2018. blood samples (n=174) were collected from apparently healthy and diseased cattle (n = 86) and buffaloes (n = 88); their age ranged from (1-2 months to over 6 years). Diseased animals showing clinical signs of theileriosis including fever (41°c), enlargement of lymph nodes, sudden decrease in milk production, tick infestation, emaciation, corneal opacity, bilateral nasal discharge and lacrimation. Blood samples were collected on EDTA as anticoagulant (1 mg/ml) from jugular vein and marked with numbered labels in the field. Sex (male and female), age and tick infestation status were recorded. The Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt agreed with the protocol of the present work.

Parasitological examination:

Thin blood smears were performed from collected blood samples. They were stained using Giemsa stain according to the method described by **Soulsby (1982).** The rest of blood samples were preserved for DNA extraction at (-20°C).

Molecular identification of *Theileria* species:

DNA extraction:

The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used for DNA extraction from examined blood samples. At 56^{O}c for 10 min, 200 µl of the blood sample suspension was incubated with 200 µl of lysis buffer and 10 µl of proteinase K. Then 200 µl of 100% ethanol was put to them. The sample was washed and centrifugated according to the manufacturer's recommendations. Finally, 100 µl of elution buffer provided in the kit was used for elution the nucleic acid.

DNA amplification by polymerase chain reaction (PCR):

After DNA extraction, Polymerase Chain Reaction (PCR) was done in An Applied Biosystems 2720 Thermal

Cycler. *T. annulata* 30 KDA gene fragment was amplified using *T. annulata* specific primer (Metabion, Germany) (forward primer; 5'-GTAACCTTTAAAAACGT -3', and reverse primer: 5'-GTTACGAACATGGGTTT -3'). *T. annulata* specific primer were analysed in a 25- µl reaction consisting of 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water and 6 µl of DNA template. The PCR reaction was made in a 25- µl containing 6 µl of template DNA, 1 µl of each primer of 20 pmol concentrations, 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan) and 4.5 µl of nuclease-free water. The reactions were started at 94˚C for5 min., 35 cycles of 94˚C for 30 s, 55˚C for 40 s, 72˚C for 45 s and finally, the extension step was done at 72˚C for 10 min. In each PCR run, *T. annulata* DNA (positive control) and sterile distilled water (negative control) were involved **(Nourollahi-Fard et al., 2015)**.

Analysis of the PCR Products:

By electrophoresis, the products of PCR including the control positive and control negative were separated on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients at 5V/cm. For gel analysis, 15 µl of the products were loaded in each gel slot. For determination the fagment size, gel pilot 100 bp ladder (Qiagen, Germany, GmbH) and a gene ruler 100 bp ladder (Fermentas, Germany) were used. For photographing the gel, a gel documentation system (Alpha Innotech, Biometra) was used. The data was analyzed using computer software.

Molecular sequencing and phylogenic analysis:

From the positive samples, only the highest bright and thick band was used for DNA sequen Biosystems 3130 Genetic Analyzer (HITACHI, Japan), the sequences similarity was established to by Gen Bank accessions, a BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul et al., 1990)** was initially used. The phylogenetic tree was established by the Meg Align module of Laser gene DNA Star version 12.1 **(Thompson et al., 1994).** The maximum likelihood, neighbor-joining and maximum parsimony in MEGA6 were used for phylogenetic analysis **(Tamura et al., 2013).**

Results

Blood smear examination:

The overall prevalence of *Theileria* sp. infection was 33.91% including 34.88% in cattle and 32.95% in buffaloes **(Table 1)**. *T. annulata* piroplasm occurred as round, oval or ring shaped forms, rod shapes and commas forms in erythrocytes. These forms undergo binary fission in erythrocytes **(Figure 2)**. This work was revealed to some risk factors affecting on the *T. annulata* infection prevalence. In association to animal's sex, *T. annulata* infection in males (44.68% &48.08%) was more than females (23.08% &11.11%) in cattle and buffaloes respectively. According to the age of animal higher prevalence was recorded in old animal (>6 years) (61.29% &48.48%) than 1-6 years (25% &37.5%), 1-2 months (18.18% & 20%) and 3-12 months (16.67% &21.62%) in cattle and buffaloes respectively. The tick infestation status showed a potential risk factor in developing infection **(Table 2).**

Polymerase chain reaction:

In the current study the PCR based technique using the specific primer 30KDA for molecular confirmation of *T. annulata* infection, the finding showed that, the PCR product length was 721 bp was obtained in *T. annulata* positive samples **(Figure 3)**.

Sequencing and phylogenic analysis:

Sequencing of the PCR products and subsequent blast analysis to detect their identities with the previous studies was done. The sequences of DNA have been put in Gene Bank using accession numbers MN251047 and MN251046 for 30 KDA in cattle and buffaloes, respectively. Phylogenetic analysis grouped the Egyptian (MN251047)_*T. annulata* _cattle_ 2019 isolate and (MN251046)_*T. annulata* _buffalo_ 2019 isolate in a separate clade and shared 100% genetic similarity with other *T.annulata* strains (GenBank Accession Numbers: Aj276654.1 *T.annulata*, Z48738.1 *T. annulata*, Af214917.1 *T. annulata* na961, Af214914.1 *T. annulata* na89d and Af214909.1 *T. annulata* na33).Also 99.7% genetic similarity with *T.annulata* strain (GenBank Accession Numbers: Af214838.1 *T. annulata* TA6) and 99.3% genetic similarity with *T.annulata* strain (GenBank Accession Numbers: Af214898.1 *T. annulata* 29d) **(Figure 4).**

Animal	Examined No.	Infected No.	Percentage
Cattle	86	30	34.88%
Buffalo	88	29	32.95%
Total	174	59	33.91%

Table1: The prevalence of *Theileria* species in cattle and buffaloes

Table 2: The risk factors (Age, Sex and Tick infestation) associated the prevalence of *Theileria* species infection.

		Cattle			Buffalo		
		Examined	**Infected**	**%**	**Examined**	**Infected**	**%**
Sex	**Female**	39	9	23.08%	36	4	11.11%
	Male	47	21	44.68%	52	25	48.08%
Age	**1-2 months**	11	2	18.18%	10	2	20%
	3-12 months	24	4	16.67%	37	8	21.62%
	1-6 years	20	5	25%	8	3	37.5%
	>6 years	31	19	61.29%	33	16	48.48%
Tick infestation	**Positive**	31	zero	zero%	24	zero	zero%
	Negative	55	30	54.55%	64	29	45.31%

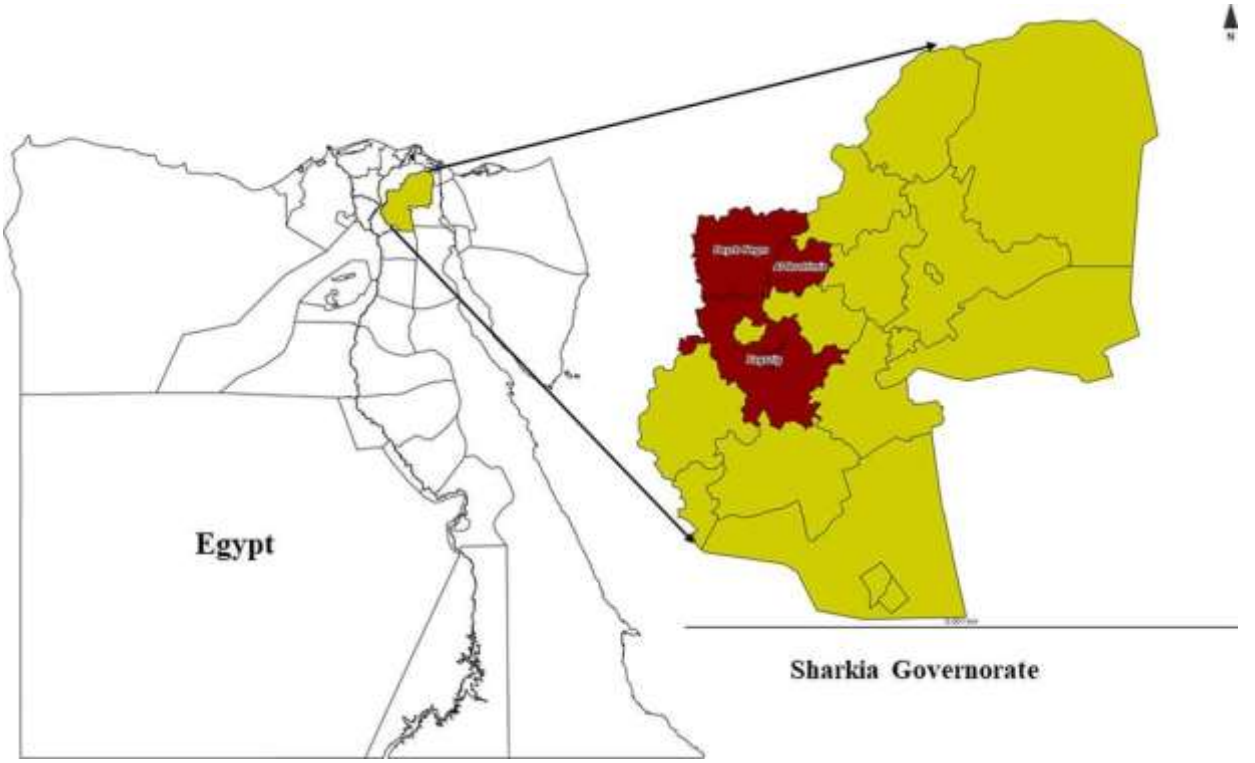

Figure 1: Sharkia Governorate location in Egypt and the location of the three cities (Red colour) involved in the study.

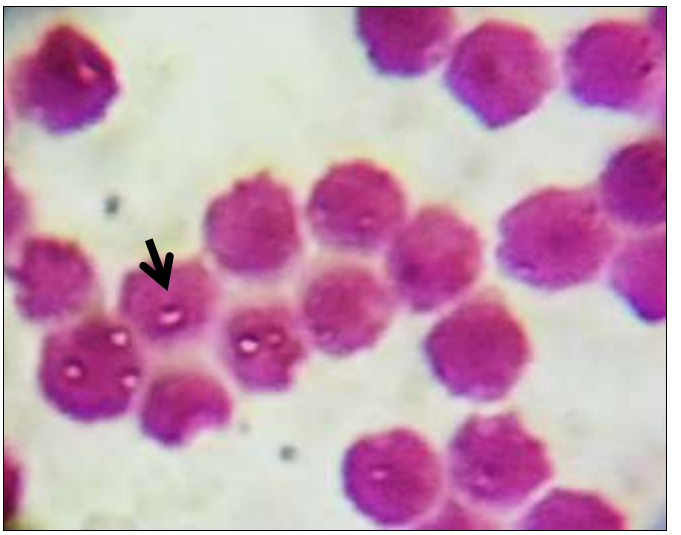

Figure 2: *Theileria* species piroplasms in blood smear stained by Giemsa stain (X1000)

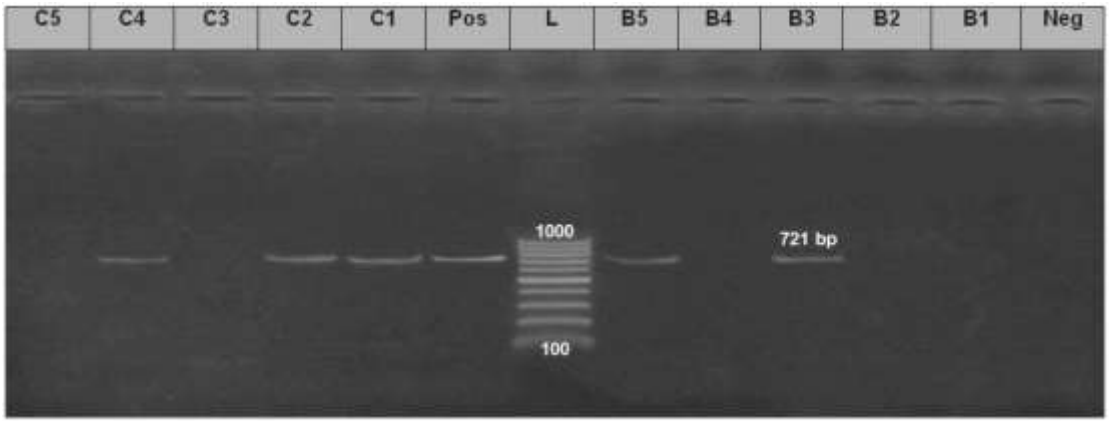

Figure 3: Showed PCR results for amplification of *T. annulata* 30KDA gene using *T. annulata* specific primer, The molecular weight of the PCR products was 721 bp. Lane L indicated molecular weight marker, Lane C1, C2 & C4 represented the positive samples from cattle, Lane B3& B5 indicated positive samples from buffaloes.

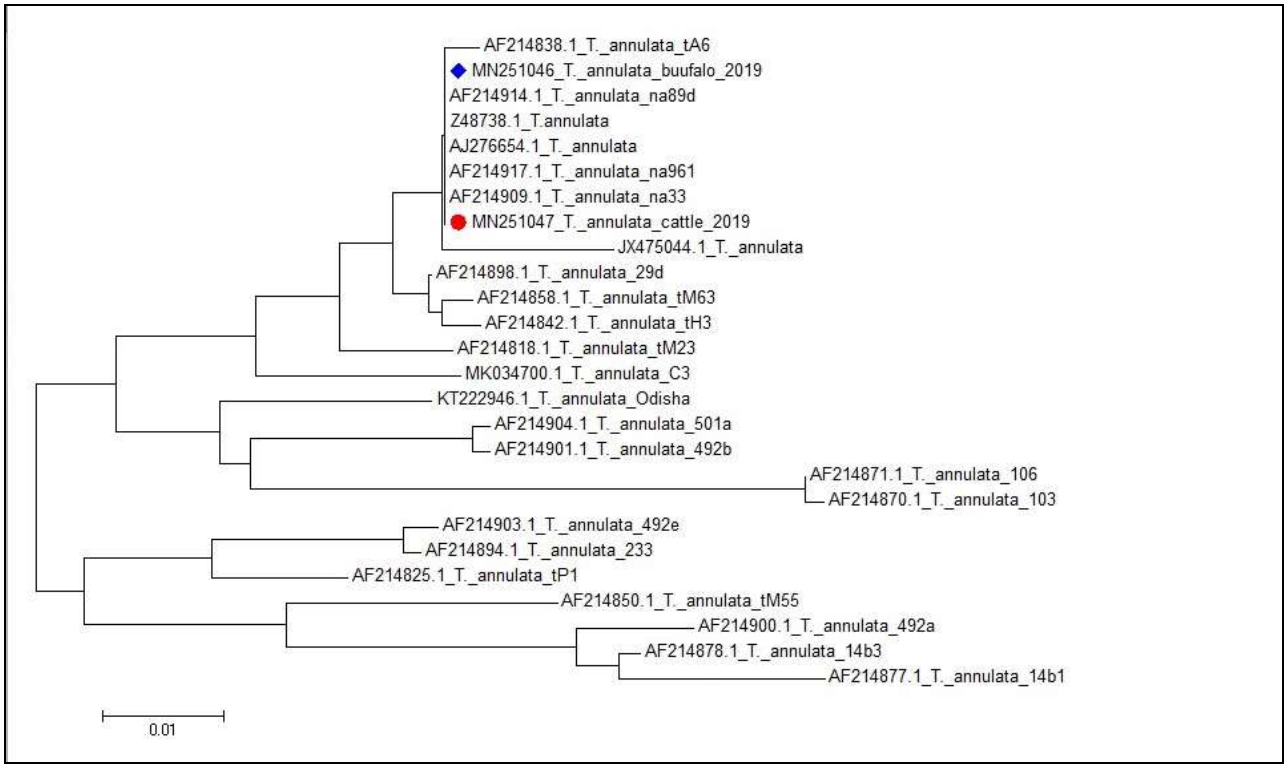

Figure 4: Illustrate the evolutionary relationships of MN251047_*T. annulata* _cattle_ 2019 and MN251046_*T. annulata* _buffalo_ 2019 with reference sequences of other *T. annulata* inferred by distance analysis (500 bp) using Phylogenic by 30KDA sequences. At the left of the support node, the percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is showed.

DISCUSSION

Theileriosis (*T. annulata* infection) is tick-borne disease that considered one of the most economically significant diseases of cattle and buffaloes in Egypt (**Osman and Gaabary, 2007** and **Ali and Radwan 2011)**. Clinically, the infected cattle and buffaloes with *T. annulata* had pyrexia, lymph node enlargement, diarrhea, corneal opacity, respiratory signs and drop in milk production. This result was agreed with that reported by **El-Deeb and Younis (2009).**

Microscopically, the morphological feature of *T. annulata* piroplasms from the examined blood films was described as **Rezai and Dalir-Naghadeh (2006) and Kundave et al. (2015).** In the present study, the overall prevalence of *T. annulata* was 33.91%, where cattle and buffaloes were 34.88% and 32.95%, respectively. This result was higher than that mentioned by **Abdel-Rady et al., (2010)** in Egypt who recorded the percentage of infection of *T. annulata* by blood smear was 25.3% and 8.6% in cattle and buffaloes respectively. Morever **Farooqi et al. (2017)** in Pakistan recorded the overall prevalence of *T. annulata* was 18.88% including 23.79% in cattle and 13.30% in buffaloes and **Narimani et al. (2017)** in Iran registered that such infection was 13% in cattle and 1.4 in buffaloes. However, **Waskel and Gaur (2015)** in India mentioned the higher prevalence of *T. annulata* for cattle's and buffaloes were 51.92% and 47.91%, respectively. The variation in the prevalence of infection may be due to the differences in management and hygienic conditions, agroecology, climate, the immune state of the host, sample size, sampling period, tick prevalence and breed.

The prevalence of *T. annulata* in relation to the animal's sex was higher in male (44.68% &48.08%) than female (23.08% &11.11%) in cattle and buffaloes respectively. The result was similar with that recorded by **Farooqi et al. (2017)** in Pakistan. The main cause is due to the less careness to male stock which mainly used for meat and draught purposes. In relation to age, the prevalence of *T. annulata* infection was higher in old animal (>6 years) (61.29% &48.48%) than 1-6 years (25% &37.5%), 1- 2 months (18.18% & 20%) and 3-12 months (16.67% &21.62%) in cattle and buffaloes respectively. These outcomes are in agreement with **Sallemi et al. (2018)** in Tunisia. The reason may be due to the decrease in the immunity with the increasing of age. Tick infestation considered the most risk factor affecting on the prevalence of *T. annulate*, this result was in line with **Inci et al. (2008)** in Turkey and **Farooqi et al. (2017)** in Pakistan.

Owing to, the higher specificity and sensitivity of the PCR rather than conventional techniques that had been performed in a number of studies on a wide range of parasites, The PCR method is more accurate for detection of *T. annulate* in blood samples (**Habibi et al., 2007**). In the present study, the PCR technique using the specific primers for *Theileria annulate* (30KDA) gene, the PCR products length was 721 bp in *T. annulate* positive samples using the specific primer 30 KDA. This result was in agreement with **Nourollahi-Fard et al. (2015)** in Iran and **Sallemi et al. (2018**) in

Tunisia, who used the same primer for *T. annulata*.

In this study, *T. annulata* 30KDA (Gen Bank Accession Numbers: MN251047 and MN251046) in cattle and buffaloes, respectively were identified. Phylogenetic analysis grouped the Egyptian MN251047_*T. annulata* _cattle_ 2019 isolate and MN251046_*T. annulata* _buffalo_ 2019 isolate in a separate clade and shared 100% genetic similarity with other *T.annulata* strains (GenBank Accession Numbers: Aj276654.1 *T.annulata*, Z48738.1 *T. annulata*, Af214917.1 *T. annulata* na961, Af214914.1 *T. annulata* na89d and Af214909.1 T. annulata na33).Also 99.7% genetic similarity with *T. annulata* strain (GenBank Accession Numbers: Af214838.1 *T. annulata* TA6) and 99.3% genetic similarity with *T. annulata* strain (GenBank Accession Numbers: Af214898.1 *T. annulata* 29d). Also, **Sallemi et al. (2018**) in Tunisia recorded the GenBank Accession Number: KU145624 of *T. annulata* Tams1 gene and the data for the Nucleotide sequence similarity revealed that the Tunisian *T. annulata* strain share 100% with with Mauritania and Egypt (AF214819 and AB917290, respectively). While, Bahrain (AF214795) was 98.2% and Turkey (U22888) was 97.1% nucleotide identity.

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الملخص العربي

التقنية الجزيئية والفحص الميكروسكوبى لعزلات ثايلريا الماشية فى محافظة الشرقية ، مصر

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*****كلية الطب البيطري، جامعة الزقازيق – قسم الأمراض المعدية.**

الثايليريا هي طفيليات أولية تنقلها القراد تصيب العديد من الحيوانات الأليفة والبرية في جميع أنحاء العالم. ثايليريا أنيولاتا (*Theileria annulata*) هي النوع الأكثر أهمية من الناحية الاقتصادية التي تؤثر على الأبقار والجاموس. تم جمع ١٧٤ عينة دم (٨٦ من الأبقار و٨٨ من الجاموس) بشكل عشوائي من الحيوانات السليمة والمصابة ظاهرياً سريريًا وتم فحصها باستخدام الميكروسكوب لشرائح الدم المصبوغة بصبغة جيمسا. تم عمل هذه الدراسة خلال الفترة الممتدة من يناير ٢٠١٨ حتى ديسمبر ٢٠١٨في مواقع مختلفة من محافظة الشرقية. كان معدل انتشار طفيل الثايليريا ٣٤.٨٨٪ و ٣٢.٩٥٪ في الأبقار والجاموس على التوالي. كانت نسبة الإصابة بالعدوي أعلى في الذكور من الإناث. فيما يتعلق بعمر الحيوان، فانتشاره مع > ٦ سنوات من العمر أكثر من ممن هم يتراوح أعمارهم بين ١–٢ أشهر و٣–١٢ شهرًا و١–٦ سنوات. كانت الإصابة بالقراد عامل مساعد لاحتمال الإصابة بالعدوى بالثايليريا. وباستخدام تفاعل البلمرة المتسلسل (PCR) تم معرفة الوزن الجزيئي لنواتج استخلاص (DNA) بكتلة ٧٢١ زوج من القواعد من عينات الثايليريا أنيولاتا الإيجابية. و بإجراء التتابع الجينى وإستخدام التمثيل الشجرى الجينى لعزلات الثايليريا تم الكشف عن درجة القرابة مع الدراسات السابقة.