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The molecular prevalence of *Theileria equi* in the Arabian stallion in Egypt with special reference to biochemical parameters

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Abstract

Theileria equi, a blood parasite spread by ticks, is thought to be a severe infectious disease that impacts the health of horses. The study's main goal was to determine the prevalence of *T. equi* in Arabian stallions in Egypt by comparing microscopic examination (ME) and conventional polymerase chain reaction (cPCR) and compare them. The study aimed to determine the liver and kidney functions of infected Arabian stallions with *T. equi*. Out of the 100 Arabian stallions in Egypt tested for *T. equi* infection, only 30 (30%) were positive through microscopic analysis, and 38(38%) were positive through a cPCR test. Arabian stallions were divided into two groups: the first group, positive *T. equi* infection (infected group) (n = 30). In the second group, negative *T. equi* infection (healthy group) (n = 10) and liver and kidney function were detected for both groups. Serum analysis revealed significant changes in the infected group's liver and kidney function parameters. Total bilirubin, direct bilirubin, indirect bilirubin, GGT, GOT (AST), urea, and creatinine were significantly increased in the serum of the infected group compared with the healthy group; total protein, albumin, and globulin were found to be decreased in the serum of the infected group when compared to the healthy group. The ALP and albumin: globulin (A: G) ratio changes weren't significant in this study. Studying the molecular prevalence of *T. equi* in the Arabian stallion in Egypt with special reference to biochemical parameters will help evaluate the prognosis and treatment of theileriosis.

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Introduction

Theileria equi is an infectious disease spread by ticks that causes equine theileriosis. (1). Four possible forms occur in the equine infected with *T. equi:* acute, chronic, or, most commonly, inapparent form (2). Clinical signs include fever, anemia, inappetence, edema, icterus, hepatomegaly, splenomegaly, and, in rare cases, mortality (3,4). The disease, which has been added to the OIE list, is global in scope, as treatment costs, abortions, loss of activity, and death are the most common economic losses (4,5). In Egypt, the prevalence of *T. equi* varies according to the age, sex, months, seasons, and geographic location of the horses (5-8). Diagnosis of *T. equi* by microscopic examination (ME) of the blood smear ispossible in the acute phases with high enough numbers of parasites in the circulation and observed intraerythrocytic (2); otherwise, diagnosis by Conventional Polymerase Chain Reaction (cPCR) is sensitive and highly specific than any other method and the best accurate diagnosis choice in *T. equi* infection seroprevalence in all phases (9,10). Numerous equine studies have examined the biochemical alterations linked to *T. equi* infection (5). Total

protein, globulin, bilirubin (total, direct, indirect), and enzyme activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) were found to be significantly higher with the study by Ozdek *et al.* (11). Ahmadpour *et al.* (12) found increased urea and creatinine in horses naturally infected with *T. equi* compared to the healthy group (12). *T. equi* was found in an experimental infection in the blood and other tissues like the lungs, livers, spleens, and bone marrow (12,13). A histological investigation revealed microthrombi in the liver and lung as well as centrilobular necrosis of the liver (4,14). As a result of decreased blood flow to the liver, hyperbilirubinemia is frequently seen along with elevated levels of the liver enzymes alkaline phosphatase (ALP), sorbitol dehydrogenase (SDH), gamma-glutamyl transpeptidase (GGT), and aspartate aminotransferase (AST) (5,11,15). In addition, acute renal failure was observed by Adam *et al.* (16); histopathological investigation has revealed renal tubular necrosis with hemoglobin casts and reticuloendothelial cell growth in the kidney, liver, lymph nodes, and lungs (2,4); renal insufficiency can result in azotemia and abnormalities in urine consistent with changes in kidney function; pigmenturia produced by either hemoglobinuria or bilirubinuria occurs with significant systemic involvement concurrent with continuous hemolytic events (17); the variable differences in biochemical parameters reported by different studies can be attributed to the *T. equi* infection disease (4,11), and these biochemical parameter changes may be useful in the evaluation of the prognosis and treatment of theileriosis.

The Egyptian Arabian stallion breed improves other horse breeds and provides the world's most expensive, finest, and purest horse breed. So, this study aims to determine the prevalence of *T. equi* in Arabian stallions in Egypt by ME and cPCR and evaluate the changes in liver and kidney function of infected stallions to decrease economic losses from this disease.

Materials and methods

Ethical approval

This investigation was conducted according to standard protocols, with no horse pain or injury. Additionally, the experiments' procedures were approved by the Ethics Committee of the Veterinary Medicine Faculty, Benha University, Egypt (BUFVTM 08-10-23).

Animals and location

This study, which involved 100 Arabian stallions aged 3 to 15, was conducted in Cairo, Egypt, between September 2022 and August 2023. Each stable had a bed of rice straw, water, and mineralized salt. Stallions feed grains twice a day and forage three times a day. The stallions were released into the yard daily and exposed to daylight. Every stallion had a vaccination and deworming program and was used for breeding. Clinically, while some horses were healthy, the majority were emaciated and exhausted. Horses also had a history of being affected by ticks.

Blood collection and microscopic examination

Following the technique, one hundred blood samples were taken from each stallion from the jugular vein and put into two sterile vacuum biochemistry tubes. Then, they were brought to the laboratory in the cold chain. The first tube, the EDTA K2/K3 tube (lavender-top tube), was used right away to generate thin blood smears, which were subsequently fixed with absolute methanol, stained with Giemsa, inspected for *T. equi* evidence under a light microscope; these samples of whole blood were preserved at -20ºC until molecular analysis (cPCR). The second tube, no additive (Red Cap Plain tubes), was centrifuged by a compact centrifuge (HERMEL Z 206 A, Germany) for serum samples at 3000 rpm for 10 minutes after the blood was allowed to coagulate at room temperature; these serum samples were stored at -20ºC until biochemical parameter estimation. Once *T. equi* were detected in all samples, we divided the stallions into two groups: the first group (n = 30) was an infected group (positive *T. equi* by microscopic and cPCR), and the second group (n = 10) was healthy (negative *T. equi* by microscopic and cPCR). We then evaluated total protein, albumin, bilirubin, ALP, GGT, AST, urea, and creatinine for both groups (infected and healthy) from serum samples.

DNA extraction

The QIA amp DNA Mini kit (Qiagen, Germany, GmbH) was used to extract DNA from blood samples, with certain modifications made by the manufacturer's instructions. In summary, 10 µl of proteinase K and 200 µl of lysis buffer were added to 200 µl of the sample suspension and incubated for 10 minutes at 56 °C. Following incubation, the lysate was mixed with 200 µl of 100% ethanol. The manufacturer's instructions were then followed for washing and centrifuging the sample. 100 µl of the elution buffer in the kit was used to elute the nucleic acid.

Oligonucleotide primer

The primers used by Metabion in Germany are mentioned in table 1 (16).

Table 1: Primers sequences for theria 16S rRNA

Primers sequences	Size (bp)
CATCGTTGCGGCTTGGTTGG CCAAGTCTCACACCCTATTT	664

PCR amplification

A 25 µl reaction comprising 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water, and 5 µl of DNA template was used to use the primers. A 2720 thermal

cycler from Applied Biosystems was used to carry out the reaction.

Analysis of the PCR products

The PCR products were separated by electrophoresis employing gradients of 5V/cm on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. 15 µl of each product was put into a gel slot for gel analysis. The fragment sizes were measured using a generuler 100 bp ladder (Fermentas, Germany). A gel documentation system (Alpha Innotech, Biometra) took pictures of the gel.

Biochemical studies

Kits for measurement of Total protein, Albumin, Bilirubin (Total and direct), ALP, GGT, AST, Urea, and Creatinine were obtained from Centronic GmbH, Germany.

Statistical analysis

The results data were examined by using the program Statistical Package for the Social Sciences (SPSS) version 26.0 (IBM Corp., Chicago, USA. Significant results levels (at levels P≤0.05) indicated a potential risk of infection.

Results

Microscopic examination and cPCR

Under a microscope, giemsa-stained blood smears from stallions revealed intra-erythrocytic, rather small, less than 2 µm long, pear- and ring-shaped *T. equi* merozoites. These four pear-shaped *T. equi* merozoites form a tetrad called the maltese cross in the erythrocytes. *Theilerial* schizogony forms, which are irregularly shaped structures called macroschizonts or microschizonts that contain tiny chromatin granules, were disclosed by lymphocytes (Figure 1). Of the 100 blood samples from Arabian stallions tested for *T. equi* infection, 38% (38/100) were positive by cPCR (Figure 2) and 30% (30/100) by microscopic examination (Figure 1). The prevalence of *T. equi* determined by molecular assay (cPCR) was significantly higher (P<0.000) than the microscopic examination (ME) (very strong) (Table 2).

Biochemicals parameters

It was found in our study that in Arabian stallions naturally infected with *Theileria equi*, bilirubin (total, direct, and indirect), AST, GGT, urea, and creatinine statistically significantly increased; however, total protein, albumin, and globulin statistically significantly decreased compared to the healthy group (P<0.05). It was found that, compared to the healthy group, variations in the ALP and albumin: globulin (A: G) ratio in Arabian stallions naturally infected with *T. equi* were not statistically significant (Table 3).

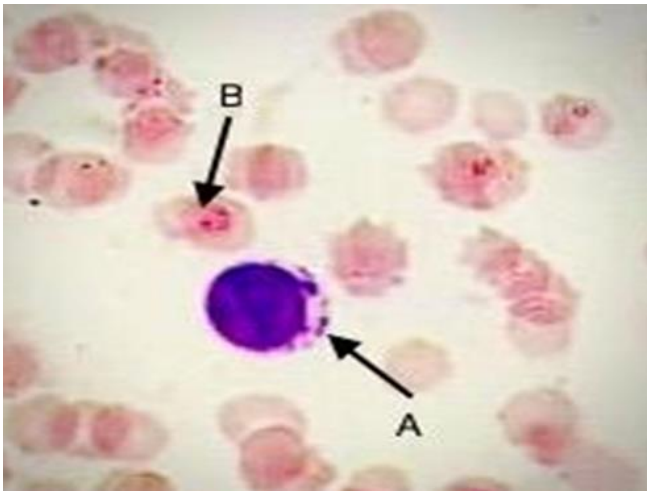

Figure 1: Blood smear of an equine infected with *Theileria equi*, stained with Giemsa: (A) Theileria schizogony forms in lymphocyte and (B) merozoites in erythrocyte.

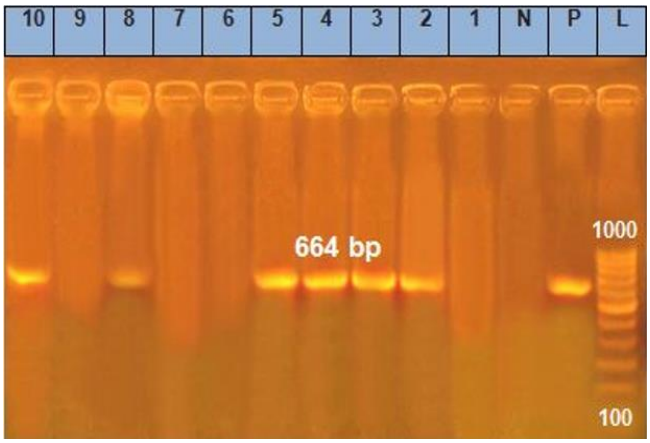

Figure 2: Agarose gel electrophoresis of PCR (664 bp) is specific for the characterization of *Theileria equi*. Lane L: 100 bp DNA marker. Lane P: Positive control for *T. equi* gene. Lane N: Negative control. Lanes 1,6,7 and 9: Negative *T. equi* DNA. Lanes 2, 3, 4, 5, 8 and 10: Positive *T. equi* DNA.

Table 2: The prevalence of *Theileria equi* infection in arabian stallions by microscopic examination and cPCR

Methods	Examined stallions (n)	Positive samples (n)	Negative samples (n)	Prevalence (%)
Microscopic	100	30	70	(30%)
cPCR	100	38	62	(38%) ***

*** Subscript significance differences between between the same columns (***P<0.05).

Table 3: Biochemical changes in arabian stallions infected with *Theileria equi* and healthy Arabian stallions

Parameter	Group		P Value
	Healthy (A) n=10	Infected (B) n=30	
Total bilirubin (mg/dl)	1.08±0.11	1.52±0.06**	0.001
Direct bilirubin (mg/dl)	0.49±0.09	0.74±0.03**	0.002
Indirect bilirubin (mg/dl)	0.61±0.07	0.76±0.03*	0.038
ALP (U/L)	276.10±30.05	223.67±14.4	0.092
GGT (U/L)	14.50±1.72	22.97±1.30**	0.001
AST(GOT) (U/L)	213.4±4.16	234.2±2.26***	0.000
Total protein (g/dl)	6.51±0.14	4.47±0.09***	0.000
Albumin (g/dl)	3.37±0.12	2.32±0.06***	0.000
Globulin (g/dl)	3.14±0.08	1.63±0.17***	0.000
Albumin: Globulin ratio	1.08±0.05	0.83±0.09	0.112
Urea (mg/dl)	19.60±0.87	30.43±0.45***	0.000
Creatinine (mg/dl)	0.62±0.04	1.46±0.05***	0.000

*P<0.05, **P<0.01, ***P<0.001 Subscript significance differences between rows.

Discussion

Arabian horses have travelled the world via trade and warfare throughout history, where they used Arabian stallions to improve other horse breeds by adding speed, refinement, endurance, and strong bone. *Theileria equi* is a blood parasite disease spread by ticks that is endemic in Egypt (17). It causes piroplasmosis, also known as theileriosis, affecting the Arabian stallions' performance and horse industry. Since Arabian stallions are a highly prized breed in Egypt, this study aims to ascertain the prevalence of *T. equi* by using cPCR and microscopic examination (ME), respectively, and to compare their prevalence. Additionally, it seeks to assess how *T. equi* affects the liver and kidney function of Arabian stallions by measuring total bilirubin, direct bilirubin, indirect bilirubin, ALP, GGT, GOT (AST), total protein, albumin, globulin, albumin: globulin (A: G) ratio, urea, and creatinine levels, which in turn affect the performance of stallions.

The prevalence of *T. equi* by ME in the current study (30%) was higher than the prevalence found by ME in Egypt by Mahdy *et al.* (18) 27.4% and Kuraa *et al.* (7) 14%. In addition, the prevalence was also higher than some other countries found, like Ethiopia 12.2% by Gizachew *et al.* (19) and Costa Rica 24.6% by Posada-Guzmán *et al.* (20). In the present study, cPCR detected that the prevalence of *T. equi*-infected Arabian stallions in Egypt was 38%, which was higher than Mahmoud *et al.* (21) 36.4%, and lower than that detected by Mahdy *et al.* (18) 60.8%; however, it was the same result as Kuraa *et al.* (7) 38%. In comparison with some other world countries, the prevalence of *T. equi*-infected Arabian stallions in our study was higher than that reported by Osman *et al.* (22) in Sudan 13.9% of horses and Sumbria *et al.* (23) in India 14.14% of horses, less than Habibi *et al.* (24) study 96.77% of healthy horses and mules in Iran and Sgorbini *et al.* (25) study in Italy 41% of horses, and nearly the same as Ahedor *et al.* (10) study in Paraguay 38.14%. Different prevalence observations have been reported from various areas of the world due to factors such as the variations in the sensitivity of different diagnostic tests used in different epidemiological studies, the presence and abundance of competent tick vectors, the activity of the host, management practices, and the effectiveness of vector control programs (4).

ME is most helpful when an equine has an acute *T. equi* infection; cPCR, on the other hand, is the most sensitive approach for diagnosing animals with a chronic *T. equi* infection. However, to ensure that the prevalence result of ME in our study differed from previous studies and that cPCR was higher in the prevalence study than ME due to cPCR's greater sensitivity and accuracy (26,27), careful examination of the smear is necessary, necessitating an experienced operator to prevent false-negative results.

Numerous studies have examined risk factors, including age, sex, breed, animal species, castration status, tick presence or absence, location, origin, and activity (1,28). These factors, which impact the frequency of *T. equi* infections, can be categorized as extrinsic/environmental or intrinsic host-related (4). Al-Ani and Yousif (29) found the prevalence of Babesia caballi higher in Arabian horses than in Thoroughbreds and Crossbreds. Moretti *et al.* (30) found that *T. equi* persists with increasing age; they also found a relation between the breed and the rate of piroplasmosis seropositivity. Piroplasmosis was found to be more prevalent in males than females in many studies, as in cattle with *Theileria* species (31) and equine with *Babesia caballi* (29,32); sex hormone levels have been linked to susceptibilities to *T. equi* infection and mice used in experiments showed that higher testosterone levels made them more susceptible to piroplasmosis infection and tick infestations (33,34). In general, the prevalence in our study differed from that of other studies conducted in Egypt, possibly due to several factors, such as age (adult horses ranged from 3 to 15 years old), breed (Arabian horses), sex

(stallions), and location (Arabian horses were found in Egypt).

The current investigation revealed that infected Arabian stallions had significantly increased serum bilirubin (total, direct, and indirect) levels. This notable rise in bilirubin levels may be caused by hemolysis and hepatic dysfunction of parasite erythrocytes (6). Various investigations reported an increase in total bilirubin levels (11,35) or no change in levels (36). Takeet *et al.* (37) also noted decreased direct bilirubin levels. Decreased blood flow to the liver with *T. equi* infection causes hyperbilirubinemia, which is frequently seen along with elevated levels of liver enzymes (5,11,15). In our investigation, blood levels of liver-specific enzymes (AST and GGT) were significantly higher in infected stallions with *T. equi* infection than in healthy stallions; however, ALP levels remained unchanged. Studies have reported that hepatocyte damage caused by hypoxia related to anemia may cause this increase in enzyme activity (5,15). The higher level of unconjugated bilirubin resulted in raised liver enzymes; liver enzyme levels rose as a result of the ongoing hemoglobin release and the creation of unconjugated bilirubin, as well as the liver's limited ability to conjugate bilirubin (38). Furthermore, other investigations found that theileriosis infections in horses did not alter liver enzyme levels (37,39).

According to Bozukluhan *et al.* (40), acute phase protein response (negative acute phase protein) and/or liver dysfunction are considered to be the causes of the reduction in albumin level. Extensive protein breakdown occurs during *T. equi* infection due to digestive disruption and protracted fever (41). Major laboratory results in this state include hypoproteinemia, hypoalbuminemia, and hypoglobulinemia, as found in our study; however, the albumin: globulin (A:G) ratio has not changed. This hypoproteinemia is also expected when there is a reduction in body weight due to fat and muscle mass (Cachectic states) (42). This was also found in other studies on *T. equi* infection, such as those conducted by Al-Obaidi *et al.* (15) and El-Sherif *et al.* (5). Studies by Zaeemi *et al.* (35) and Özdek *et al.* (11) found an increase in albumin and protein.

In our work, *T. equi* infection causes hemoglobin-induced pigment nephropathy, and systemic reactions of severe inflammation increase urea and creatinine (2). Renal insufficiency can result in azotemia and abnormalities in urine consistent with changes in kidney function with *T. equi* infection; pigmenturia produced by either hemoglobinuria or bilirubinuria occurs with significant systemic involvement concurrent with continuous hemolytic events; finally, increases in urea and creatinine parameters, which were found by Mohammed *et al.* (17) and Ahmadpour *et al.* (12) and agree with our study; on the other hand, Özdek *et al.* (11) did not find alteration statistically significant in urea and creatinine. The differences in studies may vary according to the stage of infection, either acute or chronic.

Conclusion

Theileri equi prevalence in Arabian stallions in Egypt is 30% by microscopic examination (ME) and 38% by cPCR. Total bilirubin, direct bilirubin, indirect bilirubin, gamma-glutamyl transpeptidase (GGT), aspartate aminotransferase (AST or GOT), urea, and creatinine levels increase with *T. equi* infection in Arabian stallion; otherwise, total protein, albumin, and globulin decrease. There was no alteration of alkaline phosphatase (ALP) and albumin: globulin (A: G) ratio levels with *T. equi* infection in Arabian stallions. Accurate diagnosing *T. equi* infection helps correct treatment while being aware of the prognosis. More studies are needed to understand the role of immunity in the pathophysiology of theileriosis and how to decrease its prevalence.

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Conflict of interest

The authors affirm that they do not have any conflicts of interest.

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الانتشار الجزيئي للثيليريا الخيلية علي أفحل الخيول العربية في مصر مع الإشارة الي بعض التغيرات الكيمائية

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الخلاصة

الثيليريا الخيلية، طفيل دموي ينتشر بواسطة القراد، ويعتبر مرض معدي خطير يؤثر على صحة الخيول. الهدف الرئيسي من الدراسة هو تحديد مدى انتشار الثيليريا الخيلية في أفحل الخيول العربية في مصر عن طريق استخدام الميكروسكوب المجهري وتحليل تفاعل البلمرة المتسلسل التقليدي والمقارنة بينهم كذلك تهدف الدراسة الى قياس وظائف الكلى والكبد لأفحل الخيول العربية المصابة بالثيليريا الخيلية. فمن أصل ١٠٠ فحل عربي في مصر تم اختبار الإصابة بالثيليريا الخيلية، فكان ٣٠ فحل عربي (٣٠ في المائة) إيجابي من خلال التحليل المجهري، وكان ٣٨ فحل عربي (٣٨ في المائة) إيجابي من خلال تحليل تفاعل البلمرة المتسلسل التقليدي. وتم تقسيم أفحل الخيول العربية الى مجموعتين، المجموعة الأولى إيجابي الإصابة بالثيليريا (مجموعة المصابين) (عددهم ٣٠)، المجموعة الثانية سلبي الإصابة بالثيليريا إكواي (مجموعة الأصحاء) (عددهم ١٠) وتم قياس وظائف الكلى والكبد للمجموعتين. وتبين من تحليل سيروم الدم أن هناك تغيرات كبيرة في وظائف الكلى والكبد للمجموعة المصابة. وقد وجد أن مجموع البيليروبين والبيليروبين المباشر والبيليروبين الغير مباشر وأنزيم ناقلة الأمين الكلوتاميت وأنزيم ناقلة أمين الأسبارتيت واليوريا والكرياتينين قد زادوا زيادة كبيرة في مصل الدم للمجموعة المصابة بالمقارنة بالمجموعة الأصحاء، أما البروتين والألبومين والكلوبيولين قد قل بشكل كبير في سيروم الدم للمجموعة المصابة بالمقارنة بالمجموعة الأصحاء. أما التغيرات في أنزيم الفوسفتيز القاعدي والنسبة من الألبومين الى الكلوبيولين لم تكن كبيرة في الدراسة. دراسة الانتشار الجزيئي للثيليريا الخيلية علي أفحل الخيول العربية في مصر مع الإشارة الي بعض التغيرات الكيمائية يساعد في تقيم تطورات الحالة المرضية وعلاج الثاليريوزيس.