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Comparative Diagnosis Utilizing Molecular and Serological Techniques of *Theileria equi* Infection in Distinct Equine Populations in Egypt

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Abstract : The prevalence of *Theileria equi* infection was studied in 301 equine samples (133 donkeys and 168 horses) from Giza and Cairo governorate using microscopic examination (ME), nested (nPCR), competitive ELISA (cELISA) and indirect ELISA (iELISA). The used antigen in iELISA was prepared from blood of naturally infected splenectomized donkey at the peak of parasitemia In ME, the parasite was detected in 79 (26.2%) equine blood samples; 33 donkeys and 46 horses with a prevalence rate (24.8% and 27.4%), respectively. The prevalence rate in equine samples using iELISA was (33.5%) from which 71 donkeys and 30 horses were infected (53.4% and 17.9%), respectively. The *T. equi* antibodies were detected with cELISA in 60 (19.9%) equine serum samples, where 34 donkeys and 26 horses with a prevalence rate (25.6% and 15.5%), respectively. The nPCR based on the *T. equi* merozoite antigen gene (EMA-1) allowed the visualization of species-specific amplified product in 171 (56.8%) equine blood samples, 67 donkeys and 104 horses with a prevalence rate (50.4% and 61.9%), respectively. Approximately 229 bp of the ema-1 gene from 3 Eqyption samples were sequenced and BLASTN analysis confirmed all sequences to be merozoite surface protein genes, with an identity of 100% to previously published *Babesia equi* merozoite antigen- 1 ema-1 gene reference sequence (our GenBank Accession number KX262963). Statistical analysis using Chi square indicated significant differences ($P< 0.05$) between ME and nPCR; microscopic examination and cELISA and between nPCR and cELISA on the detection of parasite carriers. In conclusion, the most sensitive technique in diagnosis of *T. equi* infection is nPCR, followed by cELIZA, iELISA and ME. The combination of ELISA and PCR was recommended for detection of acute and chronic stage.
Keywords: Equine, *Theilria equi*, Antigen, iELISA, cELISA, Immunoblot, SDS-PAGE, nPCR.

Introduction

Blood protozoal diseases are considered of great importance parasitic infections affecting equine in Egypt. Equine piroplasmosis (EP) affects the development of equine industries worldwide, including Egypt, especially in the acute phase. EP causes abortions, loss of performance, death and restrictions in meeting international requirements related to exportation or participation in equestrian sporting events [1,2,3]. Equine theilerosis (ET) is a tick-borne disease caused by the hemoprotozoan parasite *Theileria equi* (*Babesia equi*).

Based in part on finding an extra-erythrocytic stage within equine lymphocyte, *B. equi* was reclassified as *T. equi* [4]. Molecular phylogenetic investigations indicated that *T. equi* possesses characteristics of both *Babesia* and *Theileria* lineages, possibly placing it between the two[5]. Infection with ET can cause varying degrees of hemolytic anemia and associated systemic illness (fever, Jaundice, red urine, oedema, loss of appetite, weakness) [6].

In acute stage of *T.equi* infection can be easily detected inside the erythrocytes by ME. However, animals which overcome the acute stage of the disease and survived remain carriers and no parasites are readily demonstrable in the blood; at that point, a serologic test is needed to detect them [7]. One of the serological tests is the complement fixation test CFT which is a very specific test, but it lacks sensitivity in chronic infection or after treatment [8]. Another serological test is the indirect immuno-fluorescent antibody tests (IFAT) that demonstrates the high specificity, but it lacks sensitivity. However, IFAT, is considered more sensitive than the CFT [9]. It is one of the prescribed tests for equine piroplasmosis recommended by the OIE**.** The enzyme-linked immuno-sorbent assay (ELISA) is used to detect dominant antibodies to both *T. equi* and *B. caballi*. However, cross-reactivity between them may occur [9] but the sensitivity of the test has been shown to be greater than that of the CFT, when whole merozoites or even parasitised red cells were used as antigen in an indirect assay for detection of antibodies [10]. A competitive inhibition ELISA (cELISA) was developed for *T. equi* infection by using (equimerozoite antigen-1) EMA-1 and specific monoclonal antibodies [11]. This cELISA was later improved by the use of a recombinant protein instead of culture-derived whole parasites [12]. The cELISA has detected latent infections of experimentally infected horses that were not detected by CFT [9]. Since 2004, cELISA has been one of the regulatory tests prescribed by the OIE for international horse transport. Primary PCR assays for detection of parasite DNA have been developed to detect both *T.equi* and *B. caballi* DNA in horses [13]. PCR was able to detect parasitemia levels as low as 0.0083% for *T. equi* and 0.017% for *B. caballi*. A nested PCR for *T. equi* based on the sequence of the EMA-1 gene has increased sensitivity and may be more reliable for the diagnosis of subclinical infection, detecting an equivalent calculated parasitemia of 0.000006% [14]. In a field study using the nested PCR for *T. equi*, the test was able to detect 3.6 times more infections than microscopy analysis and 2.2 times more than with primary PCR[3].

In Egypt, The incidence of *T. equi* in horses and donkeys was discussed in Egypt by [15] using (ME and hemagglutination test), [7]using (ME and IFAT), [16] (ME**),** [1] using (PCR and IFA) and [17] (ME, IFAT and CFT), [18] using (ME, ELISA and PCR) and [19] **(**ME, iELISA). The cELISA and nPCR was used by [20]. More studies using sensitive and specific diagnostic techniques are still required to apply control measures. Therefore, the aim of this study was to update the information about ET in Egypt by using recent, sensitive and specific diagnostic techniques (nPCR and cELISA) in comparison with conventional method (ME and iELISA).

Materials and Methods

Blood samples

A total of 301 blood samples were collected from 133 scarified donkeys at the zoo and 168 horses at the Equestrian police station, Giza and Cairo governorates, respectively. Each sample was assigned into two parts; one in the tube with EDTA as an anticoagulant for preparing of blood films examination and DNA extraction, while the second one in a tube without anticoagulant for serum separation for serological tests.

Microscopical examination

The blood smears were made to clean, dry slide, air dried, fixed in methyl alcohol for 10 mints and stained with Diff. Quick® stain as manufacture instruction [21]. The stained blood smears were examined by light microscopy (OLYMPUS CX41) for the presence of infections.

Splenectomy of infected donkey:

Three *T.equi* naturally infected donkeys with parasitemia varied from 2-3% were splenectomized. The spleen was exteriorized following the technique of [22]**.** The infected blood with *T.equi* was collected from the third one on 10% EDTA at the peak of parastaemia for antigen preparation, as the first one was died during the splenectomy and the second one died 2 days after the splenectomy operation. The nPCR was performed and followed by sequencing for confirmation of the purity of *T.equi* Egyptian strain.

Antigen preparation

Preparation of lysate *T. equi* crude antigen (L1)

The antigen was prepared according to[23,24]**.** The blood was collected from the naturally infected splenectomized donkey at the peak of parasitaemia (15%), centrifuged at 1500 rpm (1000g) for 10 min and re-suspended in 0.15 M PBS pH 7.2 for 3 times. The packed erythrocytes were mixed with cold distilled water at 1:5 ratios. The suspension was re-centrifuged at 10.000g for 60 min at 4ºc. The supernatant lysate antigen (L1) was removed and stored at -20ºc until used in ELISA.

Preparation of lysate antigen without hemoglobin (L2)

Hemoglobin was removed from part of the supernatant lysate antigen, which prepared in the previous step, according to the procedures of [25]. Initially, 1 ml of lysate was mixed with 1 ml 40% (v/v) ethanol and 0.5 ml chloroform for 1 min and then centrifuged at 12,000×g for 5 min. The chloroform layer and membranous interface were discarded; supernatants were recovered and kept at−20 °C

Preparation of the sonicate *T. equi* antigen (S).

For preparation of sonicate *T. equi* antigen, infected erythrocytes were processed in the same manner as described above. After removal of (L1) the sediment was re-suspended in equal amount of distilled water vortexes and sonicated at ultrasonicat (SONICS Vibra cell) 100W for five times for 30 s each on ice at an interval of 1 min. after complete sonication ultracentrifugation was done at 40.000 rpm for 30 min The supernatant antigen (S) was collected and stored at -20°c until used in ELISA [26]

Indirect Enzyme Linked Immuno-sorbant Assay (iELISA)

The ELISA was performed according to [27] on 301 equine serum samples. The lysate crude antigen (L2) was the antigen of choice which gave high titer with less dilution of serum was determined after checker board titration. The plates were coated with antigen after measurement of protein according to [28]**.** The cutoff values and the ELISA levels (EL) were calculated. The cutoff value will determine as being 2.5 times the mean absorbance value of negative controls, where readings above the cutoff value were considered positive, the immunological activity of each serum was calculated by determining the sample to positive serum ratio (S/P), considering positive and negative sera as a reference, using the following equation:

S/P = (mean sample absorbance − mean absorbance of negative serum reference) / (mean absorbance of positive reference serum- mean absorbance of negative serum reference). S/P values were grouped into ELISA levels (EL), which ranged from 0 (lowest level) to 8 (highest level).

Competitive Enzyme Linked Immunossorbant Assay (cELISA)

Theileria equi antibody test kit, cELISA from VMRD Inc. (Pullmann, WA, USA) was performed on 301 equine serum samples following the manufacturer's instructions. The principle of the test is that serum antibodies to *T. equi* inhibit primary monoclonal antibodies of the detection system from binding to the antigen-coated plate. The binding of primary monoclonal antibody is detected with horseradish-peroxidase-labelled secondary antibody. Optical density (OD) values will be determined using EL_X 800 UV Universal Microplate Reader Bio-tek instruments, INC ELISA reader. The percent of inhibition (I%) will be calculated, I%= 100 − [(sample O. D. ×100) / (mean negative control O. D.)]. Samples were classified as positive if the I% value was above 40% and negative if the I% value was less than 40%. The manufacturer of the kit established these cutoff values [29]**.**

Molecular characterization of Babesia species by nPCR and sequencing of PCR product

DNA was extracted from all blood samples of 301 animals using Whatman FTA® Elute cards (Cat. No. WB120410) following the manufacturer's instructions. Then, the nPCR was performed in a final volume of 25 µl containing 12.5 µl KAPA 2G Fast Ready Mix PCR with dye (kk5101) (KAPA BIOSYSTEMS), (10 pmol) of each primer, forward primer and reverse primer there were specific primer for *T.equi* [30] and another for *B. caballi* [31] (Table 1) and 8.5 µl sterile water (The nested PCR for *B.caballi* was performed for splenctomized donkey only to confirm the purity of the strain). Two microliter of template DNA was used for

the primary PCR. The nested PCR utilized 1 µl of primary PCR product as a template. Amplification was performed in a thermocycler (TECHNE TC-3000G PCR). The conditions for *T.equi* and *B.caballi* primary PCR were 95°C for 3 min followed by 25 cycles, each consisting of denaturation at 95 °C for 15 sec, annealing at 60 °C for 15sec, and extension at 72 °C for 15 Sec. then a final extension step at 72 °C for 5 min. reactions were cooled to 4 °C. The conditions for *T.equi* and *B.caballi* nested PCR were 95°C for 3 min followed by 25 cycles, each consisting of denaturation at 95 °C for 5 sec, annealing at 60 °C for 5sec, and extension at 72 °C for 5 Sec. then a final extension step at 72 °C for 5 min. reactions were cooled to 4 °C. Positive controls, were obtained from the OIE Equine piroplasmosis reference lab located in Pullman, WA, and negative control (sterile water) were always included for PCR amplification. Amplification products were electrophoresed on 1.5% agarose gel stained with SybrSafe (Invitrogen) using 100 bp DNA ladders as a size marker (Fermentas, Germany). They were visualized under UV trans-illuminator and photographed using gel Documentation system (Bio-Rad).

(Table 1):Oligonucleotide primer pairs used in PCR amplifications for detection of *Babesia* species in equines.

Parasite	**Primer name**	**PCR reaction**	**Gene name**	**Primer sequence**	***Reference***
T. (B.)equi	Beq-F	External	EquiMerozoite Antigen-1gene(ema-1gene)	5'-GAGGAGGAGAAACCC AAG-3'	
	Beq-R			5'-GCCATCGCC CTTGTAGAG-3'	*(Baptista et al., 2012)*
	BeqN-F	Nested		5'-TCAAGGACAACAAGCCATAC-3'	
	BeqN-R			5'-TTGCCTGGAGCCTTGAAG-3'	
B. caballi	Bca-F	External	B.caballi Rhoptry associated protein gene (RAP-1gene)	5'-GATTACTTGTCGGCTGTGTCT-3'	***(Schwintet al., 2008)***
	Bca-R			5'-CGCAAGTTCTCAATGTCAG-3'	
	BcaN-F	*Nested*		*5'-GCTAAGTACCAACCGCTGA-3'*	
	BcaN-R			*5'-CGCAAGTTCTCAATGTCAG-3'*	

Sequence analysis

Purified amplified DNA fragments were submitted for sequence confirmation in a (GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers. Comparisons with sequences deposited in the Gen-Bank were done using the basic local alignment search tool (BLAST) and Percent sequence identity of Egyptian isolates with *Babesia*spp reference strains will be calculated.

Statistical analysis

Data were analyzed using SPSS version 14 computer program. The Chi-square test was applied at probability of $p<0.05$ to compare the infection rate with regard to hosts and techniques [32]

Results

Microscopic examination: ME revealed that 33 donkeys and 46 horses were infected with equine theileriosis with a prevalence rate of (24.8% and 27.4%) respectively, and overall prevalence79 (26.2 %). Statistical analysis of these data using a $\chi 2$ test results shows that there is no significant difference in the rate of *T.equi* infections among donkeys and horses those exhibited positive *T. equi* infection by ME (Table 2).

(Table 2): Comparison of the positivity of equine theileriosis using the four diagnostic techniques: ME, iELISA , cELISA and nPCR in donkeys and horses.

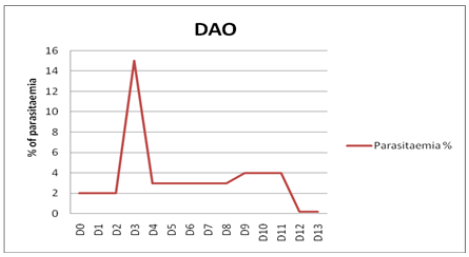
Animal	No. of inspected animals	ME		iELISA		cELISA		nPCR	
		No.of infected animals	Infection(%)	No. of infected animals	Infection (%))	No.of infected animals	Infection (%))	No. of infected animals	Infection(%))
Donkeys	168	33	24.8	71	53.4	34	25.6	67	50.4
Horses	133	46	27.4	30	17.9	26	15.5	104	61.9
χ^2		2.513		16.644		1.067		8.006	
Sig.		0.113		0.000		0.302		0.005	

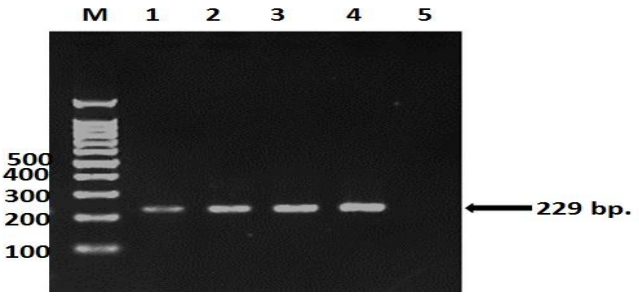
Data was analyzed by $\chi^2$and Sig. represents statistical significance for *P<0.05

Splenectomy of the naturally infected donkey:

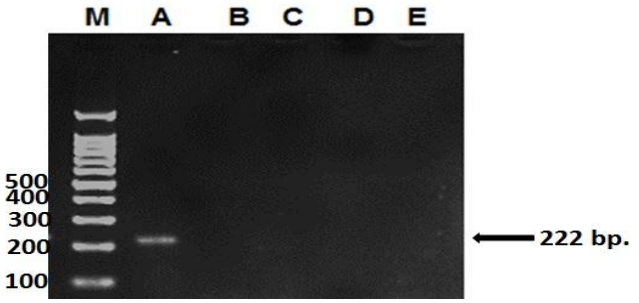
The microscopical examination of blood samples taken from splenectomized donkey during infection revealed that the parastemia began to increase gradually from 2% at the first day after the splenectomy operation (DAO) and reached 15% at third DAO then decrease again 0.2% at 14 DAO (chart 1). The nPCR and sequencing confirmed only the presence of *T.equi* pure strain in the naturally infected splenectomized donkey (Fig. 1 A -B).

(Chart 1) Percentage of parastemia on different days after operation (DAO).





(Fig. 1A): SYBR safe –stained agarose gel of PCR amplified fragments; 100-bp DNA ladder run in lane M; lane 1: represents positive control; lanes 2-4: represent positive equine blood samples with T.equi from the three splenectomized donkeys. (PCR amplified fragment (229- bp), lane 5 negative controls



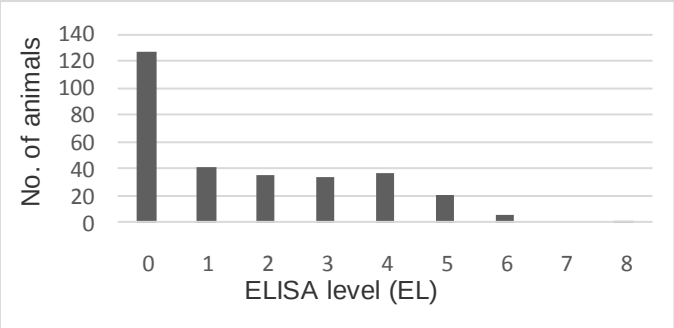
(Fig. 1B): SYBR safe –stained agarose gel of PCR amplified fragments; 100-bp DNA ladder run in lane M; lane A positive control of B.caballi, lane B,C,D represent negative blood samples from the three splenectomized donkeys. PCR amplified fragment (222- bp), lane 5 negative controls

Serological diagnosis of *T. equi* using iELISA: IELISA analyses showed that out of 301 tested samples, 101 (33.5%) were positive for *T.equi* antibodies of which; 71 (53.4%) donkey, and 30 (17.9%) horse were positive for *T.equi* antibodies. Statistical analysis of these data using a $\chi 2$ test results shows significantly higher differences in the rate of *T.equi* infections among donkeys and horses using iELISA (Table 2) and the calculated (EL) were shown in (Table 3 & Chart 2)

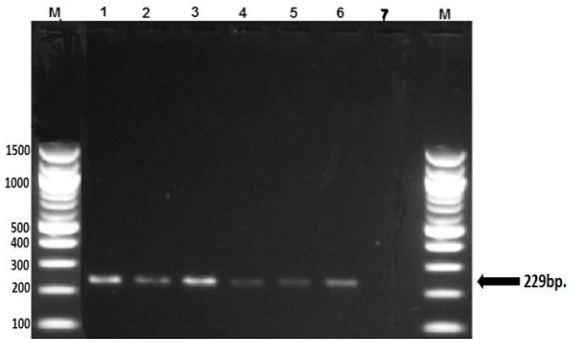
(Table 3) ELISA levels and positive serum ratio (S/P) for the detection of IgG antibodies to *T. equi*.

ELISA level (EL)	S/P	No. of animals
0	0.000-0.215	127
1	0.216-0.290	41
2	0.291-0.392	35
3	0.393-0.529	34
4	0.530-0.714	37
5	0.715-0.964	20
6	0.965-1.301	6
7	1.302-1.756	0
8	1.757-2.371	1

(Chart 2) ELISA levels and positive serum ratio (S/P) for the detection of IgG antibodies to T. equi



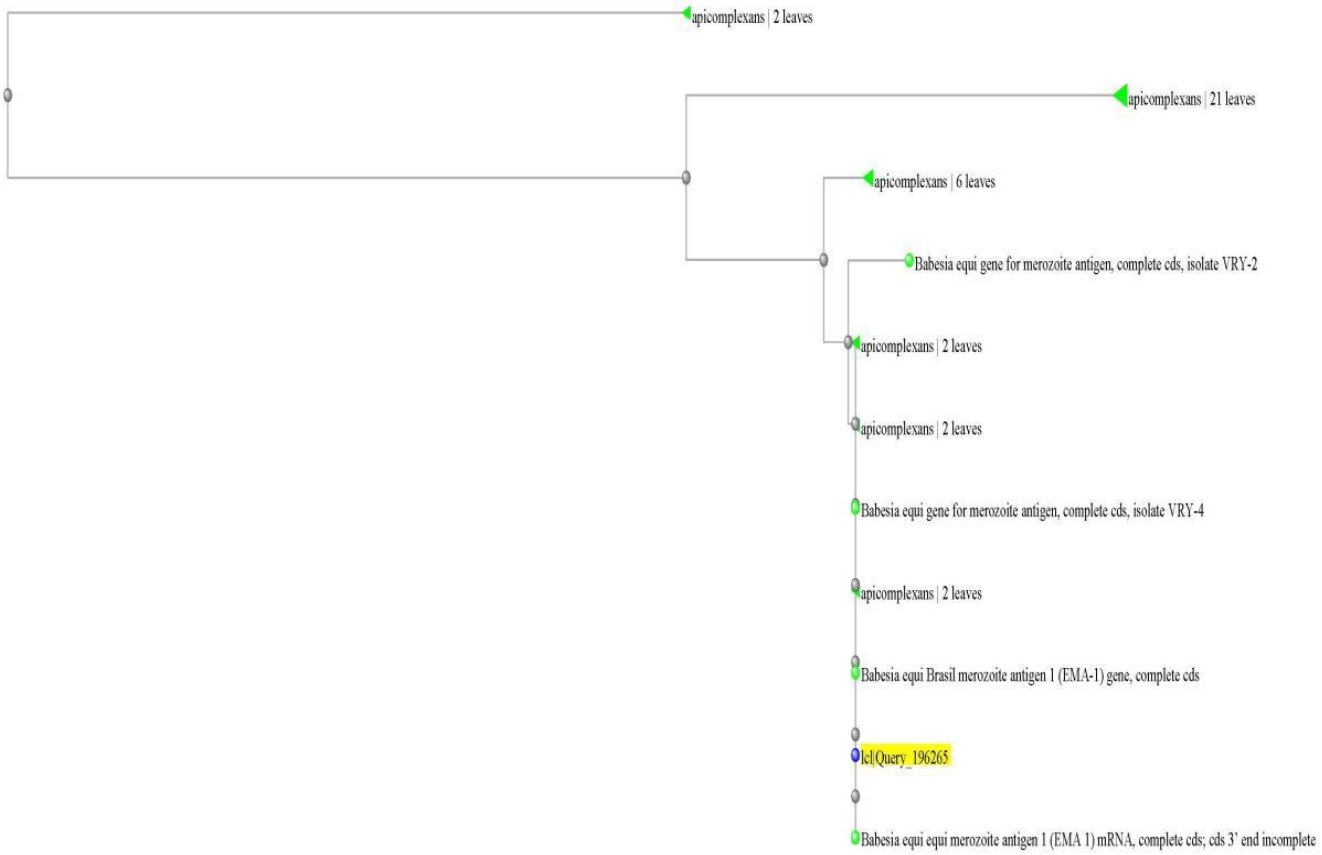
Serological diagnosis of *T. equi* by cELISA: cELISA analysis showed that 60 (19.9%) equine sera were positive for specific antibodies of *T.equi* where; 34 (25.6%) and 26 (15.5%) donkey and horse sera were positive for *T.equi*, respectively. Statistical analysis of these data using a $\chi 2$ test results shows that there are no significant difference in the rate of *T.equi* infections among donkeys and horses those exhibited positive *T. equi* infection by cELISA (Table 2).



(Fig. 2): SYBR safe –stained agarose gel of PCR amplified fragments,100-bp DNA ladder run in lane M; lane 1: represents positive control; lanes 2-6: represent positive blood samples; lane 7: represents negative control (double distilled water); the arrow showed T.equi PCR amplified fragment (229- bp).

Molecular diagnosis of *T. equi* using nPCR: 171 (56.8%) samples were positive for the presence of *T.equi*. Where; 67 (50.4%) and 104 (61.9%) donkey and horse samples, respectively were positive for *T.equi*. The PCR product was detected in agarose gel at 229 bp (Fig. 2). Statistical analysis of these data using a $\chi 2$ test results shows that there are significant difference in the rate of *T.equi* infections among donkeys and horses those exhibited positive *T. equi* infection by nPCR (Table 2).

Sequencing: Sequence analysis of PCR products positive for *T. equi* gave 231 nucleotides for each and each of them recorded 100% similarity with *Babesia equi* merozoite antigen 1 under (our GenBank Accession number KX262963). The phylogentic tree of their Sequencing was illustrated in (Fig.3)



(Fig.3): Phylogenic tree of sequencing of PCR product from donkeys

Comparison of the diagnostic performance of the four diagnostic techniques: ME, iELISA, cELISA and nPCR,: The diagnostic performances of cELISA, nPCR and iELISA used in our study were then statistically compared using a χ^2 test. The results of this statistical analysis are shown in (Table 4). The results confirmed that cELISA technique detected significantly higher *T. equi* infection rates compared to nPCR and iELISAin donkeys and horses and also significant differences between nPCR compared to iELISA in donkeys and horses. However, no significant differences were found among the ME and iELISA tests for the detection of equine theileriosis.

(Table 4). Representation of statistical analysis comparing number of infected animals for *T. equi*and by ME, cELISA, iELISA andnPCR .

Animal	cELISAvsnPCR		cELISAvsiELISA		nPCRvsiELISA		iELISAvs ME	
	χ^2	Sig	χ^2	Sig	χ^2	Sig	χ^2	Sig
Donkeys and horses	53.338	0.000	10.441	0.001	18.015	.000	2.955	0.086

Data was analyzed by $\chi^2$and Sig. represents statistical significance for *P<0.05

Discussion

The goal of this study is to establish the suitability of currently available equine theileriosis diagnostic methods. The diagnosis based exclusively on clinical signs it is not possible since it can easily be confused with other conditions [3]**.** Thus, the presence of *T. equi* infection in equines in Egypt was evaluated in this study using microscopical examination, cELISA, iELISA and nPCR, methods.

In this study, the overall prevalence of *T. equi* infection (26.2%) by ME was lower than those recorded before (38.9 – 71.5%) in Egypt [15,7,1]. In contrary, the lower incidence (13.9% and 18.0%) was detected in Egypt by [20,18]**.** The difference might be due to variation in time of sampling where the samples might be collected at acute or chronic stage of the disease. Also, might be due to the difference in the preventive measures as treatment of infected animals or control of vectors. In other countries varied infection rates were reported in Netherlands, [14] (9.5%). In Italy, [33] (3.1%) . In Brazil, 7.19%, and 3.52% [34,35]**.** In Central Ethiopia, [36] (12.2%). In Iran, [37,38,39] reported 5%, 9.1% and 9.7% of *T. equi* and *Babesia* spp. infection, respectively. In Costa Rica, [40] (24.6%). In Sudan, [41] (2.08). It was observed that the incidence in Egypt was higher than those recorded in other countries might be due to different in environment of equine breeds (temperature and humidity) which effect on the activity of ticks , type of equine(race or working) , hygienic measurement and vector control. In the present study, It was found that no significant difference between the prevalence of infection with *T. equi* in horses (27.4%) and donkeys (24.8%). This finding agrees with the results recorded before on horses and donkeys by [15,7,16] who recorded 51.2% & 51.6%, 78.6% &74% and 6.7% & 4.7%, respectively.

Serological diagnostic methods were also used in this study in order to assure the highest probability of identifying all equines infected with *T.equi*. In the present study, L2 antigen that used in iELISA was more sensitive and immunogenic than L1 antigen. This may be due to hemoglobin gives false high protein concentration and masking of antigen epitope. *T. equi* infection was recorded with overall prevalence 19.9% and 33.5% by using cELISA and iELISA, respectively. These differences may be due to the nature of antigens used, as cELISA depends on competition between serum antibodies and monoclonal antibodies against recombinant antigen (EMA-1). The higher prevalence recorded by iELISA may be due to the higher specificity of cELISA in diagnosis of *T.equi* than iELISA. The cross reactivity between *T.equi* and *B. caballi* in iELISA was recorded before by using crude antigen [19]. These could be explained as negative samples by cELISA were positive by iELISA and may be infected with *B.caballi.* So we recommended using of iELISA for diagnosis of *Babesia* spp. These results disagree with the results of [42] who found that the prevalence of *T.equi* by iELISA (65%) lower than that recorded by cELISA (67.7%). These may be due to the utilization of recombinant purified protein (EMA-1) in iELISA. In this study, the prevalence of *T. equi* diagnosed by cELISA was slightly (19.9%) higher than that recorded by 18% in Egypt [20], 4% in Erzurum [43], 11% in Greece [44], 14.6% in Jordon [45] and in Portugal [30], recorded 2.8% and 9.3% in Azorean and mainland Portugal, respectively. However, the percentage of infection with *T. equi* using cELIZA in the present study was lower than that recorded by 33.7% in Israel [46], 67.7% in Argentina [42], 32.4% in Dubai [47], 81.2% in Kenya [48], 88.5% in Costa Rica [40], 39.8% in Italy [49] and 75% in India [50]. In the present study, there were non-significant differences between cELISA results in donkeys and horses. These findings were disagreeing with other investigators [20]. However, the prevalence of infection with *T. equi* in donkeys was significantly higher than that of horses by using iELISA, these results were agreeing with previous investigator in Egypt, [7] who used IFAT**,** [15]who used haemaglutination test. In the present study, the prevalence of *T. equi* by iELISA was (33.5%) higher than that recorded before (9.6%, 30.0% & 8.8 %) by [51,18,52] respectively. However, the prevalence of *T. equi* was lower than that recorded by 73.9% in Brazil [53], 65% in the north and east of Argentina [42], 72.8% in Mongolia [54]and 34% in China [55].

Previous investigators used other serological tests for detection of the prevalence of *T. equi* in Egypt and they found that 90.4% by IFA [15]**,** 77.6% by haemagglutination test [7]**,** 59.6% and 63.6% by agar gel precipitation (G.P.) and passive haemagglutination (PHA) tests [56] and 50% by IFA [1].

In the present study, the *T.equi* infection was recorded by using nPCR with overall prevalence 56.8%. The prevalence of *T. equi* was higher in horses (60.8%) than in donkeys (50.4%) these could be explained by that the most of examined infected horses were in an acute or subclinical stage of infection. This result was lower than that detected in Egypt (77.8%) by [1] and higher than that recoded (26%,10.83% and 13.9%) by [18,38,57]. This is may be due to the higher sensitivity of nPCR than conventional PCR in diagnosis of subclinical infection and carrier animals. In the current work, the detected prevalence was lower than that recorded by 96.0% in Brazil [58] using nPCR, 59.7% in Brazil [34] using a multiplex real-time (PCR), 63.5% in Brazil [35] using

nPCR and in Egypt**,** 47.7% using nPCR [20], 70.3% in Italy using real time PCR [49] and 96.8% in Iran using PCR [39]. The lower *T. equi* prevalence was recorded in the Netherlands (15.2%), Brazil (15.0%), Dubai (33%), Azores and mainland Portugal (49.0%), Hungary (45.0%) , Thailand (1.25%), Turkey(2.96%) and Costa Rica(46.2%) [14,59,47,29,37,52,60,40]**.** Sequence analysis of PCR products positive for *T. equi* recorded 100% similarity to previously published *Babesia equi* merozoite antigen- 1 (ema-1 gene) reference sequence (our GenBank Accession number KX262963) . our sequence recorded 100% similarity with other authors [61,62,63,64,65] under accession number (L13784.1, U97167.1, JQ782603.1, XM_004829445.1 and KT443900.1**)** respectively.

In the present study, there are statistical difference between ME and nPCR, the incidence with nPCR was higher than ME as nPCR more sensitive than ME it can detect parasitemia 3.6 times more than ME [14] and can detect parasitemia equivalent to 0.000006 % [66]**.** *T.equi* parasite are usually not completely eliminated from blood of animals after treatment or naturally recovery and animals may remain lifelong carriers [67]**,** additionally, molecular detection of these agents requires DNA isolation from parasites that are physically present in the blood samples to a detectable level above the sensitivity threshold of the method used [68]. Therefore, failure to detect *T. equi* by nPCR is most probably due to parasite clearance from the circulating blood or reduction to a level beyond the detection sensitivity of the assay used [35]. In the present work, cELISA detected significantly lower prevalence than detected by nPCR assay. The limited correlation found among these methods may be due to the fact that these two tests detect different entities (DNA vs antibodies) and thus differ in principle. Hence, PCR can be considered reliable for the diagnosis of active infection, serological tests are usually considered as the method of choice for detecting persistently infected animals [20]. Also the data show statistically significant differences between iELISA and cELISA for the detection of *T. equi* infections in equine as cELISA is specific for *T. equi* only.

The blood smear examination from naturally infected donkey after splenectomy revealed that the parastaemia began to increase gradually from 2% on the first day and reached 15% on the third DAO due to removal of the spleen. The parasitemia decreased again 0.2% at 14 DAO. This is may be due to activation of other defense mechanism. This result agrees with the result recorded by [69] who found that the number of the detectable stages of *B. equi* in blood increased from 0.05-0.1 % to 2-20% after splenectomy of latent infected donkeys. In India, [70] found that the parasitemia reached its maximum level 80% after 3-4 days after splenectomy. The spleen is one of the peripheral organs of the immune system which responsible for defense mechanism in the body against infection, the splenectomy operation act as a stress factor on animal so after removal of spleen the parasite was spread.

Conclusion

The cELISA and nPCR are recommended in diagnosis of *T. equi* infection, especially in sub-clinical phase. The use of purified antigen in iELISA is recommended in order to avoid the cross-reactivity. The combination between serological and molecular methods will be key tools for helping the design and implementation of more effective control measures.

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