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Serological and Molecular Estimation of *Theileria equi* Infections in Horses of Baghdad, Al-Qadisiyah, and Wasit Provinces / Iraq

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Abstract

This study carried out to estimate prevalence of equine theileriosis (ET) caused by *Thieleria equi* in horses from different region of three Iraqi provinces (Baghdad, Al-Qadisiyah, and Wasit) using two diagnostic methods, competitive-ELISA (c-ELISA) and polymerase chain reaction (PCR). Therefore, a total of 130 horses of different sexes, ages and activities were selected randomly for collection of jugular blood samples during ten months (March to December/2018). Overall results were revealed on 36.92% and 5.38% positive horses with ET by c-ELISA and PCR, respectively. comparison of infection rates between the diagnostic methods were showed that 4.62% positives by both assays, 0.77% positive by PCR only, 32.31% positives by c-ELISA only, and 62.31% were negatives by both assays. An association of ET infection rates to epidemiological risk factors (age, gender, activity, location, month of season) was evaluated in the present study. Among seropositive horses, significant increases ($P<0.05$) in c-ELISA results were reported in groups of ≥11 years (66.67 %); females (32.14%); non-race horses (40.57%); Al-Qadisiyah (37.84%) and Wasit (37.70%) provinces; and August (61.54%), respectively. Concerning to PCR results, significant elevations were appeared in groups of 1-3 years (11.76%); females (7.14%); non-race horses (6.60%); Al-Qadisiyah (8.11%) province; and June (23.08%), respectively.

Keywords: Theileria equi, Horse, Iraq, c-ELISA, PCR

Introduction

Equine theileriosis (ET) is a protozoan hemoparasitic infectious disease which caused by *Theileria equi* classified under *Theileriidae* family of *Piroplasmida* order (Mehlhorn and Schein, 1998; Maharana *et al.,* 2016). Many species of ixodid ticks from *Amblyoma*, *Dermacentor*, *Hyalomma* and *Rhipicephalus* genera are implicated for transmission of infection (de la Fuente *et al.,* 2008). Globally, the disease is considered as one of the most important diseases that poses a serious threat to the horse industry and has great implication for the international movement of horses (Sumbria *et al.,* 2014). Indeed, ET is endemic in many tropical and sub-tropical areas as well as in temperate regions (Kouam *et al.,* 2010). At field, ET can be exhibited 3 forms involving a peracute, acute, and chronic. Peracute form occurs in neonatal foals in uterus after infection of their mothers; acute form occurs frequently and characterized by pyrexia, inappetence, hemoglobinuria, icterus, limb edema, and regenerative hemolytic anemia and even death; and chronic form that manifested by

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ambiguous clinical signs (Rothschild and Knowles, 2007; OIE, 2008; Salib et al., 2013). In endemic areas, most infected horses are apparently healthy without any clinical signs (subclinical) due to an endemic stability (Farkas *et al.,* 2013). However, chronic and subclinical horses can carry very low numbers of parasite in their blood circulation to act as a carrier for several years (Laus *et al.,* 2015).

Nowadays, several laboratory diagnostic techniques are available to detect *T. equi* in equdae. Light microscopy of Giemsa's-stained thin blood smears is the most conventional method applied widely and routinely in diagnosis of acute infection due to cheap cost; however, it is unreliable in chronic or subclinical infections that have very low levels of parasitaemia (Figueroa and Buening, 1995; Bashiruddin *et al.,* 1999). To overcome such problems, a number of serological assays were used mostly in large-scale epidemiological studies (Moretti *et al.,* 2010; Mujica et al., 2011). Although, ELISA is more time consuming and labor intensive, it remains more sensitive and specific than other serological assays in detection of specific anti- *T. equi* antibodies in both acute and latent infections (Ybañez et al., 2018). Also, many molecular techniques such as polymerase chain reaction (PCR), reverse line blotting (RLB), loop-mediated amplification (LAMP), and nucleic acid sequence-based amplification (NASBA) were developed. In general, none of these methods could be considered better than another as their score in diagnostic applications are greatly depend on the laboratory size (Criado-Fornelio, 2007).

However, competitive-ELISA and PCR are greatly increases the probability of specific detection of pathogen in a test sample. In addition, they are useful even in dealing with a large number of samples at a much higher sensitivity and have the required flexibility of automation and up gradation (Gasser, 2006; Afridi *et al.,* 2017). In Iraq, there are very limited information on prevalence and risk factors related to ET (*T. equi*); therefore, the present study was designed to evaluate the prevalence of ET caused by *T. equi* using a two diagnostic methods, c-ELISA and PCR among horses of Baghdad, Al-Qadisiyah, and Wasit provinces, Iraq. Also, association of ET infection rates with the risk factors (age, gender, location, activity, month of season).

Material and methods

Samples

During the period of March to December (2018), an overall 130 horses of different sexes, ages and activities were selected randomly from many regions of Baghdad, Al-Qadisiyah, and Wasit provinces, Iraq. Blood samples were obtained aseptically from jugular vein using 10 ml disposable syringes of gauge 18, and divided into two aliquots: 5 ml in EDTA tube for DNA extraction, and 5m into free-anticoagulant gel tube for serum collection. Serums were collected after centrifugation of blood samples at 4000 rpm for 10 minutes and transferred to 1.5 ml eppendorf. Both tubes of EDTA whole blood and sera samples were kept frozen until -20°C until be tested. In addition, required data were collected using a specific questionnaire paper to investigate the association of some risk factors (Age, gender, location, activity, and month of season) to prevalence of *T. equi* infections.

Serology by c-ELISA

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For detection of specific anti- *T. equi* IgG antibodies, serum samples of all study horses were examined serologically using a commercially available competitive-ELISA (*VMRD, USA*) kit that coated with the recombinant *T. equi* merozoites antigen 1 (EMA-1), a surface protein on merozoites of *T. equi*. According to manufacturer's instruction; serum samples in addition to positive and negative controls were warmed at room temperature (23±2°C), diluted, loaded into the Antigen-Coated Plate, incubated for 30 minutes at room temperature, and washed 3 times with washing solution. Primary antibody was added to each well, incubated for 30 minutes at room temperature, and washed 3 times. Secondary-conjugated antibody was added to each well, incubated for 30 minutes at room temperature, and washed 3 times. Substrate solution was added to each well, incubated for 15 minutes at room temperature, and then the stop solution was added and the optical density (OD) was read immediately using an ELISA plate reader (*BioTek, USA*) at 620 nm. Using the specific formula that mentioned by the manufacturer, inhibition percents (% I) were calculated as following: % I = [1- (Sample OD ÷ Mean of Negative Control)] × 100. Sample's results were interpreting as following: if sample is positive if OD ≥40 %I; whereas, sample is negative if OD <40 %I.

Molecular qPCR

According to manufacturer's instruction (*iNtRON Biotechnology, South Korea*), protocol A was followed to extract the DNA from a whole EDTA-blood samples. Purity and concentration of extracted DNA were evaluated by the Nanodrop (*Thermo-Scientific, UK*). Eppendorf tubes of extracted DNA were kept frozen at -20°C until be used.

Conventional-polymerase chain reaction (q-PCR) was applied to detect *T. equi* merozoite antigen using two sets of primers are 1 (EMA-1) gene; [EMA-1F (5'GCATC CATTGCCATTTCGAG-3') and EMA-R (5'TG CGCCATAGACGGAGAAGC-3')], and [*Beq*-F (5'-GAGGAGGAGAAACCCAAG-3') and *Beq*-R (5'-GCCATCGCCCTTGTAGAG-3')], with considering that the positive amplicon size is 750bp and 567bp, respectively (Alhassan *et al.,* 2005; Mahmoud *et al.,* 2016). Briefly, a total 5µl of DNA template was pipetted in a final volume of 20µl containing 2 µl of primer and 13 µl of PCR water within the PCR-Premix (*BIONEER, South Korea*), and subjected to the following cycling conditions of thermocycler (*BIO-RAD, USA*): 95°C for 3 minutes, followed by 25 cycles consisting of denaturation at 95°C for 20 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 20 seconds. Final extension cycle at 72°C for 5 minutes was performed and reactions were cooled to 15°C. Final products was kept at 4°C until be used.

A total of 10µl of each final PCR product was analysed by electrophoresis using 1-kbp DNA ladder (1000-1500bp), (*Qiagen, Germany*) on 1.5% agarose gel (*iNtRON Biotechnology, South Korea*) containing 1µg/ml of ethidium bromide (*Biotech, Canada*). The resulting DNA fragments were visualized by ultraviolet transilluminator.

Statistical analysis

All collected data of c-ELISA and PCR techniques were introduced, categorized, tabled, figured, and analysed by two computerized programs, Microsoft Office Excel (*2013*) and IBM/SPSS (*Version*[23]). Association between the two diagnostic methods was assessed by Cohen's kappa (***k***) test and McNemar (x^2) test; whereas, the relationship between the horse

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infection rates and study's risk factors (Age, gender, location, months of a season, and activity) was analysed by Chi-square (x^2) test. Statistical variations were considered as significant at a p value of < 0.05.

Results

Out of 130 blood samples, the results revealed that there were 48 (36.92%) and 7 (5.38%) positive horses by c-ELISA and PCR, respectively, (Table 1).

Table (1): Total results for study horses tested by c-ELISA and PCR

Test	Total No.	Positives	Negatives
c-ELISA	130	48 (36.92%) *	82 (63.08%)
PCR	130	7 (5.38%)	123 (94.62%)

Significance * (P<0.05)

Comparatively, the results showed that 4.62% were positives by both c-ELISA and PCR, 0.77% positive by PCR only, 32.31% positives by c-ELISA only, and 62.31% were negatives by both assays, (Table 2).

Table (2): Comparison of the performance of c-ELISA and PCR for diagnosis of ET

PCR	c-ELISA		Total
	Positive	Negative	
Positive	6 (4.62%)	1 (0.77%)	7 (5.38 %)
Negative	42 (32.31%)	81 (62.31%)	123 (94.62%)
Total	48 (36.92%)	82 (63.08%)	130

Of all examined horses, ODs of positive samples by c-ELISA were reported significant variation in their values, (Figure 1).

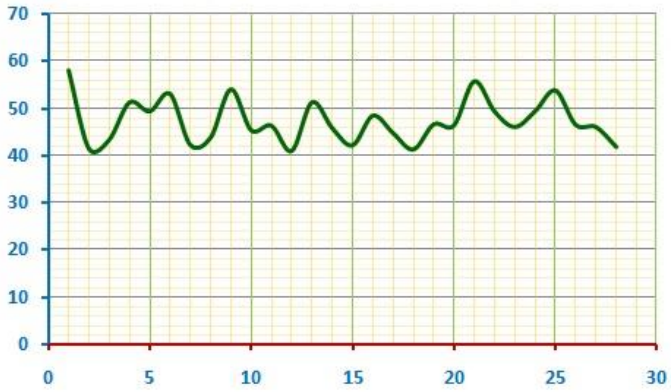

Figure (1): Levels of titers in seropositive horses by c-ELISA

An association of ET infection rates to epidemiological risk factors (age, gender, activity, location, month of season) was evaluated in the present study. Among seropositive horses,

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significant increases (P<0.05) in c-ELISA results were reported in groups of ≥11 years (66.67 %); females (32.14%); non-race horses (40.57%); Al-Qadisiyah (37.84%) and Wasit (37.70%) provinces; and August (61.54%), respectively, (Table 3).

Table (3): Association of c-ELISA positive ET infection rates with risk factors

Risk factors	Groups	Total No.	Seropositive [No. (%)]	Seronegative [No. (%)]
Age / Year	≤ 1	8	0	8 (100%)
	1- 3	17	3 (17.65 %)	14 (82.35%)
	4 - 6	61	24 (39.34 %)	37 (60.66%)
	7 - 10	32	13 (40.63 %)	19 (59.38%)
	≥ 11	12	8 (66.67 %) *	4 (33.33%)
Gender	Female	84	27 (32.14%) *	57 (67.86%)
	Male	46	11 (23.91%)	35 (76.09%)
Activity	Race	24	5 (20.83%)	19 (79.17%)
	Non-race	106	43 (40.57%) *	63 (59.43%)
Location	Baghdad	32	11 (34.38%)	21 (65.63%)
	Al-Qadisiyah	37	14 (37.84%) *	23 (62.16%)
	Wasit	61	23 (37.70%) *	38 (62.30%)
Month	March	13	3 (23.08%)	10 (76.92%)
	April	13	4 (30.77%)	9 (69.23%)
	May	13	7 (53.85%)	6 (46.15%)
	June	13	4 (30.77%)	9 (69.23%)
	July	13	3 (23.08%)	10 (76.92%)
	August	13	8 (61.54%) *	5 (38.46%)
	September	13	5 (38.46%)	8 (61.54%)
	November	13	3 (23.08%)	10 (76.92%)
	October	13	4 (30.77%)	9 (69.23%)
	December	13	3 (23.08%)	10 (76.92%)

Significance * (P<0.05)

Concerning to PCR results, significant elevation (P<0.05) was seen in groups of 1-3 years (11.76%); females (7.14%); non-race horses (6.60%); Al-Qadisiyah (8.11%) province; and June (23.08%) respectively, (Table 4).

Table (4): Association of PCR positive ET infection rates with risk factors

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Risk factors	Groups	Total No.	Positive [No. (%)]	Negative [No. (%)]
Age / Year	≤ 1	8	0	8 (100 %)
	1- 3	17	2 (11.76 %) *	15 (88.24%)
	4 - 6	61	5 (8.20 %)	56 (91.80%)
	7 - 10	32	0	32 (100%)
	≥ 11	12	0	12 (100%)
Gender	Female	84	6 (7.14%) *	78 (92.86%)
	Male	46	1 (2.17%)	45 (97.83%)
Activity	Race	24	0	24 (100%)
	Non-race	106	7 (6.60%) *	99 (93.40%)
Location	Baghdad	32	1 (3.13%)	31 (96.87%)
	Al-Qadisiyah	37	3 (8.11%) *	34 (91.89%)
	Wasit	61	3 (4.92%)	58 (95.08%)
Month	March	13	0	13 (100%)
	April	13	0	13 (100%)
	May	13	1 (7.69%)	12 (92.31%)
	June	13	3 (23.08%) *	10 (76.92%)
	July	13	2 (15.38%)	11 (84.62%)
	August	13	1 (7.69%)	12 (92.31%)
	September	13	0	13 (100%)
	November	13	0	13 (100%)
	October	13	0	13 (100%)
	December	13	0	13 (100%)

Significance * (P<0.05)

Discussion

Worldwide, ET is a severe intraerythrocytic protozoan disease of equidae which endemic in most temperate, tropical and sub-tropical countries (Wise et al., 2013; Prochno et al., 2014). The growth of international equine sport and horse trading has increased the likelihood that parasites and pathogens will be introduces into previously unaffected areas (Baptista et al., 2013). For epidemiological studies, combination of both serological c-ELISA and molecular PCR methods would be used. In international trading, c-ELISA is considered as official test because it shows both current and past exposures of the parasite; while PCR targets only the

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DNA of *T. equi* and shows the current infections (Munkhjargal et al., 2013; Sumbria and Singla, 2015).

In this study, two diagnostic methods (c-ELISA and PCR) were used to estimate the prevalence of infection and revealed on 21.54% and 5.38% positive horses, respectively. In Iraq, the present results were lower than reported serologically by (Alsaad et al., 2012) (71.73%); whereas molecularly, it was in agreement with (El-Seify et al., 2018) whose detected (5.76%), and lower than results (32%) reported by (Saleem and Al-Samarai, 2018). Globally, it recorded that the seroprevalence of ET was 10.14% in Saudi Arabia (Alanazi et al., 2012), 14.6% in Jordan (Abutarbush et al., 2012), 14.8% in Egypt (Mahmoud et al., 2016), 32.4% in United Arab Emirates (Jaffer et al., 2010), 39.8% in Italy (Del Pino et al., 2016), 44% in Spain (Cortés et al., 2017), 48% in Iran (Abedi et al., 2014), 51.16% in China (Wang et al., 2013), 63.5% in Sudan (Salim et al., 2013), 70.5% in Venezuela (Mujica et al., 2011), and 78.3% in Brazil (Vieira et al., 2013). Molecularly, it detected that the prevalence was 8.8% in Turkey (Guven et al., 2017), 14.14% in India (Sumbria et al., 2016), 18.8% in Jordan (Qablan et al., 2013), 22.5% in Central Balkans (Davitkov et al., 2016), 35.95% in Sudan (Salim et al., 2013), 36.4% in Egypt (Mahmoud et al., 2016), 45% in Iran (Abedi et al., 2014), 78.8% in Brazil (Heim et al., 2007), and 80% in France (Fritz, 2010).

It has been showed that the seropositive results by c-ELISA were higher than detected by PCR. This finding might be attributed either to the previous exposure of seropositive horses to *T. equi* as the antibodies can persist for many weeks, months or even years; parasite clearance from the circulating blood of the host; or to presence of PCR inhibitors. However, samples that prove to be negative by c-ELISA but positive by PCR might be explained by the ability of PCR to detect of early infections prior to development of specific antibodies. Since, the prepatent period of *T. equi* is 12-14 days; sampling time can play a critical role in detection of circulating haemoparasites (Jaffer et al., 2010; Baptista et al., 2013).

Variation in levels of IgG antibody titers in seropositive samples might be related to the kinetics of specific immunoglobulin isotypes that classified as cytophilic and non-cytophilic antibodies (Kana et al., 2018). It showed that *T. equi* specific cytophilic antibodies, IgGa and IgGb, were developed during acute infections suggested to be correlated with parasitemia control; whereas, non-cytophilic IgG (T) was developed after resolution of acute parasitemia to block the protective effect of cytophilic antibodies by the competitive binding to antigen (Lunn et al., 1995; Cunha et al., 2006). However, higher IgG antibody titers detected in this study may reflection for a frequent exposure of horses to *T. equi*.

As reported by many authors, identification of risk factors associated with ET could play an important role in adoption of control measures, and facilitate a better understanding for mechanisms by which *T. equi* is spread in host population (Santos et al., 2011; Costa et al., 2019). In this study, significant differences in frequency of ET were showed among the epidemiological risk factors (age, gender, activity, location, and month of season). The marked relation for ET prevalence was observed with the ages of study animals both diagnostic methods. While c-ELISA reported significant increases in ET among older horses (≥11 years), PCR results found that the younger horses (1-3 years) were more susceptible. However, the absence of *T. equi* infection in a group of ≤ 1 year, by both diagnostic methods,

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might be attributed to presence of active maternal immunity as well as good management. However, several studies suggested that the presence of parasites and parasite-specific antibodies were significantly related to age (De Campos et al., 2013; Ayala-Valdovinos et al., 2017). The abundance of tick increased with the age, since the older animals could have been exposed to ticks for a longer period than younger animals (Rüegg et al., 2007). Also, it showed that the infected horses might remain lifelong carriers of ET caused by *T. equi*, and treatments do not completely eliminate *T. equi* from the infected horse (Brüning, 1996). Regarding to PCR, the study reported an absence of *T. equi* in older age groups (7-110 and ≥11 years) which could be attributed to increase the sequestration of parasite in microvasculature with rising age (Rüegg et al., 2007). On other hand, the high prevalence of ET in an age group of (1-3 years) might be related to either decrement of maternal immunity or depressed their resistance to tick infestation. Other reasons include changes in sex hormones since many hormonal alterations can occur during this period which leading to increase the susceptibility of mare or stallion for infection (Alexander and Stimson, 1988; Roberts et al., 1996).

Significant high prevalence of ET was reported in females of this study as compared to males by both techniques. These findings were similar to that concluded by (Sevinc et al., 2008; Bahrami et al., 2014; Afridi et al., 2017) and incompatible with (Amir et al., 2012; Abedi et al., 2014; Cortés et al., 2017). However, possible causes for increased susceptibility of females are attributed to immunosuppression as a result of pregnancy, lactation, nutritional and environmental stresses (Afridi et al., 2017). Other reasons involved the partial selection of females over male counterpart for their draught; and strict living standard provided to the males utilized for breeding purposes (Moretti et al., 2010; Sumbria et al., 2014).

Analysis of activity factor revealed that the non-racing horses were more positivity than racing horses by both diagnostic assays. These findings concurrent with that reported by (Peckle et al., 2013; Benfenatki et al., 2016), and disagreement with that detected by (Abedi et al., 2014; Bahrami et al., 2014). It probably that the non-racing horses are kept outdoors under poor living condition for daily transport and farm/work activities, thereby being more exposed to tick. Also, racing horses received more balanced meals in addition to proper private and veterinary care, and kept in suitable stables. Advanced management, with diseases and tick control programs can reduce the chance of infection in horses kept for racing purposes (Peckle et al., 2013; Sumbria et al., 2016).

Regarding to location factor, significant increases were found serologically in Al-Qadisiyah and Wasit provinces, respectively; and molecularly, in Al-Qadisiyah province. This could be explained by the presence of biotic and abiotic characteristics and their interactions which determine the tick population and consequently the level of exposure to the pathogens (Del Pino et al., 2016). There are many factors that essential for constant maintenance of parasites and vectors such as soil factors (type, pore size and humidity), climate and microclimate sources; tick related factors (distribution, abundance, behavior, adaptation to new environments), urbanization, and contact with other domestic animals at pasture (Pfaeffle et al., 2013; Scoles and Ueti, 2015). However, subclinical infections have particular relevance to the horse-industry where the geographical movement of presumably healthy horses may

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aid in spread of parasite or where the subclinical infections might be negatively affected the animal's performance (Baldani et al., 2010; Bahrami et al., 2014).

Significant elevation in prevalence of ET among summer months by both diagnostic methods, serologically at August and molecularly at June was detected in current stusy. These results were similar to findings reported by (Moretti et al., 2010; Del Pino et al., 2016) and incompatible to that detected by (Rüegg et al., 2007) who concluded that neither date of sample collection nor abundance of tick infestation have a significant influences and assumed that the parasites and/or their specific antibodies are present in the host in absence of tick, and can be detected serologically. However, climatic changes over recent decades have probably led to a wider spatial distribution of vector ticks and extension in their activity; and effectively, an increasing the incidence of ET (Del Pino et al., 2016). In some conditions, stressful factors due to summer months might be broken the immunity leading to reactivation of latent infections as a result of stress.

Conclusion

In Iraq, information about the prevalence of ET in horse populations is essential as a contribution to partial knowledge about dynamic of ET to control the disease and reducing the economic losses generated. It is assumed that the regions of Al-Qadisiyah, Wasit, and Baghdad provinces, respectively, are enzootic areas to ET (*T. equi*) since the high positive horses by both c-ELISA and PCR. Also, it concluded that diagnosis of ET can greatly enhanced by utilization of recent diagnostic procedures. The epidemiological risk factors (age, gender, activity, location, and months of season) appeared significant variations ($P<0.05$) with the prevalence of positive horses to ET.

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