Comparing the efficacy of three diagnostic tools (microscopic examination, conventional PCR, and Real-Time PCR) in detecting *Theileria equi* infection among Egyptian equines

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Abstract

Equine theileriosis represents one of the main and serious health problems affecting equines industry globally, that is caused by tick-borne protozoan parasite called *T. equi*. This study aimed to assess the sensitivity of three diagnostic tools named: microscopic examination of a blood smear, conventional PCR, and Real-Time PCR (qPCR) to detect *T. equi* among equine population (n = 116) raised in Giza Governorate, Egypt. Microscopic examination of Giemsa-stained blood smears revealed the infection of 16.4% (19/116) of examined equines by *T. equi* while conventional PCR and qPCR revealed that 29.3% (34/116) and 43.1% (50/116) of examined equines were infected with *T. equi* respectively. Our results demonstrated that the qPCR had the highest sensitivity (100%) followed by conventional PCR (68%) while microscopic examination had the lowest sensitivity (38%). Furthermore, the negative predictive value (NPV) of qPCR was the highest (100%) compared to conventional PCR and microscopical examination (80.49% and 68.04% respectively) which revealed that all negative cases detected by qPCR were certainly correct compared to the other two diagnostic assays. Therefore, it is highly recommended to incorporate PCR diagnostic assays (conventional PCR and qPCR) alongside microscopic examination to evaluate the epidemiological status of equine theileriosis.

Introduction

Globally, the equine industry suffers from a tick-borne protozoan parasite named *Theileria equi* (*T. equi*). This parasite has endemicity in tropical, subtropical and some temperate zones of the globe (Ozubek & Aktas, 2018; Souza et al., 2019). Several tick vectors can transmit *T. equi*, the most vectors are those belonging to genera *Dermacentor*, *Boophilus*, *Rhipicephalus*, and *Hyalomma* whose distribution have a direct relation to the prevalence of *T. equi* (Bhagwan et al., 2015). Economically, *T. equi* has a significant effect on equine causing severe economic losses with impairment of equine movement. Equine theilerioses has several nonspecific symptoms such as anemia, icterus, edema, loss of condition, and even death. Survived animals remain carriers with low parasitemia for their lifetime thus representing a potential source of infection to surroundings (Malekifard et al., 2014; Mahmoud et al., 2016).

The standard technique for diagnosis of acute equine theilerioses is the microscopic examination of stained blood smears but due to low parasitemia in carrier animals, it is of low sensitivity (Kim et al., 2008; Elseify et al., 2018). Molecular methods are proved to have the highest specificity and sensitivity in the recognition of parasitic DNA. Polymerase chain reaction (PCR) can encounter parasitic nucleic acid in carriers with a parasitemia of 0.000001% (Alhassan et al., 2005; Davitkov et al 216).

This study aimed to detect the prevalence of *T. equi* among equines from Giza province, Egypt by microscopic examination of Giemsa-stained blood smear, conventional PCR, and Real-Time PCR (qPCR) and to differentiate between the sensitivity of these methods of diagnosis in the detection of *T. equi*.

Materials And Methods

Animals and sample collection

We collected 116 blood samples by jugular venipuncture method using EDTA tubes from equines reared in Giza Governorate, Egypt during the first semester of the year 2019. All collected samples were transferred to Animal Health Research Institute (AHRI) Dokki, Egypt for examination.

Microscopic examination

Giemsa-stained thin blood films were prepared from the collected whole blood and were inspected with a light microscope using an oil immersion lens (100X) for observation of intraerythrocytic merozoites belonging to *T. equi* according to (Levine, 1982).

Molecular examination

DNA extraction

Genomic DNA was isolated from all microscopically positive and negative samples using Thermo Scientific™ GeneJET Genomic DNA Purification Kit (Cat No #K0722) according to the manufacturer's instruction. The resulted DNA was preserved at -80°C till used in the downstream examination.

Conventional PCR

PCR technique was performed using specific primers for *T. equi*, (Bec-UF2) F, 5-TCGAAGACGATCAGATACCGTCG-3 and (Equi-R) R, 5-TGCCTTAAACTTCCTTGCGAT-3 established by (Alhassan et al., 2005). PCR reactions were performed using GoTaq® G2 Flexi PCR Kit (Promega, USA) with a total volume of 25 µl containing 5X Green GoTaq® Flexi Buffer (10 µl), 25mM MgCl₂ Solution (2 µl), PCR Nucleotide Mix (dNTPs) 10 mM each (1µl), Primer mix 10 pmol (1µl), 1.25 u GoTaq®G2 Flexi DNA Polymerase template DNA (5 µl), DNase/RNase free water (6 µl). The thermal condition was 950C for 5 min, followed by 35 cycles repeated of denaturation at 960C for 1 min, annealing at 600C for 1 min, and extension at 720C for 1 min. then a final extension at 720C for 5 min, after that holding stage at 40C for infinite time. 1.5 % ethidium bromide-stained agarose gel was prepared. After that 8 µl of the generated PCR products were loaded in it and allowed to migrate under constant volt of 80 V for 40 min. visualization of agarose gel by Gel documentation system (XR) (Bio-Rad.UK). positive samples for *T. equi* showed a band of molecular weight of 392 bp that were compared to a positive control sample obtained from the National Research Center (NRC), Egypt.

Real-Time PCR (qPCR)

The qPCR technique was accomplished conferring to (Kim et al., 2008) using specific primer and probe targeted 18s rRNA gene of *T. equi*, (Be18SF) a forward primer with a sequence 5-GCGGTGTTTCGGTGATTCATA-3, (Be18SR) a reverse primer with a sequence 5-TGATAGGTCAGAACTTGAATGATACATC-3 and (Be18SP) a fluorescent TaqMan probe with a sequence 5-AAATTAGCGAATCGCATGGCTT-3 that was labeled at its 5- end with a 6-carboxyfluorescein reporter dye and at 3- end with 6-carboxy-tetramethylrhodamine quencher dye.

The qPCR 20 μ l reaction consists of 4 μ l Mix stable qPCR 5x, 1 μ l *T. equi* detec-qPCR mix, 5 μ l DNA template and 10 μ l nuclease free water. The qPCR was executed in a 96-well optical plate (Stratagene Mx3005 P) following this thermal profile: Activation at 95⁰C for 15 min, followed by 45 cycles repeated of denaturation at 95⁰C for 20 sec, hybridization, extension and data collection at 55⁰C for 1 min.

Statistical Analysis

Results were compared by one-way analysis of variance. Differences were determined by Fischer’s least square difference test. The obtained results of the microscope, PCR, and qPCR were compared by using the x2 test. All significant alterations were detected by a p-value < 0.05.

Assessment parameters include specificity, sensitivity, likelihood ratio, positive predictive value (PPV) and negative predictive value (NPV) (Thrusfield, 2005).

Results

By microscopic examination, *T. equi* appeared as small single round, double round, single pyriform and maltase cross shapes (Figure. 1). While, by conventional PCR, the PCR product of *T. equi* were 392 bp (Figure. 2) and by qPCR, the samples were considered positive when they displayed a positive FAM signals, on the other side were considered negative when they displayed a negative FAM signals. Positive results were represented by S-shape amplification curve. The inter section point between amplification curve and the threshold line called cycle threshold (Ct). Ct of our positive samples were ranged from 20 to 35 (Figure. 3).

Examination of Giemsa-stained blood smears confirmed the *T. equi* infection among equines in 19 (16.4%) while conventional PCR showed 34 (29.3%) and qPCR presented 50 (43.1%). So, qPCR results are significantly higher than that of conventional PCR and microscopic examination as illustrated in (Table 1).

Table 1

Infection rate of *T. equi* among equines by microscopic examination, conventional PCR and qPCR.

Techniques	Number of infected samples	%
Microscope	19/116 ^c	16.4
Conventional PCR	34/116 ^b	29.3
Real time PCR (qPCR)	50/116 ^a	43.1
Data is presented as samples. total infected samples/total number of samples and total percent of infected. Present superscript letters (^{a-c}) differ significantly.		

Assessment parameters include specificity, sensitivity, negative predictive value (NPV) and positive predictive value (PPV) are illustrated in (Table 2) that showed (100%) specificity for all used 3 methods

for diagnosis but qPCR had the highest sensitivity (100%) over conventional PCR (68 %) and microscopical examination (38 %). Likelihood Ratio results are illustrated in (Table 3).

Table 2

Assessment of Giemsa-stained blood smears, Conventional PCR and qPCR for detection of *Theileria equi* amongst Equines.

Techniques	Total	TP	TN	FP	FN	Sensitivity	specificity	PPV	NPV
Microscopic examination	116	19	66	0	31	38	100	100	68.04
Conventional PCR	116	34	66	0	16	68	100	100	80.49
Real-time PCR (qPCR)	116	50	66	0	0	100	100	100	100
TP: True Positive, TN: True Negative, FP: False Positive, FN: False Negative, PPV: Positive Predictive Value and NPV: Negative Predictive Value									

Table 3

Estimation of the likelihood ratio (LR) for assessment and differentiation between microscopic examination, Conventional PCR, and qPCR for detection of *Theileria equi* amongst Equines.

Techniques	likelihood Ratio + ve (LR+)	likelihood Ratio -ve (LR-)
Microscopic Examination	Infinite (Inf)	0.62
Conventional PCR	Infinite (Inf)	0.32
Real-time PCR (qPCR)	Infinite (Inf)	0.00

Discussion

Equine theileriosis is an important worldwide distributed tick-borne protozoan disease affecting equines that's can lead to serious health and economic impacts. Moreover, most infected animals showing no clinical manifestations and develop into asymptomatic carriers thus they can potentially spread the infection (Ueti et al., 2008). Due to low parasitemia in carrier animals, traditional methods (microscopic examination of blood smears) usually fail to precisely identify those carriers. For this reason, tests that depend on detecting genomic fingerprints of pathogens proved as more promising tools for the diagnosis of equine theileriosis (Malekifard et al., 2014).

This study aimed to evaluate the sensitivity of microscopic examination of a blood smear, conventional PCR, and Real-Time PCR (qPCR) in determining the prevalence of *T. equi* among equines from Giza Governorate, Egypt.

In the current study, the prevalence of *T. equi* by microscopic examination was 16.4% (19/116). This was in agreement with some former Egyptian studies that recorded 19.8% (Radwan, 2009), 18 % (Mahmoud et al., 2016), and 13.9 % (Ibrahim et al., 2011). And lower than that recorded in Egypt by (Salib et al., 2013)

who recorded 34%, and (Farah et al., 2003) who recorded 38.9%. The obtained results were higher than some recorded in other countries such as Abedi et al. (2015) in Iran 3.8%, Malekifard et al. (2014) in Iran 6.25%, and Sumbria et al. (2015) in India 4.17%. Such difference may attribute to the vector activity in the study area, environmental conditions, and the disease stage -acute or chronic- at sampling (Mahdy et al., 2016).

According to the present study, the prevalence of *T. equi* by conventional PCR was 29.3% (34/116). This result was higher than those stated previously by (Ibrahim et al., 2011) in Egypt (26%), (Davitkov et al., 2016) in Central Balkan (22.5%), (Hosseini et al., 2016) in Iran (11.1%), and (Guyen et al., 2017) in Turkey (8.8%) while it was lower than that's reported by (Mahmoud et al., 2016) in Egypt (36.4%).

Concerning real-time PCR (qPCR), our results reported the prevalence of *T. equi* was 43.1% (50/116) which's agreed with (Alanazi et al., 2014) in Saudi Arabia (42%). However, our result was lower than the result stated by (Bhoora et al., 2009) (80%) in South Africa while it was higher than the result reported by (Jaffer et al., 2010) (36.2%).

Current results declared that qPCR had the highest sensitivity (100%) in detecting *T. equi* infection followed by conventional PCR (68%) while microscopic examination of blood smears was the lowest sensitive technique (38%). These results strongly agreed with previous results reported by (Ibrahim *et al.* 2011; Malekifard et al. 2014; Mohamed *et al.* 2016; Guven et al. 2017) who stated that molecular diagnostic tools had higher sensitivity in detecting *T. equi* compared to traditional methods.

According to the current study, the sensitivity of qPCR assay is 100% followed by conventional PCR 68% then Microscopical analysis 38% in the detection of *T. equi*. The NPVs of microscopical examination and conventional PCR were relatively low as 68.04% and 80.49 %, respectively when compared with qPCR that revealed 100 % NPV. These results indicate 100 % ability of qPCR to detect true negative when compared with the other two assays for diagnosis. So, it is recommended to combine PCR techniques with microscopic examination for studying the epidemiology of equine theileriosis, especially in carrier cases.

In the current study, results of likelihood Ratio for positive samples (LR+) were infinite for all three techniques while for negative samples (LR-) were 0.62, 0.32 and 0.00 for microscopic examination, conventional PCR and qPCR, respectively. The LR + is a quantifiable proposal of the power of a positive outcome. The perfect diagnostic test would have an LR + equal to infinity (detecting all true positives, and producing no false positives), and the top test for sensing a disease is therefore the one with the highest LR+. While, the ideal diagnostic test would have an LR- equal to zero (producing no false negatives, but detecting all true negatives), and the topmost test for ruling out a disease is therefore the one with the lowest LR- (Thrusfield, 2005). According to this rule the qPCR technique is the best one in diagnosis of *T. equi* followed by conventional PCR then microscopic examination.

Conclusion

In conclusion, to conduct an epidemiological survey on equine theileriosis, it is highly recommended to associate conventional methods of diagnosis with molecular diagnostic tools due to their superior sensitivity and accuracy especially in subclinical and chronic phases of infection.

Declarations

Compliance with ethical standards

Ethical statement: All experimental conditions for animals were performed according to the guidelines approved by the Animal Care and Use Committee of Cairo University, Giza, Egypt.

Conflict of interests: Authors declared that there is no conflict of interest.

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Figures

Figure 1

Microscopically stained blood smears showed different forms of *Theileria equi* merozoites (x100) A, B and C.

Figure 2

Results of Conventional PCR amplification for detection of *T. equi* at 392 bp fractionated on 1.5% agarose gel. Lane M: 50 bp DNA ladder, Lane 1: *T. equi* negative control, Lane 2: *T. equi* positive control, Lane 3-14 *T. equi* field positive samples.

Amplification Plots

Figure 3

qPCR results for *T. equi*: A) positive control, B) negative control, C) negative field samples and D) positive field samples.